CHARACTERIZATION OF A CANDIDATE EFFECTOR OF WHEAT YELLOW RUST TARGETING CHLOROPLAST WITH A NOVEL TRANSIT PEPTIDE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYŞE ANDAÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

JANUARY 2019

Approval of the thesis:

CHARACTERIZATION OF A CANDIDATE EFFECTOR OF WHEAT YELLOW RUST TARGETING CHLOROPLAST WITH A NOVEL TRANSIT PEPTIDE

submitted by **AYŞE ANDAÇ** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Assoc. Prof. Dr. Can Özen
Head of Department, **Biotechnology** _____

Prof. Dr. Mahinur S. Akkaya
Supervisor, **Biotechnology, METU** _____

Assoc. Prof. Dr. Bala Gür Dedeoğlu
Co-Supervisor, **Biotechnology, Ankara University** _____

Examining Committee Members:

Assoc. Prof. Dr. Çağdaş D. Son
Biology, Middle East Technical University _____

Prof. Dr. Mahinur S. Akkaya
Biotechnology, METU _____

Assist. Prof. Dr. Urartu Şeker
UNAM, Bilkent University _____

Assoc. Prof. Dr. Aslıhan Günel
Chemistry Department, Ahi Evran University _____

Assoc. Prof. Dr. Mehmet Somel
Biology, Middle East Technical University _____

Date: 15.01.2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Ayşe Andaç

Signature:

ABSTRACT

CHARACTERIZATION OF A CANDIDATE EFFECTOR OF WHEAT YELLOW RUST TARGETING CHLOROPLAST WITH A NOVEL TRANSIT PEPTIDE

Andaç, Ayşe
Doctor of Philosophy, Biotechnology
Supervisor: Prof. Dr. Mahinur S. Akkaya
Co-Supervisor: Assoc. Prof. Dr. Bala Gür Dedeoğlu

January 2019, 104 pages

Fungal pathogen, *Puccinia striiformis* f. sp. *trtici*, is the causative agent of stripe disease of wheat which causes disruption on wheat yield in many parts of the world. Fungal pathogens secrete effector molecules into host plant cells to suppress host immunity via virulence to colonize plants. Ongoing efforts are being made to identify and characterize effector proteins in many fungal plant pathogens. Nevertheless, the precise biological and biochemical functions of many effectors, such as their trafficking from the pathogen to the host cytoplasm, have yet to be fully understood. In this study, we show that an effector candidate (PstCTE1) of *Puccinia striiformis* f. sp. *tritici* localizes to chloroplasts when expressed in planta, although it has no transit signal region that can be detected by widely accepted prediction tools, indicating that it must be carrying a unique localization signal. Moreover, N-terminal tagging has no effect on the chloroplast localization of PstCTE1. It has been also observed the entrance of the effector to the chloroplast even in the presence of an intact signal peptide on the N-terminus of the transit peptide region, a result that supports possible host cell re-entry.

Keywords: Puccinia striiformis, Plant-Pathogen interactions, Wheat, Effector, Subcellular localization, Chloroplast, Transit peptide

ÖZ

KLOROPLASTA YENİ BİR TRANSİT PEPTİT İLE LOKALİZE OLAN BUĞDAY SARI PAS PATOJENİNİN KARAKTERİZE EDİLMESİ

Andaç, Ayşe
Doktora, Biyoteknoloji
Tez Danışmanı: Prof. Dr. Mahinur S. Akkaya
Ortak Tez Danışmanı: Doç. Dr. Bala Gür Dedeoğlu

Ocak 2019, 104 sayfa

Puccinia striiformis f. sp. *tritici*, buğdayda sarı pas hastalığına neden olan bir fungus patojeni olup, dünyanın bir çok yerinde buğday üretiminde büyük kayıplara neden olmaktadır. Bu fungus patojenleri bitkinin immunitesini baskılamak için kendi efektör moleküllerini konak hücresi içerisine göndererek, bitki üzerinde kolonileşirler. Patojenin bu önemli efektör proteinlerini karakterize etmek ve tanımlamak için bir sürü çalışmalar devam etmektedir. Yine de, bu efektörlere ait biyolojik ve biyokimyasal fonkiyonlar; yeterli düzeyde tanımlanamamıştır. Bu tez çalışmasında, *Puccinia striiformis* f. sp. *tritici* fungusunun bir efektör adayı olan PstCTE1 çalışılmış ve bitkide ekspres edildiğinde kloroplasta lokalize olduğu gözlemlenmiştir. Bu lokalizasyona sebep olan transit peptid en çok kullanılan biyoenformatik programlar ile aranmış fakat tespit edilememiştir ve kendine özgü bir transit peptidi olduğu düşünülmektedir. Daha sonra, bu transit peptidin N-terminal etiketlemeden etkilenmediği ve PstCTE1 efektörünün yine kloroplastta localize olduğu gözlemlenmiştir. Ayrıca, efektörün N-terminal tarafına signal peptidi birleşik şekilde ekspres edildiğinde yine kloroplasta gittiği gözlemlenmiştir ve bu bulgu da hücre içerisine tekrar grime hipotezini desteklemektedir.

Anahtar Kelimeler: Puccinia striiformis, Bitki patojen ilişkisi, Buğday, Efektör, Hücre içi lokalizasyon, Kloroplast, Transit peptid

To my family

ACKNOWLEDGMENTS

First of all, I would like to express the deepest appreciation to my supervisor Prof. Dr. Mahinur S. Akkaya for her time, guidance and support during my PhD study. Without her help, patience and knowledge, this PhD study would not have been possible. I would like to thank Assoc. Bala Gür Dedeoğlu for being my co-supervisor.

I would like to thank my thesis committee members Assoc Dr. Çağdaş D. Son and Assist. Dr. Urartu Şeker.

I want to express my special thanks to my lab members: Bayantes Dagvadorj, Zemran Mustafa, Sayit Erdoğan, Dorukcan Özköse for their collaboration and friendship. Specially, I want also to thank my husband and lab member, Ahmet Çağlar ÖZKETEN for his collaboration and help to overcome the stressful situations during my PhD study.

I would like to acknowledge Dr. Kamoun labs for providing the plasmids. I am grateful to Dr. T. Bozkurt (Imperial College, London, UK) and Assoc. Prof. C. D. Son (METU, Ankara,Turkey) for their microscopy resources.

I would like to thank to TÜBİTAK (113Z350, BIDEB-2211/C) and METU-BAP financial supports.

I would like to express my deepest gratitude to my beloved parents, Teslime Andaç and Zafer Andaç; my sister, Ceren Andaç; and my grandparents, Ayşe Andaç and Galip Andaç for their endless support, encouragement and love.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

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

PAMP: Pathogen-associated molecular patterns

PCD: Programmed cell death

PCR: Polymerase chain reaction

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

CHAPTER 1

INTRODUCTION

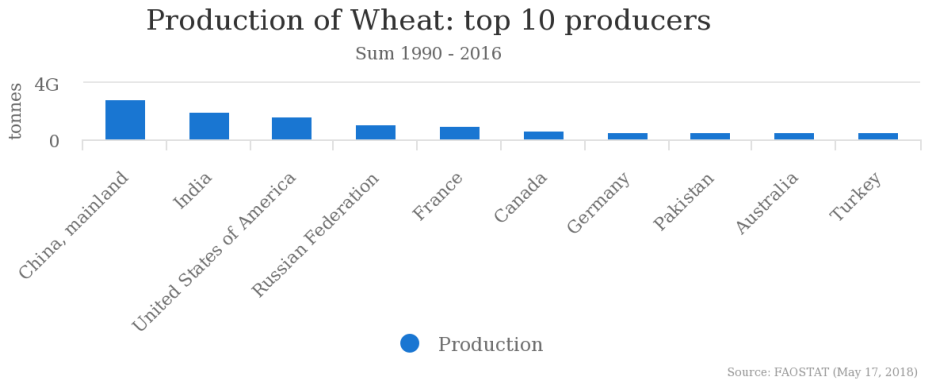
1.1. Wheat

Wheat is the most broadly grown crop in the world which has been produced thousands of years by humans. Since it is a key source of starch and energy, it provides nearly two-thirds of the human diet. Also, it is an important source for animal feed and industrial products such as paper additives or alcohol production (Curtis, 2002). According to Food and Agricultural Organization, wheat is the most generally harvested crop after rice and its annual production exceeds about 600 million tons. In 2016, the production exceeds about 700 million tones and the demand will be expected to increase by 60% in 2050 because of the increase in the world population (FAO, 2016). Wheat is the major grown crop in Turkey in which production exceeds 20 million tons annually and it is the 10^{th} wheat producing countries in the world (FAO, 2016; TürkStat, 2017) (Figure 1A & 1B).

Among the other cereals, wheat is a main source of starch and energy; also, it offers many vital or valuable components for health such as protein, vitamins, dietary fiber and phytochemicals (Shewry and Hey 2015). Moreover, its agronomic flexibility, easiness of packing and easiness of translating grain into flour makes wheat a primary component of diet (Curtis, 2002). The main wheat species grown in the world is *Triticum aestivum* which is a hexaploid species, known as 'bread wheat'. However, *T. turgidum* var. *durum* which is a tetraploid species, known as 'pasta wheat' or 'durum wheat', is included in the total world wheat production because of its adaptiveness to hot dry conditions such as Mediterranean Sea (Dinu et al. 2018; Shewry and Hey 2015).

Since wheat is an essential dietary component, it is important to sustain the wheat production or even increase the yield to satisfy the increasing human population needs. In order to do this, the yield loss due to climate change and many diseases such as rust should be prevented by develop resistant wheat varieties by genetic development.

A



B

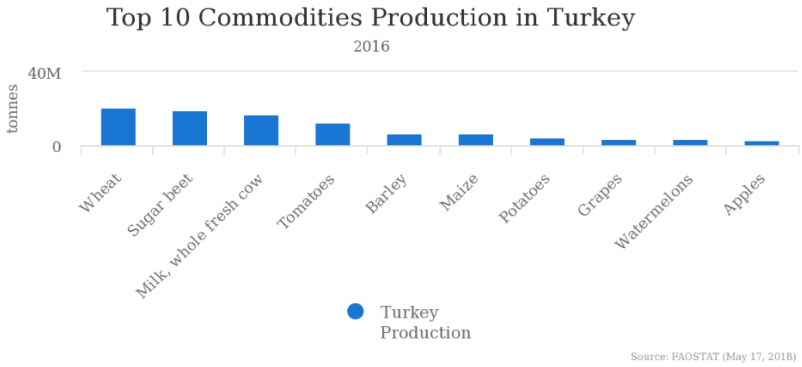


Figure 1.1. The ranking of (A) wheat producing countries and (B) wheat production rates in Turkey.

(Source: Food and Agriculture Organization 2018)

1.2. Stripe rust

Rust diseases are the oldest plant diseases and still, it is the most destructive disease of wheat and most crucial limitation of the global wheat production. There are three different types of rust diseases of wheat: leaf rust (brown rust or orange rust), stripe rust (yellow rust), and stem rust (black rust) (Roelfs 1992). They show similar disease symptoms and need similar conditions for infection. Infection of rust diseases normally confine to wheat but sometimes they can be seen in other cereals and grasses (Murray *et al*., 2005). Rust fungi are obligate parasites which means they can only grow on living host tissue and cannot be grown on artificial media (Chen et al. 2014).

Table 1.1. *The types of rust diseases, their hosts and pustule location.*

Disease	**Pathogen**	**Primary hosts**	**Pustule location**
Leaf rust	*Puccinia triticina*	Bread and durum wheats, triticale	Upper leaf surface and rarely on leaf sheaths
Stem rust	*Puccinia graminis* f.sp. *tritici*	Bread and durum wheats, barley, triticale	Upper and lower leaf surfaces, stem and spikes
Stripe rust	*Puccinia striiformis* f.sp. *tritici*	Bread and durum wheats, triticale, a few barley cultivars	Leaves and spikes and rarely on leaf sheaths

Source: (Curtis, 2002)

Figure 1.2. The difference between A) Stem rust B) Leaf Rust C) Stripe Rust based on their shapes and distributions on the wheat.

(Wolf *et al*., 2010)

Stripe rust which is caused by *Puccinia striiformis* f. sp. *tritici (Pst)* fungus is the greatest detrimental disease of wheat associated to leaf and stem rust and it is also known as yellow rust. It infects rye, barley and many grass species (Wellings 2011). It is thought that this disease emerged before wheat was cultivated as food. The stripe rust disease was first defined in Europe by Gadd in the year 1777 and its origin is thought to be Transcaucasia where the grasses were the principal host and then, the pathogen spread into Europe to China and eastern Asia (Eriksson and Henning, 1896). Since then, stripe rust has been described in over 60 countries and it has been spread through the world except Antarctica (Chen, 2005; Bux *et al.*, 2012).

Stripe rust disease development is very sensitive to weather conditions and require low temperatures for infection in comparison to other rust diseases. The best

temperature for germination is between 7-12 ºC but it can also occur between 0-23 ºC (Chen 2005; Chen et al. 2014). However, the stripe rust disease was seen in warmer zones lately where the disease is absent or rare before. This means stripe rust has begun to adapt high temperature conditions (Mboup *et al.*, 2009; Hovmøller *et al.*, 2010). The moisture is another vital condition for infection conditions. In the germination, it requires high humidity but also it affects spore survival unfavorably. Since spores can maintain their viabilities in dry conditions, dry spores can survive longer than moist ones and scatter long distances. The pathogen grows about 14 days and the initial symptoms are chlorotic patches on the upper leaves. Then, yellow to orange uredinia grows on these patches and in optimum conditions the spores can cover the entire leaf surface (Roelfs *et al.*,1992).

Spores can be spread by the help of wind over long distances. Nevertheless, the spores can be affected by UV radiation highly, so their dispersal is restricted to small distance (Chen, 2005; Murray *et al.*, 2005). Interestingly, it was reported that yellow rust spores were spread 800 km by the wind in viable state in Europe (Maddison and Manners 1972). Also, wind dries the spores which increase its viability duration.

Stripe rust cause reduction in grain yield and quality, because the seeds produced from diseased crops have low vigor, smaller size and poor germination rate. In other words, stripe rust decreases the green part of the leaf and this declines the sugar supply to the developing seed (Murray *et al.*, 2005) . Profit losses caused by stripe rust varies from 10% to 70% due to wheat cultivar susceptibility rate, infection time, disease progress rate and duration of the disease. In favorable conditions the yield losses can be 100% if the infection starts very early and the disease development endures during growing season of the wheat (Chen, 2005; Sharma-Poudyal & Chen, 2011). For management of wheat rusts, planting resistant cultivars can be the most efficient solution; however, rusts are evolving to overcome this resistance. For instance, a new wheat stem rust gene strain, *Ug99*, appeared in Uganda in 1999 which makes the commercial wheat

varieties vulnerable again. Also, the emergence of two highly aggressive and virulent strains of yellow rust (PstS1 and PstS2) pose a severe threat to the wheat stock of the world. Since, the aggressive strains which can endure complex temperatures are still evolving, it seems that the epidemics of rust diseases will continue (Hovmøller et al. 2010).

A

B

Figure 1.3. A) The spores of stripe rust disease on the leaves. B) The image of wheat field infected with stripe rust disease.

(Barb Ziesman, 2016)

1.3. Plant immunity

Plants are exposed to massive range of pathogen attacks like animals; however, they lack circulatory system, mobile immune cells like macrophages and adaptive immune

system. Instead plants have evolved to different rapid and efficient defense mechanism against a vast majority of pathogens together with bacteria, fungi, viruses and nematodes which is called innate immunity (Chisholm *et al*., 2006; Jones and Dangl, 2006).

Plants have two types of defense mechanism; pathogen-associated molecular pattern (PAMP) -triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is the basal defense of the plant which is evoked by the stimulation of pattern recognition receptors (PRRs). These receptors are found on the surface of the plant cell and they recognize pathogen associated molecular patterns (PAMPs) such as bacterial flagellin, peptidoglycan, fungal chitin or lipoteichoic acid upon pathogen attacks (Dodds and Rathjen, 2010). Basal defenses are stimulated against incompatible interactions with non-host pathogens which are often adequate to eliminate pathogens and stop their growth.

Successful pathogens have evolved to suppress PTI by carrying pathogen virulence molecules called effectors into the plant apoplast or cytoplasm to initiate pathogenicity. By deployment of effectors, pathogens destroy the resistance signaling or resistance responses and eliminate the first immunity defense layer which is called effector triggered susceptibility (ETS) (Miller *et al*., 2017; Zipfel 2008). In response to this, plants have evolved specialized cytoplasmic resistance (R) proteins to identify specific effector proteins; that is, effectors trigger the plant immune responses and activates the second layer of defense, effector triggered immunity (ETI) (Cui *et al*.,2015; Jones and Dangl, 2006).

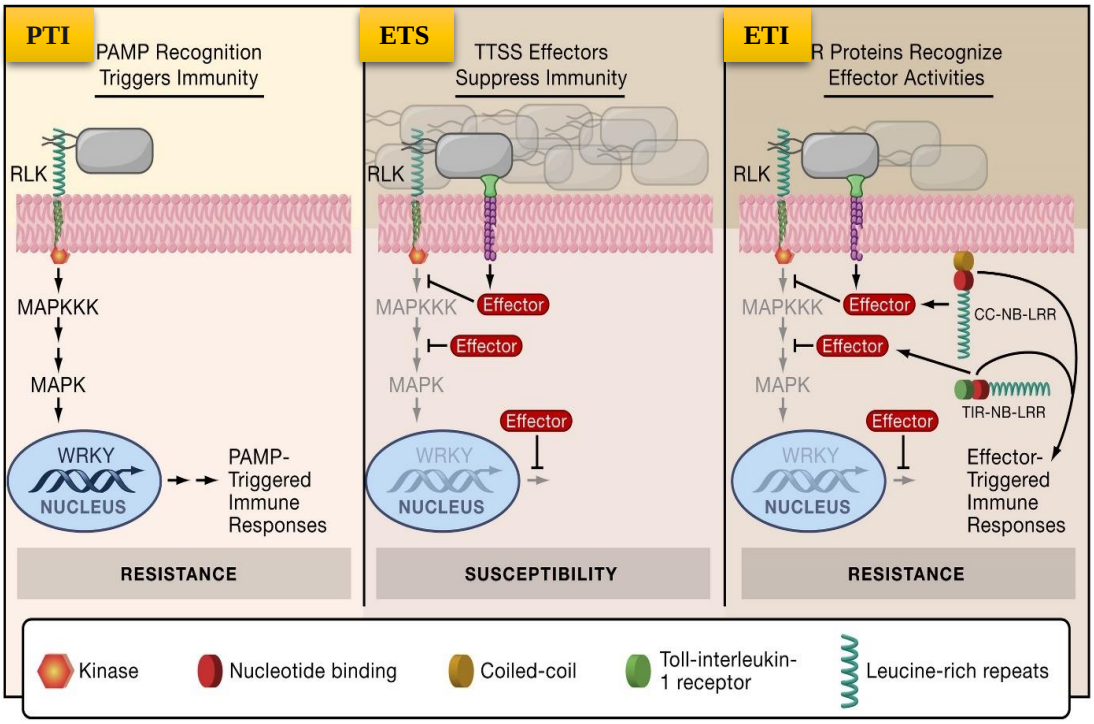


Figure 1.4. The overview of plant immune system. In PAMP triggered immunity (PTI), when the pathogen attacks plant, pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin are recognized by pattern recognition receptors like receptor like kinases (RLK) which evokes the basal defense system of the plant. This recognition results in PTI in the end of downstream signaling through MAP kinase cascades and also regulates WRKY transcription factors for transcriptional reprogramming. In effector triggered susceptibility (ETS), pathogen overcomes the basal defense system of the plant by releasing its effectors and pathogens can accumulate inside the plant cell which leads to disease development. In effector triggered immunity (ETI), there are plant resistance (R) proteins such as NB-LRR proteins which detects effector action in the plant cell and develops resistance against them (Staskawicz *et al*., 2006).

Both defense mechanisms show similar responses; however, ETI give more powerful and faster responses which involves hypersensitive response (HR), a kind of localized cell death happening in the infection spot. HR causes cytoplasmic shrinkage, mitochondrial swelling, vacuolization and chloroplast disruption in the infected cell (Stael *et al*., 2015). Thus, HR prevents pathogen proliferation by limiting their access to nutrient sources of the plant. PTI is commonly active against non-adapted

pathogens which is called non-host resistance whereas ETI is effective against adapted pathogens (Coll *et al*., 2011).

All bio-trophic plant pathogens have specialized infection structure called haustorium which is also needed for successful transfer of effector proteins. This structure forms after penetration of the pathogen and it develops at the inner side of the cell wall. Through haustoria, the pathogen takes nutrients through plant for their growth and reproduction. *Avr* effector proteins are expressed in the haustorium and they are delivered to the host plant cell (Underwood 2012). Bacterial pathogens utilize different secretion systems, type II and type III (T3SS), to deliver their effectors; however, the exact delivery mechanism of cytoplasmic effectors from filamentous pathogen into plant cell is poorly understood (Wang et al. 2017).

Several physiological and biochemical changes in the plant are initiated to occur from the onset of pathogen invasion. The earliest cellular events are Ca^{+2} invasion across the plasma membrane which is required for production of oxidative burst and HR; and intracellular pH changes. Oxidative burst which is the generation of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) is another early defense reaction. Increase in ROS amount cause the cell wall establishment, HR formation and expression of defense genes (Cohn *et al*., 2001; Scheel 1996). These defense genes encode in the biosynthesis of phytoalexins and pathogenesis related (PR) proteins such as glucanases, chitinases, defensins etc., which both have antimicrobial and antifungal properties (Van Loon and Van Strien 1999).

Many of these changes and responses in plant are caused by interaction of effector molecules with specific *R* proteins in plants. In many pathogen invasions, HR production starts by this 'gene for gene' model which was first proposed by Flor in 1942. When both *R* protein in plant and corresponding *avr* gene in the pathogen are present, the disease resistance occur but either of them is absent, it results in disease (Dangl and Jones 2001). So far, many *R* proteins are identified and the largest class of these proteins are NB-LRR which are composed of nucleotide binding (NB) domain

and a leucine-rich repeat (LRR) domain. NB-LRR proteins are controlled by complex interactions between NB and LRR domains (Cohn *et al*. 2001.). Each *R* protein has conserved NB site which is important for ATP- or GTP- binding and it contains three ATP/GTP binding motifs. It is shown the specific binding and hydrolysis of ATP for the NB domains of two tomato *R* genes, so it is thought that ATP hydrolysis leads to conformational changes that regulates signal transduction in immunity (McHale et al. 2006; Tameling et al. 2002). Comparative sequence analysis showed that the recognition specificity of the *R* proteins comes from LRR domains which is consisted of tandem LRRs and they are found at the carboxy termini of plant NB-LRR proteins. These domains may have roles in detecting effector molecules and also in protein-protein interactions (McHale et al. 2006; Moffett et al. 2002; Warren et al. 1998).

1.4. Chloroplast

Chloroplast is a large plant cell organelle which is bounded by a double membrane with an intermembrane space (Cooper and Hausman 2007). It is an important organelle for plants since photosynthesis takes place in here and also, this organelle plays a major role in plant immunity because of regulating biosynthesis of many defense related molecules, together with hormones such as nitric oxide (NO), salicylic acid (SA), and jasmonic acid (JA) and secondary messengers during plant-pathogen interaction. Moreover, chloroplast is the major site of generation of reactive oxygen species (ROS) initiating signal transduction for immunity (Torres *et al*., 2006; de Torres Zabala *et al*. 2015). Changes in the ROS production is observed during biotic and abiotic stress in plants. Higher levels of ROS can lead to initiation of HR formation which is toxic to plant cell as well as pathogen and lower levels can lead to acquired resistance which means this level enhances the production of defense molecules and prepares the plant cell for future attacks *(*Padmanabhan 2010; Torres *et al*., 2006). Chloroplast act as a defense organelle both in PTI and ETI through ROS production (Göhre 2015).

During pathogen attack, it is thought that plants should demand higher photosynthesis rate since the biosynthesis of defense genes requires more energy. However, instead of increased level of photosynthesis, it is reported that infected plants limit the photosynthetic functions to suppress the pathogen growth (Serrano, Audran, and Rivas 2016; Swarbrick, Schulze-Lefert, and Scholes 2006). Taken together, chloroplast is a signaling organelle which can sense biotic and abiotic stresses at the cellular level and respond by producing intracellular signals to block pathogen development and also to communicate with other organelles (Serrano et al. 2016). Chloroplast, nucleus, cell membrane and endoplasmic reticulum (ER) have vital roles during plant response against pathogen attack and the efficient signaling between organelles is very crucial for defense response. The establishment of this signaling is enabled by chloroplast as a sensor and deliver the intracellular signals between organelles (Caplan et al. 2008; Nomura et al. 2012). Therefore, it is not surprising that chloroplasts are targeted by pathogen effectors to disrupt the immune signaling and hormone balance to promote virulence in plants.

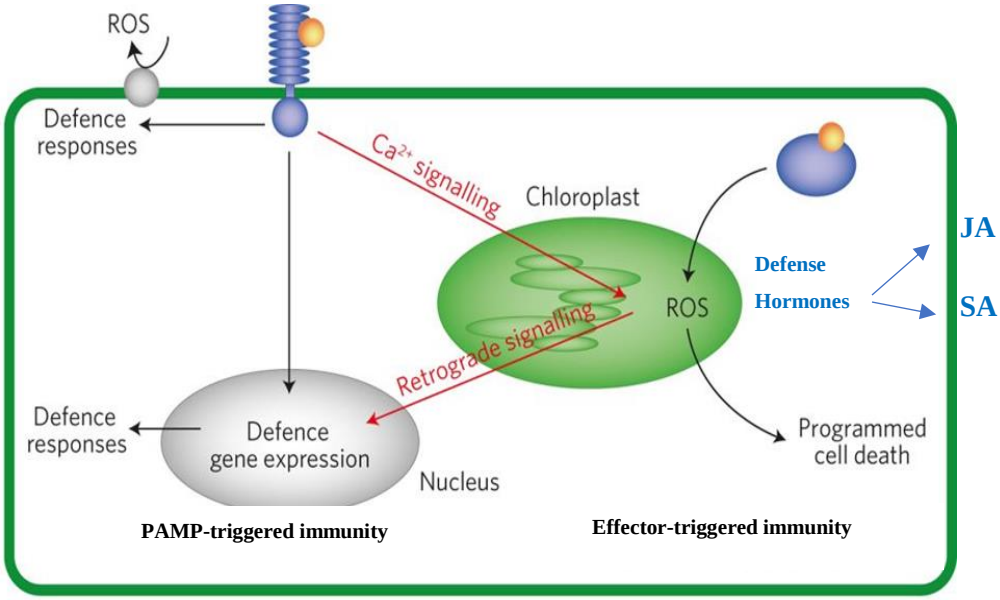


Figure 1.5. Chloroplast during pathogen attacks as one of the major defense organelle. In the beginning of plant defense system PAMPs are recognized by receptors which is found at the plasma membrane (black arrows). In this stage, they can cause a defense response such as an apoplastic burst or the defense response signal is transmitted to nucleus for causing transcriptional reprogramming damage which results in defense responses lead to PAMP-triggered immunity. Otherwise, the pathogen overcomes the PAMP-triggered immunity by releasing its effector proteins in the cytoplasm. In this case, effectors are detected by resistance proteins which leads to effector-triggered immunity. The diseased cell cause changes in the ROS production which induces hypersensitive response, in other words programmed cell death. Chloroplast mediates the synthesis of the major defense related hormones like jasmonic acid (JA) and salicylic acid (SA); also, secondary messengers like calcium. ROS production activities are not only involved in PTI to initiate HR but also, they have significant role in PTI (red arrows). After pathogen attack and their recognition at the plasma membrane cause calcium signals transmission to the chloroplast, which collects signals from the environment and give feed back to the nucleus to trigger expression of defense proteins (Göhre 2015).

1.5. Chloroplast effectors

Determining the functions of effectors is significant to understand the mechanism of pathogenicity and also for protection the plants from disease. Identification of localizations of effectors in the host cell compartments are important to understand their functions (Alfano 2009). Many effectors of fungal pathogens have been found to localize different compartments in the host cell such as plasma membrane, cytoplasm, endoplasmic reticulum, vacuole and nucleus (Caillaud et al. 2012; Petre,

Saunders, and Sklenar 2015; Rafiqi et al. 2010). Though, there are only a few fungal effectors are known which target to chloroplast and they are discovered recently (Petre, Lorrain, et al. 2016; Petre et al. 2015). Before that, chloroplast targeting effectors were reported in a bacterial plant pathogen *Pseudomonas syringae* pv *tomato* (Jelenska et al. 2007; Li et al. 2014; de Torres Zabala et al. 2015).

Chloroplast targeting effectors have N-terminal transit peptide which mediates the chloroplast entry. Chloroplast effectors with transit peptides are detected by the canonical import pathway and received to chloroplast from the cytoplasm through TOC/TIC (translocons of the outer and inner chloroplast membranes). After their import to chloroplast, the transit peptide is cleaved off (Petre, Lorrain, et al. 2016; Sowden, Watson, and Jarvis 2018). However, there are chloroplast targeting effectors which lacks N-terminal signal peptide and chloroplast targeting information lies in the mature protein. In this situation, neither the N-terminal nor the C-terminal part of the protein is important but the whole mature protein is significant for targeting. Thus, these chloroplast targeting proteins do not use the canonical import pathway and they have non-canonical transit peptides. These chloroplast targeting effectors are directly merged with the lipid bilayer of chloroplast outer membrane by unidentified interactions. They usually target outer membrane of the chloroplast whereas, the other effectors which have N-terminal transit peptides target to inner membrane and intermembrane space of the chloroplast (Miras et al. 2002).

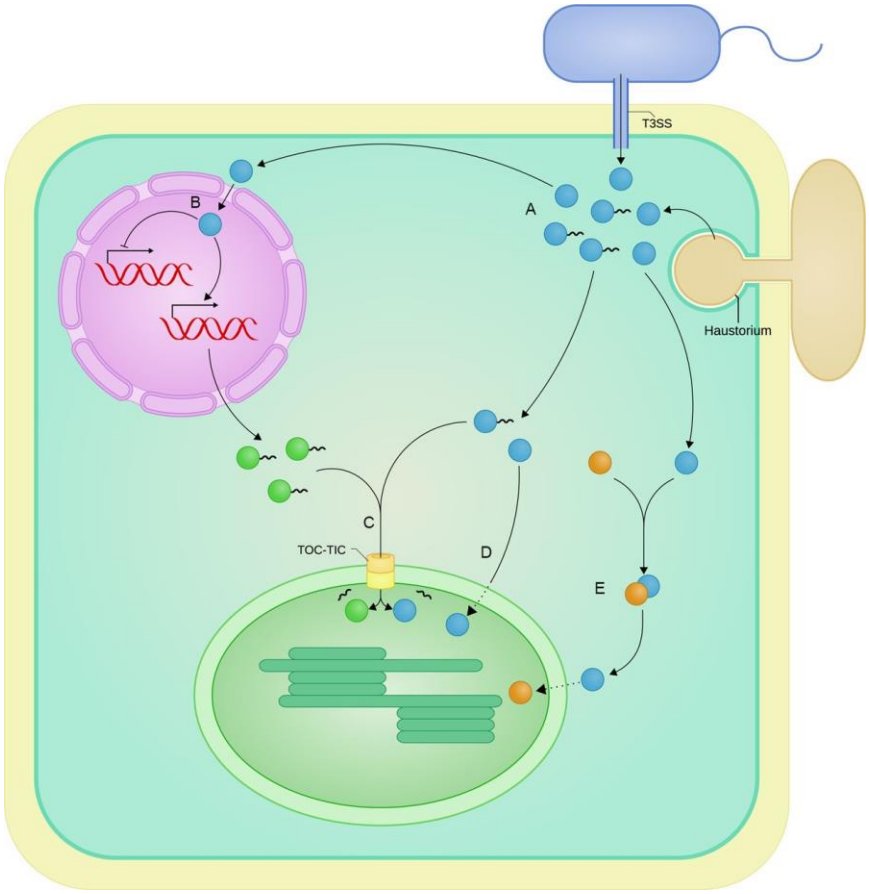


Figure 1.6. Pathogen effectors target mechanism to chloroplast for evading the plant immune system. **A)** Pathogens release effectors (blue circles) into plant host cells by using type III secretion system (T3SS) or structures like haustoria. **B)** Some effectors localize to nucleus to change the gene transcription including nuclear encoded chloroplast targeted genes (NECGs) (encoded genes are represented by green circles) which produces enzymes for hormone biosynthesis. **C)** The effectors and host NECG products have transit peptides (black wavy lines) which are detected by the canonical import pathway and the translocons of the outer and inner chloroplast membranes (TOC–TIC). After their transportation to chloroplast, the transit peptide is cleaved off. **D)** The effectors which have non-canonical transit peptides which lacks cleavable N-terminal transit peptides enter into the chloroplast by non-canonical way. **E)** Effectors may have the ability to change the localization of non-chloroplastic host proteins (orange circles), and lead them to target chloroplast. The entry mechanisms of E and D are still unknown and required to be further identification (shown by dotted branches) (Sowden et al. 2018) .

1.5.1. N-terminal transit peptides of chloroplast effectors

The effector proteins that target chloroplast have transit peptides in their N-terminus as an import signal and after entry it is cleaved by processing peptidases (Teixeira and Glaser 2013). Since pathogen lacks chloroplast, it is thought that effectors imitate transit peptides of plant chloroplast proteins for targeting; however, the mechanism for this mimicry still remains unclarified (Petre, Lorrain, et al. 2016). The transit peptides have varieties in their length and composition and they lack consensus among each other but they contain a characteristic amino acid composition (Bruce 2000). Also, they may have specific physicochemical properties which is essential for recognition by import mechanism. These physicochemical properties can be environmentally sensitive or context specific, differently functioning due to pH, in a membrane-like environment, or upon receptor binding (Bruce 2000; Chotewutmontri et al. 2012). Therefore, physicochemical properties define the transit peptides rather than their sequence information. Moreover, it is thought that they may evolved *de novo* in contrast to other domains which have strict amino acid sequence similarity for functioning (Tonkin, Kalanon, and McFadden 2008).

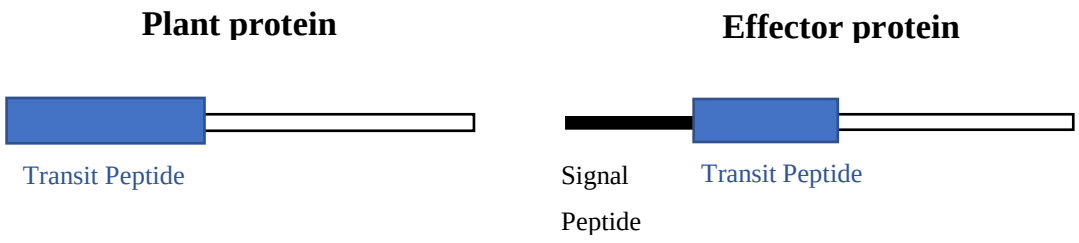


Figure 1.7. Schematic view of chloroplast proteins in plants and chloroplast targeting effector proteins.

1.6. PstCTE1 effector gene

The effector genes of *Puccinia striiformis* f. sp. *tritici*, which encode secreted proteins, were predicted from haustorial cDNA library by Yin *et al* in 2009. The haustorial cDNA library were constructed after haustorium isolation from heavily infected wheat leaves with *Pst-78* and full-length unisequences were selected (Yin et al. 2009). In this research, 15 candidate effector genes with secretion signal peptide in their N-terminus were found. PstHa12j12 (GH737467) was one of these accepted putative effector genes, which we named it PstCTE1 in our research. This effector gene is expressed in all stages of *Pst* development such as in urediniospore, germination of urediniospore and leaves with *Pst* infection; however, the highest expression was seen in *Pst*-infected leaves. The expression level of infected leaves is ten-fold higher than germinated urediniospores and also eight-fold higher than urediniospores expression levels. It has no homology in the databases according to Yin et al., 2009; however, four homologs of PstCTE1 (e-value threshold<e^{-5}) was found and three of which were recently reported (Xia C. unpublished, BioProject: PRJNA422914, 2018). Moreover; majority of effectors are small, cysteine rich proteins and PstCTE1 is also small and has six cysteine residues which is significant for protein folding processes (Lu and Edwards 2015; Yin et al. 2009).

1.7. Agrobacterium tumefaciens mediated gene transfer

Agrobacterium-mediated gene transfer is one of the most used and highly effective method for plant genetic engineering. *A. tumefaciens* is a wide spread Gram-negative soil bacterium which is capable of transfer foreign genes in host plant cell (Gelvin 2003). This soil pathogen naturally infects wound sites of dicotyledonous plants and causes an important crown gall disease by developing a tumor. It has 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 is the genetic material that is introduced into nucleus of host plant cell from Ti plasmid of agrobacterium

which results in the crown gall disease (Guo et al. 2011). The T-DNA has two types of genes: the oncogenic genes which is used in the production of auxins and cytokinins and responsible for tumour formation; and the genes used in the production of amino acid-sugar conjugates (opines) for the bacterium growth as a carbon source (Borem et al. 2014; de la Riva et al. 1998).

T-DNA region is identified by its left and right borders which are 25 bp length and very homologous sequences. These borders are the only required sequences for transfer of T-DNA. Moreover, virulence genes, located on *Vir* region of Ti plasmid, mediate the T-DNA transfer but they are not transferred themselves. The *Vir* region is composed of six vital (virA, virB, virC, virD, virE, and virG) and two nonessential genes (virF and virH). These virulence genes are involved in sensing the plant signal molecules; processing, transfer and nuclear localization of T-DNA (Guo et al. 2011; Peralta and Ream 1985; Schrammeijer 2000).

Preliminary methodologies for introducing gene of interest (goi) into *Agrobacterium* T-DNA region of Ti plasmid were required complex procedures because of the oversize of Ti plasmid. To overcome these difficulties, T-DNA and *vir* genes were separated into two different vectors, called binary T-DNA and *vir* helper. In this binary system, tumor inducing and opine synthesis genes were removed since they are harmful to plant cells and have no significant roles in transfer process (Gelvin 2003; Lee and Gelvin 2007).

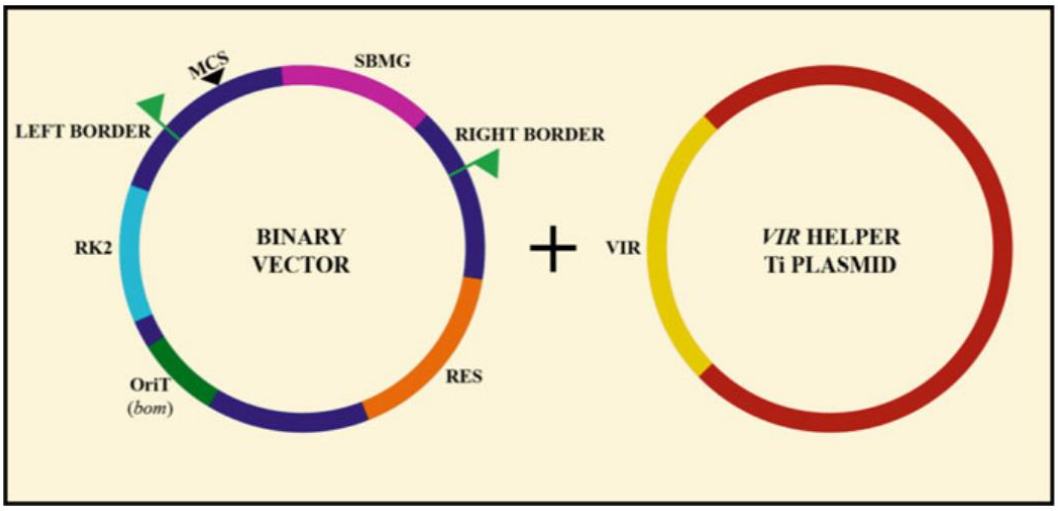


Figure 1.8. Binary vector system of Agrobacterium. Binary vector has left and right borders, multiple cloning site (MCS), selective plant marker gene (SBMG), antibiotic resistance gene (RES), origin of transfer (OriT) and replication origin (RK2). The target gene is cloned into T-DNA region which is located between left and right borders. *Vir* genes are located on a separate replicon called *vir* helper *T*i plasmid. T-DNA transfer are mediated by *vir* genes on *Ti* helper plasmid (Ozyigit 2012).

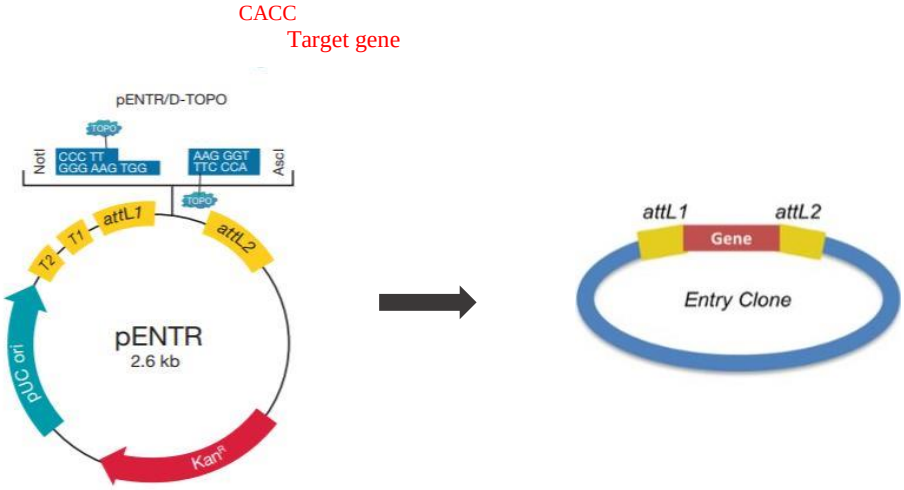
1.8. Gateway cloning

Determining functions of the genes requires the cloning the gene of interest (goi) into variable special vectors and it enables to identify gene expression, encoded protein purification, subcellular localization determination and interactions with other proteins. Each procedure needs subcloning into one or more vectors and this becomes time consuming and troublesome with conventional cloning systems which depends on restriction digestion and ligation systems. However, the development of robust site-specific recombinational cloning method, Gateway cloning, enables cloning procedures fast and reliable. Gateway cloning system utilizes site-specific recombination of bacteriophage λ in *E. coli*, which integrates and excises itself in and out of a bacterial chromosome, to transfer the gene to a new vector (Hartley *et al*., 2000; Karimi *et al*., 2007).

In this cloning system, the gene of interest is cloned into entry vector initially, then the gene can be transferred into many vectors for different purposes. Polymerase chain reaction (PCR) is performed to add CACC sequences to the 5' of target gene and by this sequence the gene of interest is inserted into entry vector by topoisomerase which is a site-specific recombinase (Earley *et al*., 2006). The obtained plasmid has the target gene with *att*L recombination site (attL1-gene-attL2). Then, the target gene is transferred to destination vector which has *att*R recombination site (attR1-ccdB-attR2), which is called LR clonase reaction. These sites recognize each other and recombination takes place. Destination vectors have ccdB gene between *att*R recombination sites which is a killer gene and a selection marker. This gene activates gyrase-mediated cleavage of double-stranded DNA and it prevents survival of most *E. coli* strains unless they have *ccdB* resistance. Hence, the transformants having the desired constructs can be selected by *ccdB* gene as well as antibiotic resistance of destination vector (Hartley *et al*., 2000; Cheo *et al*., 2004; Karimi *et al*., 2007).

A

pENTR-D/TOPO



B

LR Reaction

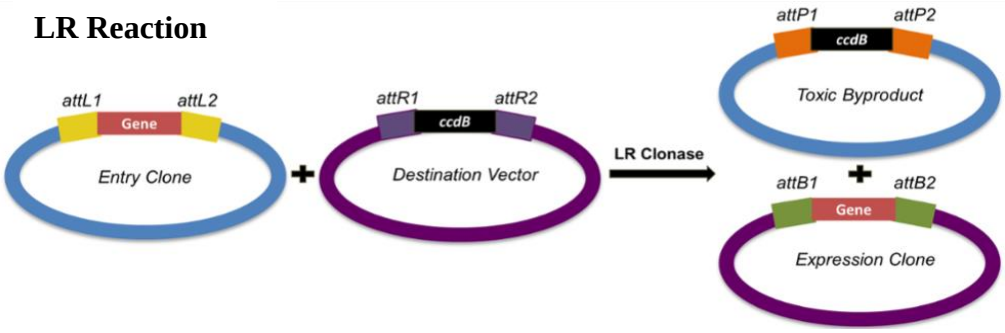


Figure 1.9. Schematic illustration of Gateway cloning system. **A)** CACC sequence is added to 5'end of the target gene by PCR and obtained product is inserted into entry (pENTR/D-TOPO) vector by topoisomerases found at its both ends. **B)** In LR clonase reaction, the gene of interest is transferred from entry vector to destination vector between *att*L and *att*R sites. In transformants, only destination vector with target sequence can survive since toxic byproduct have the lethal *ccdB* gene (Earley *et al*., 2006; Soriano 2017; Thermo, Gateway User Guide Manual, 2003).

1.9. pK7FWG2 and pH7WGR2 vector

All Gateway-compatible destination vectors have their backbone from pPZP200 plasmid which contains an origin for replication in *E. coli* (ColE1) and in *Agrobacterium* (pVS1) and also *bom* site for transferring from *E. coli* to *Agrobacterium* (Karimi, Inzé, and Depicker 2002). Destination vectors are T-DNA plasmids that are utilized in *Agrobacterium* mediated gene transfer for plant functional genomic studies. The transfer of genes from entry vector to Gateway destination vectors is fast and reliable which is occurred between *att*L and *att*R sites by LR clonase enzyme system (Karimi, Depicker, and Hilson 2007).

The destination vectors, pK7FWG2 and pH7WGR2, are used in subcellular localization studies of a gene of interest with a C-terminal GFP and N-terminal RFP fusion, respectively. Moreover, they have Spectinomycin (Spec) for plasmid selection in bacterium (*E. coli* and *Agrobacterium*) and Kanamycin (Kan) resistance for plant selection. They have *ccdB* gene between *att*L and *att*R flanking sites, so only the constructs with the goi is observed (Karimi et al. 2002). The map of the vectors is revealed in Figure 1.10 and Figure 1.11.

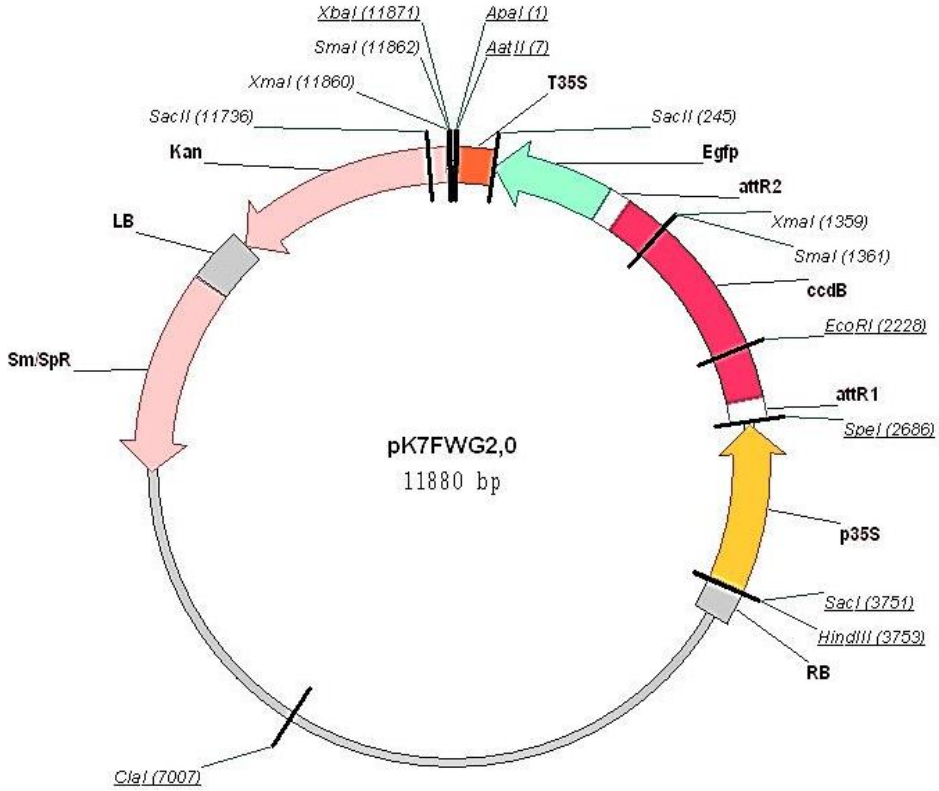


Figure 1.10. Vector map of pK7FWG2Vector map of pK7FWG2.

(Karimi et al., 2002)

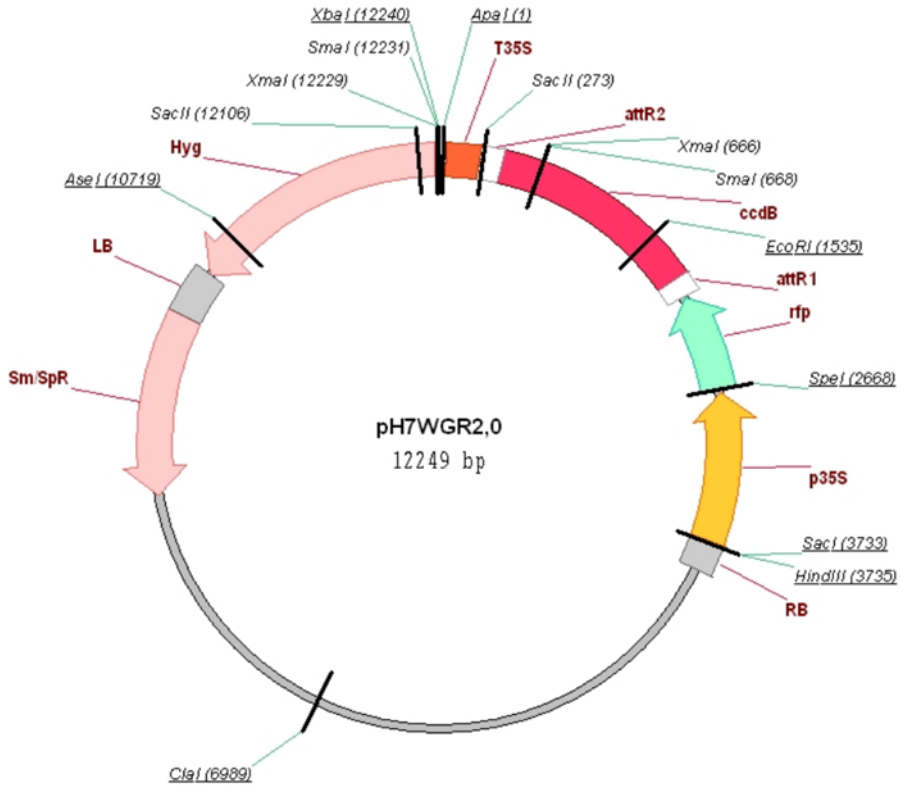


Figure 1.11. Vector map of pH7WGR2.

(Karimi *et al*., 2002)

1.10. Subcellular localization

The determination of molecular functions of effectors is important because of being key elements in understanding plant-pathogen interaction mechanisms. For this effort, subcellular localization is a successful method to detect the role of the effectors in pathogenicity and also their localizations might reveal how they affect the host immunity (Dowen et al. 2009). Through this method, many successful pathogen effector localization studies have been accomplished inside plant cell (Bozkurt et al. 2012; Rafiqi et al. 2010; Ve et al. 2013).

Many obligate biotrophic fungi like *Pst* lack stable transformation system and this limits the achievements in wheat-Pst interaction studies. Also, direct microscopic observation of effectors is not observable because of its dilute form in the cytoplasm

of plant (Bozkurt et al. 2012; Caillaud et al. 2012). Therefore, effectors fused with fluorescent proteins are used for expression in plants through *Agrobacterium*-mediated gene transfer to investigate its subcellular localization.

So far, this method is only suitable for dicot plants such as tobacco and is not functional for monocot plants such as wheat. Genetic transformation can be achieved by *Agrobacterium* in wheat immature embryos yet the efficiency is still low (Przetakiewicz et al. 2004; Wu et al. n.d.). However, there is another technique, protoplast isolation and transfection, which is an effective and fast method and it can be utilized for subcellular localizations of effector genes in the primary host, wheat. Plant protoplasts have been used widely in genetic transformation studies since they lack cell wall. Macromolecules can be delivered into protoplast by many techniques such as PEG–calcium fusion, electroporation and microinjection (Liu et al. 2016; Yoo, Cho, and Sheen 2007).

1.11. pJL48-TRBO vector

The protein of interest can be produced in plants by agrobacterium mediated transient expression in a very rapid and convenient way. In order to do this, ***T****obacco mosaic virus* (TMV)- based transient expression vectors can be used by agroinfiltration method since these vectors can express very high amounts of various proteins in plants. For producing large amounts of proteins in plant, a very high amount of agro-infiltration solution should be used but it was realized that, this amount of agrobacterium caused HR response in plants. It was found that this response came from TMV coat protein gene sequence which causes its virulence (Wroblewski, Tomczak, and Michelmore 2005). After removal of this coat protein, high-efficiency **t**obacco mosaic virus **R**NA-**b**ased **o**verexpression vector (TRBO) was developed and the agro-infection method became very efficient and more favorable (Lindbo 2007).

The TMV based pJL48-TRBO vector is an agroinfection-compatible and it has *Cauliflower mosaic virus* (CaMV) 35S promoter. The deletion of the TMV coat

protein makes this vector smaller which enables the cloning and handling this vector much easier. Moreover, it has higher agroinfection efficiency and protein expression amounts (Lindbo 2007).

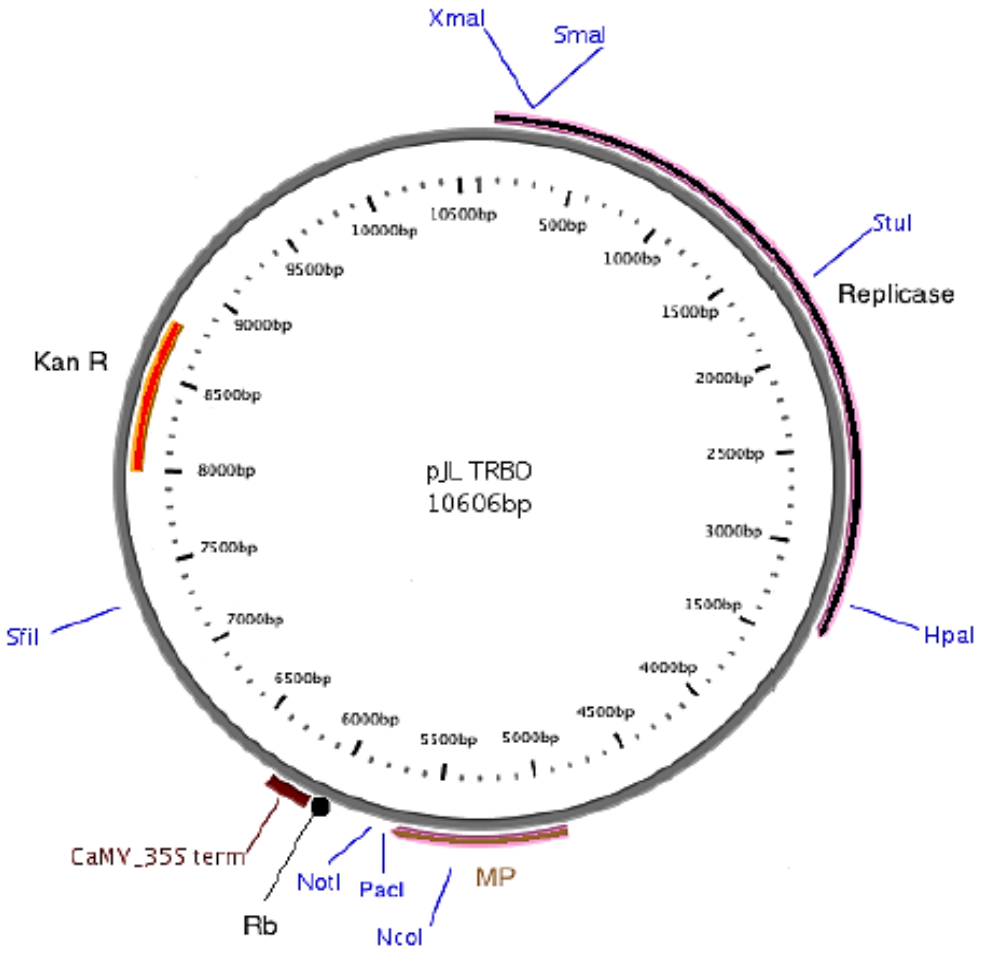


Figure 1.12. Vector map of pJL48-TRBO.

(Xiaoli Dong *et al*., 2004)

1.12. Immunoprecipitation

Immunoprecipitation is a commonly used method to separate and concentrate a specific protein from a mixture containing many different proteins. It is the small-scale affinity purification of antigens with using a specific antibody; that is, the specific antibody binds the protein of interest in the protein solution of cell or tissue lysate and then, antibody-antigen complex can be isolated from the protein mixture (Bonifacino, Dell'Angelica, and Springer 2001). The isolated sample can then be detected by many ways such as SDS-PAGE, western blotting and mass spectroscopy. Through this technique, protein determination and identification, protein-protein relations and post-translational modifications of proteins can be performed. Immunoprecipitation combined with western blotting analysis increase the sensitivity of detection of proteins by using specific antibodies. Then, mass-spectroscopy can be used to identify proteins because it is a very sensitive and accurate way of determining protein complexes (ten Have et al. 2011).

1.13. Aim of the study

Domesticated cereals like wheat, corn, rice and barley are the primary components of human diet for thousands of years. However, the cereals are subjected to the biotic (pathogens, viruses) and abiotic (environmental) stresses which causes the decrease in their quality and yield. Thus, controlling these stresses are very crucial to prevent the losses which occurs every year in the world. Chemicals are broadly used in agriculture to control the biotic stress; however, it is a temporarily solution to kill pathogens and also has many drawbacks on the environment. Thus, plant-pathogen interactions should be determined at molecular level to improve long-term and effective solutions. By this effort, genetically improved crops can be developed against pathogen disease to prevent losses. Many studies are ongoing to characterize the effectors of fungal pathogens which are key molecules in plant-pathogen interactions, but still there is

very limited information about effector secretion, their secretion routes and also entry mechanisms into host cell (Petre and Kamoun 2014).

In this study, one of the candidate effector genes of *Puccinia striiformis* f. sp. *tritici* (PstCTE1) is aimed to characterize and identify its function. To do this, PstCTE1 was cloned into pK7FWG2 Gateway destination vector to visualize its subcellular localization in *Nicotiana benthamiana* plant by GFP expression. Since genetic transformation techniques are ineffective on wheat, *N. benthamiana* is used as the host which is useful model plant to transiently express proteins. Also, protoplast isolation and transfection were performed to confirm the subcellular localization on the host plant, wheat. After determining that the effector localization, its transit peptide location was searched by prediction tools including TargetP and ChloroP; also, it was identified by experimentally.

It was searched that if N-terminus tagging change the localization of the effector like the other chloroplast targeting effectors. Also, it was expressed with its signal peptide (SP-PstCTE1) to observe whether it is localized to apoplast or chloroplast again.

Moreover, immunoprecipitation was performed to PstCTE1 and SP-PstCTE1 to determine the differences between them and also identify the probable transit peptide processing.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant materials

2.1.1. Wheat and tobacco growth

For tobacco plants, Nicotiana benthamiana seeds were planted and grown in pots at 16 h light / 8 h dark cycle at 24oC. After one week, they were sub-cultured that only one plant was planted in each pot. They became ready for agro-infiltration after 4-6 weeks. Wheat cultivar, Avocet S, were grown 16 h light / 8 h dark at 20-23°C for 10 days in growth chamber. After ten days they were ready for protoplast isolation.

2.2. *In silico* characterizations

The EST sequence of stripe rust candidate effector gene, PstCTE1 (PstHa12j12) is assigned GenBank Accession number of GH737467.1 at National Center of Biotechnology Information (NCBI) databases. The signal peptide of the sequence was confirmed using SignalP 4.1 (http://www.cbs.dtu.dk/services/ SignalP/). For the transit peptide search, ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) were used. To investigate any known homologs, NCBI database using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) web-tool was scanned. Multiple alignment analysis and phylogenetic tree of the homologs along with PstCTE1 were performed with using ClustalW tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

The relationship of the transit peptide of PstCTE1 was investigated with the remaining available Pucciniales proteome sequences which are *Puccinia striiformis* f. sp. *tritici*

(*Pst*), *Puccinia graminis* f.sp. *tritici* (*Pgt*) and *Puccinia triticina* (*Ptt*). A database was constructed with their proteome sequences which were downloaded from Puccinia Group website of Broad Institute (http://www.broadinstitute.org/). Combined proteomes of Pucciniales were filtered based on existence of signal peptide using SignalP 4.1. These candidate effectors were further analyzed with TargetP 1.1 to predict their subcellular localization. These chloroplasts targeting effectors were used to construct a combined CTPome database for 3 Pucciniales reference genome (*Pst*, *Ptt* and *Pgt*). Multiple alignment analysis was conducted using ClustalW to compare PstCTE1 within our CTPome. Then, phylogenetic tree was constructed using iTOL (https://itol.embl.de/).

2.3. PCR and cloning

The open reading frame of PstCTE1 gene had made synthesized with N-term *Pac*I and C-term *Not*I sites without its signal peptide and cloned in pUC57 vector using EcoR1 site (GeneScript). Primer sequences used in this study are presented in Table 2.1. PstCTE1 gene was amplified with Fwd-CACC-CTE1 and CTE1-Rev-NoStp primers to obtain C-term tagged constructs for subcellular localization studies. For N-terminus tagged constructs, the gene was amplified by using Fwd-CACC-CTE1 and CTE1-Rev-Stop primers. After PCR amplification, the gene have N-term CACC sequence for cloning into pENTR/D-TOPO entry vector (Invitrogen). After cloning into entry vector, the construct was transferred into destination vectors pK7FWG2 and pH7WGR2 using LR Clonase for C-term GFP and N-term RFP constructs, respectively.

To integrate signal peptide to PstCTE1, three subsequent PCRs, which were performed one after another with following primers using the products as template DNA: Fwd-CTE1-SP-1, Fwd-CTE1-SP-2 and Fwd-CTE1-SP-3. Lastly Fwd-CACC-SP-CTE1 and CTE1-Rev-NoStp primer was used for amplification with the PstCTE1 DNA. To truncate the gene, two PCRs were performed. Fwd-CACC-CTE1 and KLR-

Rev primers were used in the presence of the PstCTE1 DNA as a template in PCR for N-term part of the gene. Fwd-CACC-SGF and CTE1-Rev-NoStp were used in the PCR similarly for C-terminus fragment. To prepare a construct with the signal peptide on the N-terminus of the second half of the gene (C-terminus truncate), Fwd-CACC-SP-CTE1, Fwd-CTE1-SP-2, and Fwd-SP-SGF primers, again lastly CTE1-Rev-NoStp were used for PCR amplification with PstCTE1 DNA as a template. These PCR products using CACC forward primers were also cloned into pENTR/D-TOPO vector and recombined into Gateway destination vector pK7FWG2 using LR Clonase. The constructs were multiplied in *E. coli* TOP10 strain and stocked at -80 °C. The *Agrobacterium* GV3101 strain for tobacco infiltration assays were transformed with the plasmids by electroporation at 2.2 kV on Celljet duo, Thermo Corp. using cuvettes with 2 mm gap. pTRBO-GFP construct was used for GFP alone assays. The vectors pK7FWG2 and pH7WGR2 and the construct pTRBO-GFP were gifts from from Kamoun Lab, Sainsbury laboratory, Norwich, UK.

For cloning the PstCTE1 into pJL48-TRBO vector, PstCTE1 gene synthesized in pUC57 vector which has *Pac*I and *Not*I were amplified by PstCTE1-PacI-Fwd and PstCTE1-NotI-Rev primers. Then, this amplified product was digested with *Pac*I and *Not*I enzymes but since the gene has a small size, it was problematic to get efficient digestion. Thus, the gene of interest was cloned into pGEM-T-Easy vector (Promega) and then double digestion was performed with *Pac*I and *Not*I enzymes. Then, ligation was made with double digested PstCTE1 gene and pJL48-TRBO by T4 DNA ligase. The construct was multiplied in *E. coli* TOP 10 strain after ligation and the positive clone verified by colony PCR was stocked at -80 °C. This construct was transferred into *Agrobacterium* GV3101 strain for expression of PstCTE1 gene in *N. benthamiana*.

Table 2.1. *The primer sequences used in this study.*

Primer Names	Primer Sequences (5' to 3')
Fwd-CACC-CTE1	CACCATGCTAACTAAGACAGGGG
CTE1-Rev-NoStp	AGATGTGCTTCTCCCAACATCTTG
CTE1-Rev-Stop	CTAAGATGTGCTTCTCCCAACATC
Fwd-CTE1-SP-1	GGATTAATTAAATGCAGCTCTACATATCAATT
Fwd-CTE1-SP-2	GCTATAAGCAGTACTAATCCAATTGATATGTA
Fwd-CTE1-SP-3	TCTTAGTTAGGGTTTTCACTGTCGTTGCTATAAGCAGT
Fwd-CACC-SGF	CACCATGTCGGGATTCAAAGCAACTAAG
KLR-Rev	ACGAAGCTTGTTGCATAAAGAATC
Fwd-CACC-SP-CTE1	CACCATGCAGCTCTACATATCAATTGGA
Fwd-SP-SGF	TTTGAATCCCGAGGTTTTCACTGTCGTTGCTATAAGCAGT

2.3.1. Polymerase chain reaction (PCR)

For PCR reaction, the used reagents and their amounts are listed in Table 2.2 and Table 2.4. Before cloning, Q5 High Fidelity DNA Polymerase (M0491, NEB) was used for PCR reaction. To verify the clones after cloning by colony PCR, *Taq* DNA Polymerase (M0320, NEB) was used.

Table 2.2. *PCR reagents and their amounts used for Q5 high fidelity polymerase.*

PCR reagents (in 200µL PCR tube)	**Amount**
Template DNA	Variable (50pg-150ng)
5X Q5 Reaction Buffer	5 µL
10mM dNTPs	0.5 µL
10µM Forward Primer (10pmol/ µL)	1.25 µL
10µM Reverse Primer (10pmol/ µL)	1.25 µL
Q5 High Fidelity DNA Polymerase (2000U/ml)	0.25 µL
5X Q5 High GC Enhancer	5 µL
Nuclease Free Water	Variable
Total volume	25 µL

Table 2.3. *PCR conditions in thermocycler for Q5 high fidelity polymerase PCR.*

Step	**Temperature,** °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	

Table 2.4. *PCR reagents and their amounts used for Tag DNA Polymerase colony PCR.*

PCR reagents (in 200µL PCR tube)	**Amount**
10X Standard *Taq* Reaction Buffer	2.5 µL
10 mM $MgCl_2$	1.5 µL
25 mM dNTPs	0.5 µL
10 µM forward primer (10pmol/ µL)	0.5 µL
10 µM reverse primer (10pmol/ µL)	0.5 µL
Template DNA	1 µL
Taq Polymerase (5U/µL)	0.125 µL
Nuclease free water	18.375 µL
Total volume	25 µL

Table 2.5. *Conditions in thermocycler for Tag DNA Polymerase colony PCR.*

Step	**Temperature,** °C	**Time**	
Initial Denaturation	95	30 sec	
Denaturation	95	30 sec	35 cycle
Annealing	55	30 sec	
Extension	68	1 min	
Final Extension	68	5 min	

2.3.2. Gateway cloning

The gene of interest was amplified with CACC primers and then inserted into entry vector pENTR^{TM}/D-TOPO (Kan^{R}) by topoisomerase mediated cloning. Next, the gene of interest was transferred into pK7FWG2 (Spec^{R}) and pH7WGR2 (Spec^{R}) destination vectors by LR Clonase enzyme mix. For pENTR/D-TOPO cloning, Invitrogen pENTR^{TM} Directional TOPO^{R} cloning kit was used and the amounts of the reagents required for cloning is listed in Table 2.6.

Table 2.6. *The reagents and their amounts used in pENTR/D-TOPO cloning.*

Reagents (in 200 µL PCR tube)	**Amounts (µL)**
PCR product	1
Salt solution	1
pENTR/D-TOPO vector	0.5
Nuclease free water	2.5
Total volume	6

After mixing the reagents, it was incubated at 23 ºC for 1 hour and then all the reaction mixture was transferred into *E. coli* TOP10 competent cells. Transformed cells were plated on agar plates holding 50 µg/mL Kanamycin. The positive colonies were tested by PCR and stocked at -80 ºC and plasmid isolation was performed from positive clone of pENTR/D-TOPO reaction.

For transferring the gene of interest from entry vector to destination vectors, LR clonase reaction was performed with Invitrogen Gateway^{R} LR Clonase^{TM} II Enzyme Mix kit. The reagents were listed in Table 2.7. For LR reaction, two destination vectors, pK7FWG2 and pH7WGR2, were used.

Table 2.7. *The reagents and their amounts required for LR clonase reaction.*

Reagent (in 200 µL PCR tube)	**Sample (µL)**
pENTR-PstCTE1 (100-150 ng/µL)	1
Destination vector (150-200 ng/µL)	1
TE buffer (2X)	2.5
LR Clonase enzyme mix	0.5
Total volume	5

The reagents for LR clonase were mixed and incubated at 25 ºC for 1 hour. In order to stop the reaction, addition of 1 µL Proteinase K solution was achieved and incubated for 10 minutes at 37 °C. Then, all the reaction mixture was transformed into *E. coli* TOP 10 competent cells and plated on agar with 100 µg/mL Spectinomycin. Transformed cells were checked with colony PCR and stocked at -80 ºC. Plasmid isolation were performed from the positive clone for further experiments.

2.3.3. Cloning of PstCTE1 in pJL48-TRBO vector

PstCTE1 gene was amplified with PstCTE1-PacI-Fwd and PstCTE1-NotI-Rev primers. Then, the amplified product was first cloned in pGEM-T-Easy vector (A137A, Promega) because the gene has a small size which causes the low efficiency of digestion with restriction enzymes. For cloning procedure, T4 DNA ligase was used (M0203S,NEB) and the mixture was incubated at 16 ºC overnight (Table 2.7). After cloning, the PstCTE1 was double digested with *Pac*I (R0547s, NEB) and *Not*I (R31895, NEB) enzymes for 3 hours at 36 ºC according to Table 2.8. Also, the pJL48-TRBO vector was double digested with the same enzymes with same conditions. Then, the digested products both the PstCTE1 gene and the pJL48-TRBO vector were

loaded in 1% agarose gel in 1X TAE buffer in order to isolate the digested products. After separation on agarose gel, the products were cut from the gel and QIAquick Gel Extraction Kit (Qiagen) (Cat # 28706) was used to purify the samples by using manufacturer's protocol (Section 2.3.3.1).

The eluted DNAs from gel extraction were loaded on agarose gel again and their quality was checked. Then, PstCTE1 gene insert were ligated with pJL48-TRBO according to Table 2.9 and incubated at 16 overnight ^{o}C. Then, the cloning mixture was transformed into *E. coli* and transformed cells were plated on 50 µg/mL Kanamycin agar plates. The next day, 4-5 colonies were picked and verified by colony PCR. The plasmid isolation was performed from the positive clone and transformed into *Agrobacterium* GV3101 and stocked at -80 ^{o}C.

Table 2.8. *The reagents and their amounts used in pGEM-T easy vector ligation.*

Reagents (in 200 µL PCR tube)	**Amounts (µL)**
PCR product	5
pGEM®-T or pGEM®-T Easy Vector (50ng/µL)	1
10X T4 Ligase Buffer	1
T4 DNA ligase with 10 mM ATP (5 unit/µL)	0.1
ddH_2O	2.9
Total Volume	10

Table 2.9. *The reagents used in double digestion reaction.*

Reagents (in 200 µL PCR tube)	**Amounts (µL)**
Vector	10
*Pac*I (10,000unit/mL)	0.5
*Not*I HF (20,000unit/mL)	0.25
NEB buffer 4 (10x)	2
BSA (100X)	0.2
ddH_2O	7.05
Total	20

Table 2.10. *The reagents used in ligation reaction with pJL48-TRBO vector.*

Reagents (in 200 µL PCR tube)	**Amounts (µL)**
10X T4 ligase buffer	1
pJL48-TRBO vector	1
PstCTE1 insert	2
T4 DNA ligase with 10 mM ATP (5 unit/µL)	0.2
ddH_2O	6.4
Total Volume	10

2.3.3.1. Gel extraction

1.5 mL microcentrifuge tubes were used to store the cut gel pieces and their weights were measured. 3 volumes of Buffer QG was added to 1 volume of gel (1 mg = ~1 µL). The tubes were incubated at 50 ^{o}C for 10 min for dissolving of the gel in Buffer QC. In this step, the tubes were mixed by spinning every 2-3 min. Then, 1 volume of isopropanol was added to each tube and mixed. The samples were transferred into QIAquick spin column in a provided 2 mL collection tube to bind DNA. Centrifugation was performed 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 isolate the samples from remaining agarose. Centrifugation was performed again for 1 minute at 13,000 rpm. The flow through was discarded and the column was wash away by 0.75 mL Buffer PE. Before centrifugation, the samples were incubated at room temperature for at 3-5 min. Then, centrifugation was performed for 1 min at 13,000 rpm. The flow through was poured off and the spin column was centrifuged again about 2 min to get rid of remaining PE buffer in the column. Next, the spin column was removed from collection tube and it was placed into a new 1.5 mL microcentrifuge tube. 30 µL nuclease free water was added and it was incubated at room temperature for 3 min. Centrifugation was performed for 3 min at 13,000 rpm. Finally, the eluted DNAs were detected by separation on 1% agarose gel in 1X TAE buffer to see if the DNAs were taken out from bands.

2.4. Bacterial preparations and transformations of *E. coli*

2.4.1. *E. coli* TOP 10 competent cell preparation

A single colony of *E. coli* TOP10 was inoculated in 5 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) with no antibiotic and incubated overnight at 37 ^{o}C at 250 rpm. From overnight culture, 1 mL was inoculated into 100 mL sterile LB medium and it was incubated at 250 rpm for 2 hours until $A_{600\ nm}$ reached to 0.375. The culture was separated into two in 50

mL sterile falcon tubes and incubated at 10 min on ice. Next, the cells were centrifuged for 5 min at 5000 rpm, at 4 ^{o}C and the supernatant was discarded. The cell pellets were resuspended in filter sterilized 10 mL cold 100 mM $CaCl_2$ by gentle mixing and incubated on ice for 10 min. Centrifugation was performed again for 5 min at 5000 rpm, 4 ^{o}C and the supernatant was decanted. The cells were washed again as mentioned above. The cells were incubated on ice for 30 min and last centrifugation was performed in the same conditions above. The pellets were resuspended in 2 mL $CaCl_2$ solution which is held at 4 ^{o}C for being cold. The competent cells can be stored at 4 ^{o}C for 1 week.

2.4.2. Heat-shock transformation of *E. coli*

For transformation of ligation products into competent *E. coli* TOP10, heat shock transformation method was performed. 5-10 µL ligation product was mixed with 100 µL competent cells and incubation was completed on ice for 10 min. Next, the cells were transferred into water bath at 42 ^{o}C for 45 sec and they were immediately transferred on ice again. The cells were incubated on ice for 5 min. Then, 500 µL sterile LB medium was added to the cells and incubated at 37 ^{o}C, 200 rpm for 1 hour. After incubation, the cells were put on LB agar plates (5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 1.6 mL NaOH (0.5 M), 1.5 % w/v agar in 0.5 L ddH_2O, sterilized by autoclave) with appropriate antibiotics. The plates were incubated at 37 ^{o}C for overnight. Next day, 4-5 colonies were carefully chosen for colony PCR and grown in LB medium with appropriate antibiotics. Next day, colony PCR was performed from the grown cultures and plasmid isolation was performed from one of the positive colonies.

2.4.3. Plasmid isolation

For plasmid isolation, QIAprep® Spin Miniprep Kit (Lot# 142349895) was used and its protocol was applied. Overnight grown 4 mL cell cultures was centrifuged in 2 mL eppendorf tubes at 5000 rpm for 2 minutes. The supernatant was decanted and the pelleted cells were resuspended with 250 μL Buffer P1 with vortex. Then, 250 μL Buffer P2 was added and the tubes were mixed by inverting them for 10 times until the solution appear as viscous and in blue color. Buffer P3 was added lastly and again the tubes were inverted for 10 times until its blue color disappears. Next, centrifugation was performed at 13000 rpm for 10 min and the supernatant was collected in a QIAprep spin column. The spin columns were centrifuged at 13000 rpm for 1 min and the flow through collected in collection tubes was discarded. 750 μL PE Buffer was added to spin columns and waited for 2-3 minutes. Next, the tubes were centrifuged at 13000 rpm for 1 min and the flow through was discarded. Again, centrifugation was performed to remove remaining PE buffer. Spin columns were removed from collection tubes and placed in 1.5 mL eppendorf tubes. For elution, 30 μL ddH_2O was added to the tubes, waited for 2-3 min and centrifugation was performed at 13000 rpm for 2-3 min. Lastly, the spin columns were removed from tubes and the eluted plasmids were stocked at -20 ^oC.

2.5. Agrobacterium mediated gene transfer

2.5.1. Electro-competent agrobacterium GV3101 preparation

First, streak plate was performed from -80 stock of agrobacterium GV3101 on LB agar containing 2.5 µg/mL Tetracycline (Tet) and incubated at 28 ^{o}C overnight. Next day, a single colony was picked and grown in LB medium containing 2.5 µg/mL Tet^{+} at 28 ^{o}C with shaking at 250 rpm overnight. From the overnight culture, 1 mL was taken and inoculated in 100 mL LB medium containing 2.5 µg/mL Tet^{+}, again at 28 ^{o}C, 250 rpm overnight. The following morning, the optical density range ($A_{600\ nm}$) should be between 0.5-0.7. After having the desired optical density, the cell culture was divided into two 50 mL falcon tubes and rested on ice for 30 min. Next, the tubes were centrifuged at 3500 rpm, 4 ^{o}C for 15 min. The supernatant was discarded and the cells were resuspended by filter-sterilized 50 mL 10 % glycerol which is held at 4 ^{o}C for being ice cold. The cells were centrifuged again in the same conditions and the supernatant discarded. Pelleted cells were resuspended in filter-sterilized 50 mL 10 % glycerol which is ice cold. The last centrifugation was performed in the same conditions and the pelleted cells were resuspended in 200 µL GYT medium (10 % glycerol, 0.125 % yeast extract, and 0.25 % tryptone). The electro-competent cells were separated in 1.5 mL tubes containing 50 µL cell and stored at -80 ^{o}C.

2.5.2. Agrobacterium transformation by electroporation

For electroporation of plasmids into agrobacterium GV3101, electro-competent cell was taken from -80 ^{o}C stock and rested on ice for 5 min. 1-2 µL plasmid (~ 150 ng) was mixed with 50 µL electro-competent cell and waited on ice for 10 min. Next, the sample was transferred into electro-cuvette with 1mm gap and electro-shock was applied with 2.2 kV in electroporator (Celljject duo, Therma Corporation). Next, 1ml LB medium was added to the cuvette and transferred into 1.5 mL tubes. The cells were incubated at 28 ^{o}C for 1 hour with 200 rpm shaking. Then, the cells were put on agar plates with appropriate antibiotics and incubated at 28 ^{o}C for 2 days. After 2 days, 4-

5 colonies were picked and checked by colony PCR. Positive clones were stocked at -80 ^{o}C.

2.6. Agroinfiltration into *N. benthamiana* leaves

Agrobacterium cells with desired plasmid was taken form -80 ^{o}C stock and grown on agar plates by streak with 10 µg/mL Rifampicin (Rif), 25 µg/mL Gentamicin (Gen) for agrobacterium GV3101 and 100 µg/mL Spectinomycin for destination vectors (pK7FWG2 and pH7WGR2) for 2 days. After that, the grown agrobacterium colonies were scratched out by 1 mL sterile pipette tip and resuspended in 1 mL ddH_2O. Centrifugation was performed at 5000 rpm for 5 min. The supernatant was discarded and the cells were washed with 1 mL ddH_2O again. Centrifugation was performed 5000 rpm for 5 min. The cells were washed with 1mL agroinfiltration buffer (10mM MES, 10mM $MgCl_2$, pH 5.7) and centrifugation was performed again in the same conditions. Lastly, the cells were re-suspended in 1 mL agroinfiltration buffer and optical density ($A_{600\ nm}$) of the cells were measured. The cell concentration set to 0.2 and infiltrated into *N. benthamiana* leaves with 2 ml syringe without the needle. The infiltrated leaves were collected 2 days post infiltration (dpi) for imaging the expression of the proteins by microscope and protein isolation.

2.7. Confocal microscopy

Small leaf pieces near infiltration spots were cut and placed on distilled water. Leica 385 TCS SP5 confocal microscope (Leica Microsystems, Germany), Leica DMI 4000 equipped with andor DSD2 spinning disk confocal and Leica DM4000B microscope/ DFC 280 was used for imaging of the expression of proteins by their GFP or RFP fluorescence.

2.8. Protoplast isolation and PEG mediated transformation

Wheat strain Avocet S and tobacco plant *N. benthamiana* were grown under 16 h light / 8 h dark at 20-23°C for 10 days and 4 weeks, respectively. Healthy grown wheat and tobacco leaves were cut into small pieces (1.5-2 mm strips) using a sharp sterile blade and put into enzyme solution (1.5 % Cellulase R10, 0.5% Macerozyme R10 (Yakult Honsha, Tokyo, Japan) in 0.4 M Mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM CaCl2, 0.1% BSA). Tissues were incubated at 25 °C, 50 rpm for 4 hours at dark for digestion. After fully digestion of the leaves, the equal amount of W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES (pH 5.7)) was added into enzyme solution and the solution mixture filtered through nylon mesh (0.45 µm). Centrifugation was achieved at 100 g for 1-2 min and the supernatant was discarded. The protoplasts were counted under microscope with hemocytometer and they were resuspended in $2x10^5$/mL in W5 solution. Then, protoplasts were kept on ice for 30 min. After 30 min, the protoplast settle down and the supernatant was discarded carefully as much as possible. The cells were dissolved in MMg solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES (pH 5.7) at $2x10^5$/mL. For PEG mediated transformation, 10 µL (10-20µg) DNA was mixed with 100 µL protoplasts ($2x10^4$/ml). 110 µl 40% PEG solution (0.6 M mannitol, 100 mM CaC12, 40 % v/v PEG 4000 (Fluka)) was added into the solution and incubated at 25 °C, dark for 15 min. Cells were washed with 400 µL W5 solution and centrifugation was accomplished at 100 g for 2 min. They were resuspended at 2 mL W5 solution and incubated in 6 well plates at room temperature with no light and dark condition for approximately 2 days. Then, they were visualized under light microscope.

2.9. Protein extraction and immunoprecipitation

2.9.1. Total protein isolation

For total protein isolation, 3 dpi infiltrated *N. benthamiana* leaves were collected and put into liquid nitrogen immediately. They were grinded with mortar and pestle until

they become powder. Then, 1g of leaf powder was taken and mixed with 2 ml of ice-cold extraction buffer ((GTEN (10 % Glycerol; 25 mM Tris pH 7.5; 1 mM EDTA; and 150 mM NaCl), 2 % (w/v) PVPP, 10 mM β-mercaptoethanol, 1X Protease inhibitor (Thermo, #88666) and 0.1 % Tween-20) in 15 mL falcon tube. The solution was vortexed until it becomes homogeneous and centrifuged at 3000 g, 4 ºC for 10 min. The supernatant was transferred into new 2 mL tube and centrifuged at maximum speed (20,000 g) for 10 min to get rid of remaining solid green parts. Again, the supernatant was transferred into new 2 mL tube and centrifugation was performed until it becomes cleared from solid parts. The protein extract can be stored in -80 °C.

2.9.2. Immunoprecipitation

Immunoprecipitation was carried out using GFP_Trap_A beads (Chromotek, Munich, Germany). For one sample, 25 µL GFP trap beads was used. 1000 mL total extract was mixed with 25 µL GFP trap beads and 475 µL immunoprecipitation (IP) buffer (GTEN, 0.1% Tween). The total proteins and beads were mixed well by shaking with rotator for 1 hour at 4 °C. The mixture was centrifuged at 800 g for 30 sec and the supernatant was decanted carefully with 1.5 mL syringe with needle. The sample was resuspended in 1 mL IP buffer and rotated for 1 hour. This washing step was repeated 4 more times. At the last washing step, all the liquids were removed and the beads were stocked at -20 °C.

2.10. SDS-PAGE preparation and separation of proteins

2.10.1. SDS-PAGE preparation

SDS-PAGE separating and stacking gel was prepared according to Table 2.6 and 2.7. First, separating gel was prepared. All the reagents were mixed except ammonium persulfate (APS) and NNNN-Tetramethylehylenediamine (TEMED) because they added lastly to prevent the solidification of the gel before pouring. The solution was

poured between glass plates on a casting stand and waited for 30 min for drying. 400 µL isopropanol was added after pouring the separating gel to remove the balloons. After drying, isopropanol was removed and stacking gel was prepared in the same way as separating gel. The stacking gel solution was poured on separating gel between glass plates and the comb was placed. Again, 500 µL isopropanol was added. After 1 hour, the gel was solidified and ready for SDS-PAGE electrophoresis.

Table 2.8. *Separating gel contents and their amounts.*

Reagents	**Amounts**
Tris HCl 1.5 M pH 8.8	1.5 mL
Acrylamide (40 %)	1.8 mL
SDS (10%)	60 µL
ddH_2O	2.60 mL
APS (10%)	30 µL
TEMED	3 µL
Total volume	6 mL

Table 2.11. *Stacking gel contents and their amounts.*

Reagents	**Amounts**
Tris HCl 0.5 M pH 6.8	1.25 mL
Acrylamide (40 %)	0.5 mL
SDS (10%)	50 µL
ddH_2O	3.15 mL
APS (10%)	50 µL
TEMED	5 µL
Total volume	5 mL

2.10.2. SDS-PAGE

For SDS-PAGE, Thermo Owl P8DS electrophoresis system was used. The SDS gel which was prepared in glass plates was removed from casting stand and placed in electrophoresis tank. The inner and outer chamber of electrophoresis apparatus was filled with transfer buffer. The protein mixtures were mixed with Lane Marker Sample Buffer (Thermo, #39000) (1X) and DTT (100mM) and eluted from the beads by boiling 10 minutes at 95°C. After loading the same amount of proteins in the wells with molecular weight marker, the gel was run at 100 V (20 min) until the proteins were attained to separating gel and then at 200 V (1 h) until the dye in the sample buffer achieved to the bottom of the gel.

2.10.3. Western blot

The gel assembly was removed from the electrophoresis tank and washed with dH_2O and put into Transfer Buffer (Thermo, #84731) for equilibration. PVDF membrane (Thermo, #88520, 0.2 μm pore size) was activated with methanol for 1 min and then soaked in Transfer Buffer for 10 min for equilibration. Western Blotting Filter Paper (Thermo, #84783) was dipped in Transfer Buffer, too. The transfer sandwich was prepared on transfer apparatus as filter paper on the bottom, then PVDF, gel and another filter paper, respectively. The blot was performed on PierceG2 Fast Blotter (Thermo) at constant current of 1.3 A for 10 min. After transfer, the PVDF membrane was blocked with blocking buffer ((3% BSA in TBS-T (25mM Tris-HCl, pH 7.4, 0.137 M NaCl, 2.7 mM KCl, 0.1% Tween 20)) for 1 h. Then, the membrane was incubated in anti-GFP monoclonal mouse antibody (Thermo MA5-15256) solution as primary antibody which was diluted 1/5000 in blocking solution for 1 h. Next, the membrane was washed with TBS-T for 5 times and incubated in goat anti-mouse IgG-Alkaline Phosphatase polyclonal antibody (Chemicon International, #AP308A) as secondary antibody with a 1:10000 final concentration for 1 h. After incubation, the membrane was washed with TBS-T again for 5 times. Lastly, 1-Step™ NBT/BCIP

Substrate Solution (Thermo #34042) was added to the blot and incubated (5-15 min) until the color of bands develops for visualization.

2.10.4. SYPRO Ruby staining

For LC-MS/MS analysis, the eluted proteins were separated on 12% SDS-PAGE gel. After separation, the gel was removed from the SDS-PAGE tank and placed into a container to fix the gel with the fixing solution containing 10% methanol, 7% acetic acid for 30 min. The fixation solution was removed from the gel after 30 min and 50 ml SYPRO Ruby gel stain was added to the container containing the gel. The gel was incubated with the Ruby stain for overnight with gentle shaking. The next day, the gel was detached from the stain and placed into washing solution containing 10% methanol, 7% acetic acid for 1 hour. Then, the gel was washed with ddH_2O and visualized under UV.

2.11. Suppression assays

In this assay, pK7FWG2/PstCTE1 and pK7FWG2/SP-PstCTE1 were used to test whether they suppress the cell death caused from Inf1 and PstSCR1 separately. Inf1 and PstSCR1 are known cell death inducers in *N. benthamiana* (Kamoun et al., 1999; Dagvadorj et al. 2017). pTRBO/GFP and pTRBO/SP-GFP were used as controls.

The samples pK7FWG2/PstCTE1 and pK7FWG2/SP-PstCTE1 in *Agrobacterium* GV3101 were plated on LB agar by streak with 10 µg/mL Rifampicin (Rif), 25 µg/mL Gentamicin (Gen) and 100 µg/mL Spectinomycin. The samples pTRBO/GFP, pTRBO/SP-GFP, pGR106-INF1 and pTRBO/Pst-SCR were plated on LB agar with 10 µg/mL Rifampicin (Rif), 25 µg/mL Gentamicin (Gen) and 50 µg/mL Kanamycin (Kan). The incubation of streak plates at 28 °C for 2 days was accomplished and after that, they were prepared for agro-infiltration by the procedure which was mentioned

in Section 2.6. The agrobacteria suspension obtaining the effectors pK7FWG2/PstCTE1, pK7FWG2/SP-PstCTE1 and the controls pTRBO/GFP, pTRBO/SP-GFP were set to final O.D value of 0.4 and they were infiltrated to *N. benthamiana* leaves with a syringe without a needle. After 2 days, the infiltrated areas of *N. benthamiana* were challenged with Inf1 and PstSCR1 to measure the cell death rate. pGR106-INF1 and pTRBO/Pst-SCR were prepared by the same procedure and set to final O.D values of 0.4 and 0.3, respectively. The agro suspensions containing Inf1 and PstSCR1 were infiltrated into the same areas which was infiltrated with effectors before. The cell death results were imaged under UV light after 4 days post inoculation.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Selection of PstCTE1 from Pst candidate effectors and in-silico characterization

PstCTE1 (PstHa12j12) was chosen from the candidate effector EST sequences of haustorial cDNA library reported previously by Yin et al., 2009, since this effector candidate highly upregulated upon infection with comparison to urediniospores and germinated urediniospores (Yin et al. 2009). Moreover, PstCTE1 have 6 cysteine residues and homology search displayed no similarity to any known sequence in the database according to Yin et al., 2009. However, four homologs of PstCTE1 were reported in *Pst*, recently (Xia et al. 2018). For these homologs, multiple alignment analysis and phylogenetic tree construction were performed (Figure 3.1A & 3.1B). Multiple alignment analysis showed conserved residues in their signal peptide region and also in the transit peptide region. However, PstCTE1 effector show no homology to known proteins in other organisms. Thus, to discover the similarity of the transit peptide of PstCTE1 with the remaining available Pucciniales (*Pst*, *Pgt*, *Ptt*), predicted chloroplast targeting effectors were chosen from these Pucciniales proteome sequences and they were combined as CTPome. Based on the multiple alignment analysis, the level of genetic similarity of PstCTE1 to this CTPome was shown by phylogenetic tree (Figure 3.1C). There are three branches in the phylogenetic tree and the largest branch is the green one which PstCTE1 belongs in. Thus, PstCTE1 effector protein is unique to *Pst* and there is no homology to other organisms.

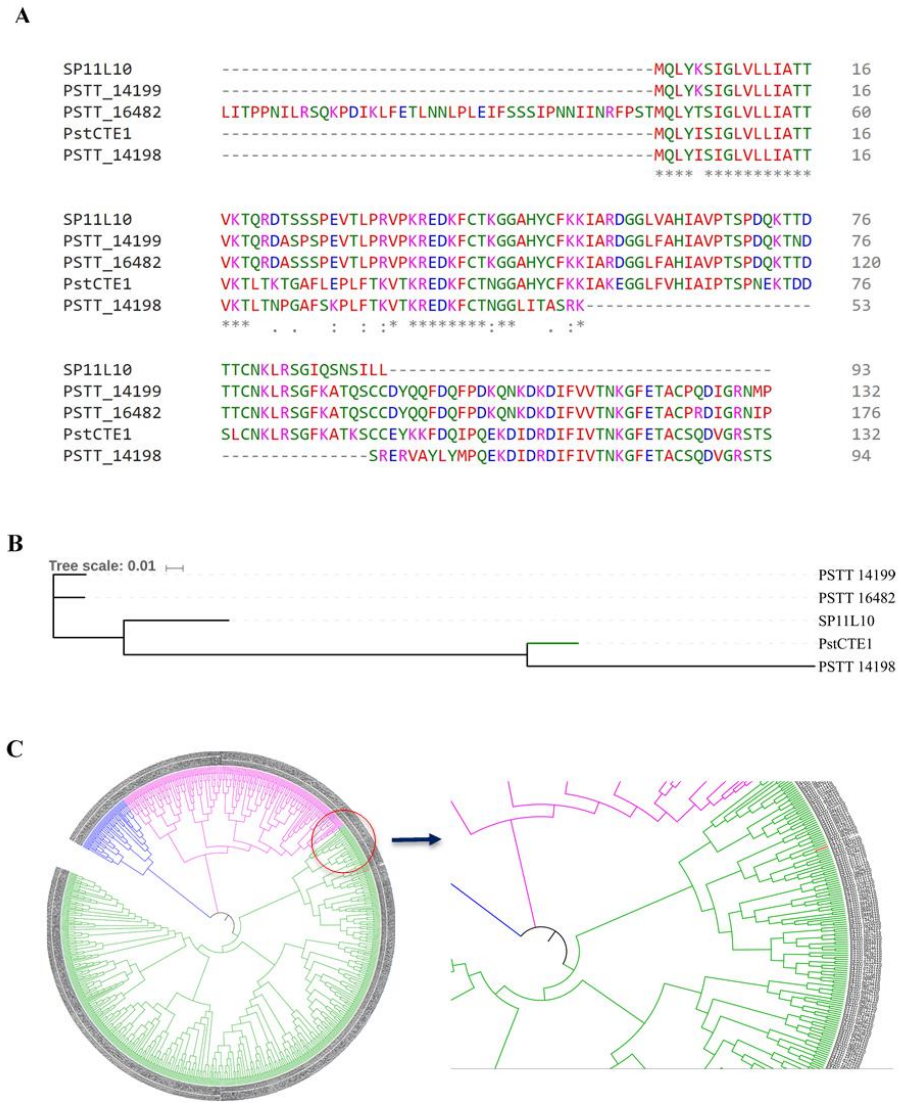


Figure 3.1. Sequence alignment of PstCTE1 homologs and phylogenetic tree of available Pucciniales proteome sequences. **A**) Multiple alignment analysis of PstCTE1 with four homologs. Fully conserved residues are indicated with asterisks. Colons show conservation between groups with strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix). Periods indicate conservation between groups with weakly similar properties (scoring =< 0.5 in the Gonnet PAM 250 matrix). **B)** Phylogenetic tree of PstCTE1 with these four homologs. **C)** Phylogenetic tree of PstCTE1 with predicted *Pucciniales* proteome sequences.

3.2. Subcellular localization of PstCTE1

The effectors are secreted from pathogens and can localize diverse subcellular compartments in the plant cell. To discover the subcellular localization, PstCTE1 effector with no signal peptide was expressed with C-terminal GFP tag in *N. benthamiana*. To do this, first the effector gene was amplified with CACC primers which are Fwd-CACC-CTE1 and CTE1-Rev-NoStp and the amplified PCR product was cloned into pENTR/D-TOPO entry vector (Section 2.3). Then, the gene of interest was transferred into pK7FWG2 destination vector by recombinational LR cloning which adds the effector C-terminal GFP (Section 2.3.2). After transforming this construct into agrobacterium GV3101, the effector was expressed in *N. benthamiana* by agro-infiltration. 2 days post infiltration, the leaves were cut and examined under confocal microscope.

According to confocal analysis, pK7FWG2-PstCTE1 shows chloroplast localization because GFP signals from pK7FWG2-PstCTE1 was overlayed with chloroplast auto-fluorescence (Figure 3.2).

In order to determine subcellular localization on the host plant wheat, protoplast isolation was performed and transfected with PstCTE1 effector. Since, transient expression or genetic transformations are not achievable in wheat, protoplast isolation is an effective method to determine subcellular localization in the host plant of *Pst*. After protoplast isolation, protoplasts were transfected with pK7FWG2-PstCTE1; and pTRBO-GFP (GFP alone) was used as control. After 2 days transfection, they were visualized under light microscope with GFP filter. For comparison, protoplast isolation was performed also from tobacco and transfected with pK7FWG2-PstCTE1 and pTRBO-GFP. From the microscope images (Figure 3.3A&3.3B), chloroplast localization was observed in the pK7FWG2-PstCTE1 transfected protoplasts of wheat and tobacco; however, GFP alone transfected protoplasts showed fluorescence in nucleus and throughout the cell. This result show that, this effector targets to wheat chloroplast.

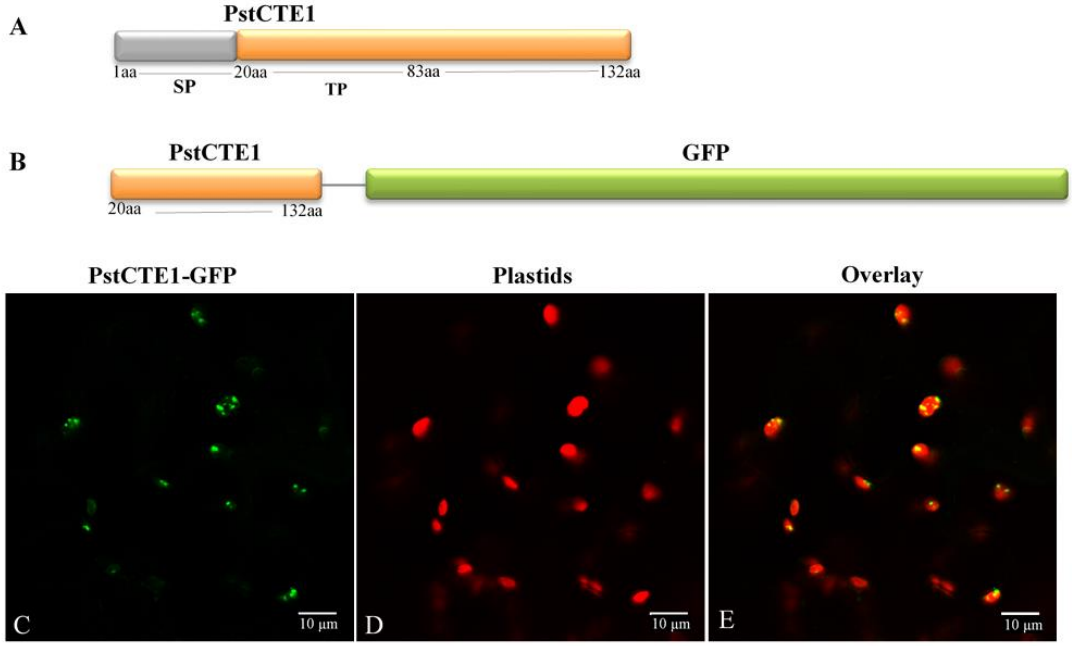


Figure 3.2. Accumulation of PstCTE1 in N. benthamiana chloroplasts. **A**) Schematic view of PstCTE1 gene, which shows the location of signal peptide and possible transit peptide. **B**) Schematic view of the construct used in this experiment. **C**) PstCTE1 effector protein with C-terminal GFP fusion expressed in *N. benthamiana*. **D)** Auto-fluorescence of chloroplasts. **E)** Overlay image of PstCTE1-GFP and chloroplasts. Images were taken at 2 days after agro-infiltration. The GFP was excited using 488 and 561 nm laser diodes, and fluorescent emissions were collected at 495-550 nm. Chloroplast auto-fluorescence was visualized by far infrared (>800 nm) excitation and emission (Leica SP40).

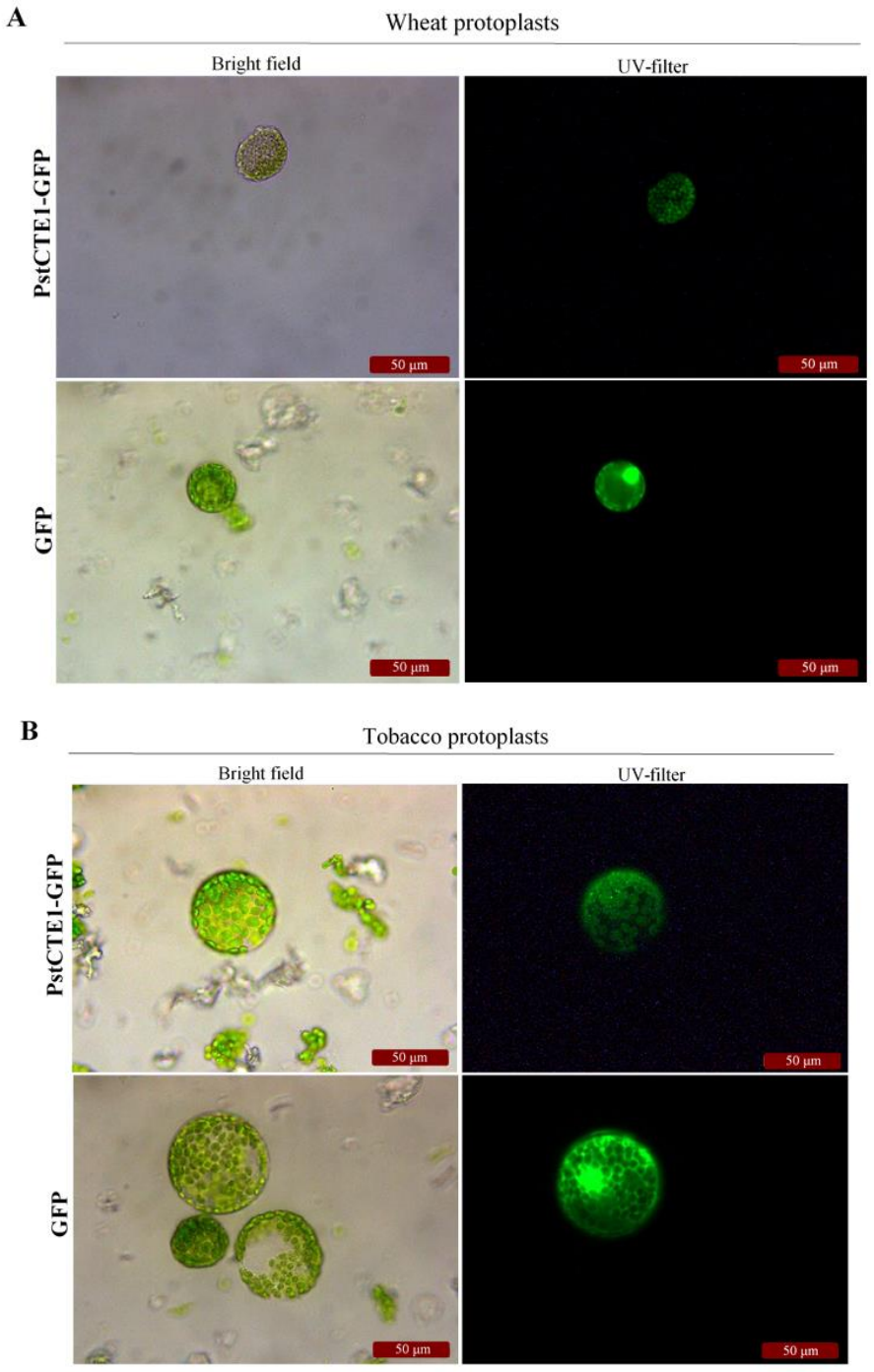


Figure 3.3. Subcellular localization of PstCTE1 in wheat and tobacco protoplasts. Transfected **A)** wheat protoplasts and **B**) tobacco protoplast with PstCTE1-GFP and control GFP constructs. Visualization was conducted under a light microscope (Leica DM4000B microscope/DFC 280 camera (40X magnification).

3.3. N-terminus of PstCTE1 is responsible for chloroplast localization

Chloroplast targeting effectors have transit peptide sequence in their N-terminus region after signal peptide sequence which directs the localization of effectors. After chloroplast targeting of PstCTE1 was observed, its putative transit peptide sequence was searched by bioinformatics tools predicting transit peptide amino acid sequences such as TargetP 1.1, ChloroP 1.1 and WolfP Sort. However, none of them predicted a sequence indicating chloroplast localization which suggests that this effector has a unique and unknown transit peptide region. Thus, it was decided to find the transit peptide region experimentally by splitting the gene.

Since intactness of domains are important for the function of proteins, first, domain analysis of PstCTE1 was performed with PSIpred program for predicting secondary structure (Buchan et al. 2013; Jones 1999). So that, if there is a domain in the sequence which is chosen to split, it can be detected by this program. PSIpred program was preferred to use because in a recent study, Zhang *et al*., 2017, it was shown that the prediction of this program highly matched with the circular dichroism (CD) data of an effector studied (Zhang et al. 2017). Moreover, transit peptides are cleaved by processing peptidases after chloroplast entry (Richter and Lamppa 1999). Therefore, it was also checked if the effector has any peptidase cleavage sites by using Prosper program to determine possible cleavage sites (Song et al. 2012). Between the predicted cleavage sites, one of the four cysteine protease cleave sites (in between amino acid 83 and 84) was found on the predicted helix domain which contains the amino acids between 76-85. In order to keep the intactness of the domain, it was decided to split the gene into two, with one part containing 20^{th} to 83^{nd} amino acids which indicating the N-terminus of the effector (no signal peptide), and other part had the rest of the gene starting from 84^{rd} amino acid and onward, indicating the C-terminal part of the gene (Figure 3.4).

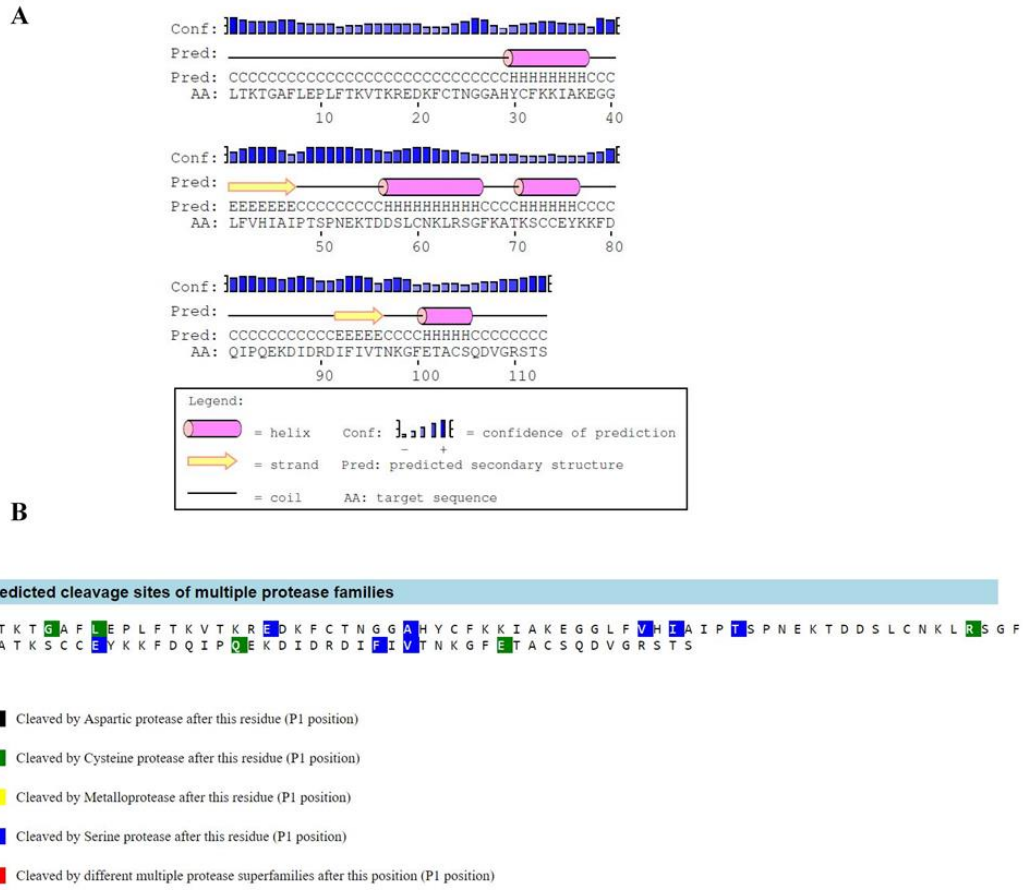


Figure 3.4. PstCTE1 secondary structure analysis and predicted cleavage sites. Prediction of secondary structure and protease cleavage sites of PstCTE1 was performed by the PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) and PROSPER programs (https://prosper.erc.monash.edu.au/home.html), respectively.

The parts of the gene were separated by PCR; the N-terminus part was amplified by Fwd-CACC-CTE1 and KLR-Rev (named as PstCTE1-KLR$_{20-83}$), the C-terminus part was amplified by Fwd-CACC-SGF and CTE1-Rev-NoStp (named as PstCTE1-SGF$_{84-132}$). After amplification with CACC primers, these products were cloned into pENTR/D-TOPO entry vector (Section 2.3) separately, and then they were transferred into pK7FWG2 destination vector which adds the effector C-terminus GFP. After transforming into *Agrobacterium* (Section 2.5), they were expressed in *N.*

benthamiana. Based on confocal microscopy results, it was observed that N-terminus part of the protein, PstCTE1-$KLR_{20\text{-}83,}$ was target to chloroplast again; because the GFP signals from PstCTE1-$KLR_{20\text{-}83}$ was merged with RFP signals from chloroplast auto-fluorescence (Figure 3.5A). However, the C-terminus part, PstCTE1-$SGF_{84\text{-}132}$ shows cytoplasm and nucleus localization which is expected for GFP alone localization (Yan et al. 2015) and can no longer target chloroplast (Figure 3.5B). Despite the prediction programs could not find the transit peptide of PstCTE1, this result shows that it has a functional N-terminal signal which has the information to target chloroplast. It can be concluded that PstCTE1 has an unknown and unique transit peptide signal.

The reasons of failure of prediction programs can be that transit peptides differ in their length and composition; they have no consensus amino acid sequences but possess specific physicochemical elements, which may be environmentally sensitive or context specific and, thus, may act differently as a result of diverse pH conditions, in a membrane-like environment, or upon receptor binding, that are required for recognition by a common import mechanism.

All described chloroplast inner and intermembrane space proteins have N-terminal cleavable transit peptide; however, most chloroplast outer membrane proteins contain the chloroplast targeting information in the mature protein itself, rather than N-terminal part of the protein. In this outer membrane proteins, neither N-terminal nor C-terminal part of the protein is important for chloroplast targeting but the mature protein is significant and if this mature protein is cleaved, neither of its parts can target chloroplast anymore (Miras et al. 2002). Since PstCTE1 was cleaved for determining its transit peptide and N-terminus have the targeting information rather than the mature protein, it can be suggested that PstCTE1 is an inner or intermembrane protein of chloroplast.

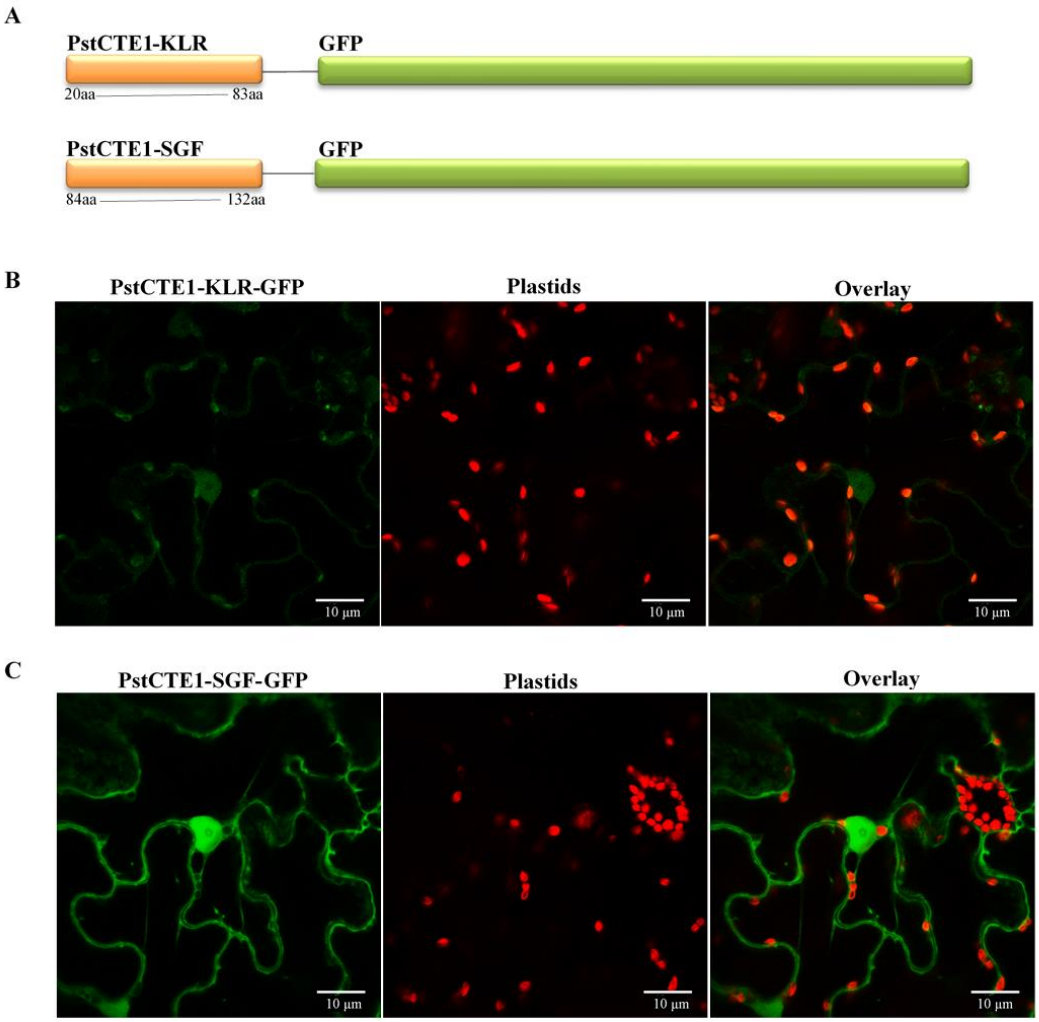


Figure 3.5. Subcellular localizations of truncated PstCTE1. **A)** Schematic views of PstCTE1-KLR20-83 and PstCTE1-SGF84-132 constructs. **B)** Image of PstCTE1-KLR20-83 fused with GFP (N-terminal part of the protein, which possesses a translocation signal targeting the chloroplast). **C)** Image of the PstCTE1-SGF84-132 truncation fused with GFP (C-terminal part of the effector protein) localized to the cell membrane and nucleus. Visualization was conducted at 2 days after infiltration of *N. benthamiana* leaves. The GFP probe was excited using 488 and 561 nm laser diodes, and fluorescent emissions were collected at 495-550 nm. Chloroplast auto-fluorescence was visualized by far-infrared (>800 nm) excitation and emission (Leica SP40).

3.4. N-terminus tagging of PstCTE1

In the literature, it was shown that when N-terminus chloroplast targeting protein tagged with a reporter gene, the protein can no longer target chloroplast because the reporter gene disrupt transit peptide functionality (Carrie et al. 2009). Thus, this protein shows nucleus and cytosol localization instead of chloroplast. To test the functionality of the transit peptide with N-terminus tagging, PstCTE1 was amplified with Fwd-CACC-CTE1 and CTE1-Rev-Stop, then cloned into pENTR/D-TOPO entry vector (Section 2.3). Next, the gene of interest was transferred into pH7WGR2 destination vector which adds the effector N-terminus RFP and transformed into *Agrobacterium* (Section 2.5). RFP-PstCTE1 was co-infiltrated with PstCTE1-GFP in *N. benthamiana* in order to co-express these two constructs in the same cell and check the colocalization in chloroplasts. Interestingly, these two constructs co-localized to chloroplasts which is a different result other than the literature reports (Petre, Lorrain, et al. 2016). In the Figure 3.6, it is observed that GFP signals from pK7FWG2/ PstCTE1-GFP and RFP signals from pH7WGR2/RFP-PstCTE1 was merged in the chloroplasts of the cell. This result show that N-terminus tagging have no effect on the transit peptide function and it can be suggested that this signal has a robust activity.

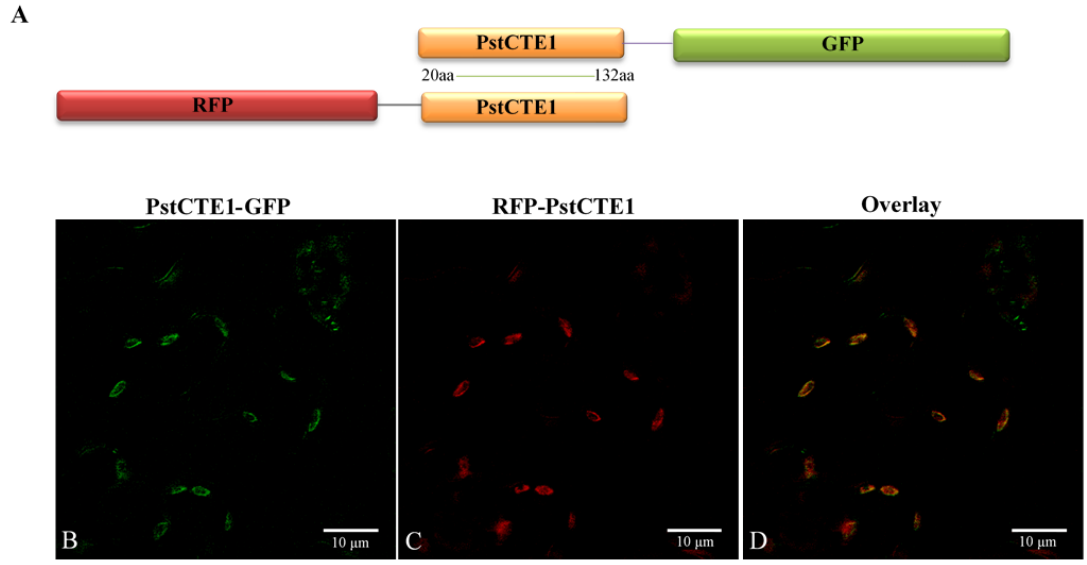


Figure 3.6. Co-expression of PstCTE1 with fused reporters in N. benthamiana. **A)** Schematic view of PstCTE1-GFP (C-terminal fusion) and RFP-PstCTE1 (N-terminal fusion). **B)** Image of PstCTE1-GFP targeting chloroplasts. **C)** Image of RFP-PstCTE1 targeting chloroplasts. **D)** Overlay images of B and C. Images were visualized at 2 days after infiltration using a Leica DMI 4000 equipped with an Andor DSD2 spinning disc confocal.

3.5. SP-PstCTE1 may re-enter to chloroplast

Signal peptide is a short peptide found at the N-terminus of the newly synthesized protein which directs the protein to its destination in the cell. After entry, the signal peptide is cleaved from the protein by signal peptidases. In this study, PstCTE1 was expressed with its signal peptide to analyze if it re-enters into chloroplast. This assay is called cell re-entry assay and it is widely used to assess pathogen entry into host cell. In this assay, the effector protein with their signal peptide intact are expressed in the plant cell and the protein is secreted to apoplast where they re-enter their destinations in the cell (Petre, Kopischke, et al. 2016). Normally, if a protein is expressed with signal peptide intact, it carries the protein into apoplast. Though, a current model suggested that the effectors of filamentous plant pathogens have N-terminal entry domains or translocation signals which are essential and sufficient to enter plant cells (Dou et al. 2008; Rafiqi et al. 2010; Whisson et al. 2007). Thus, cell re-entry assay is used commonly in effector biology to test re-entry for identifying any N-terminal entry domain in effector proteins. However, there are some arguments on this assay about its validity since the effector entry mechanism is still unsolved question in plant pathology (Petre and Kamoun 2014). It is a robust and specific assay but it is uncertain that effector protein re-enters its destination from the apoplast; because it may be escaped from the secretory pathway through retrograde transport or it may undergo translation at alternative sites which resulted in a protein lacking its signal peptide (Bos et al. 2006; Petre, Kopischke, et al. 2016).

In this study, signal peptide was incorporated to PstCTE1 by three PCRs which were performed one after another with Fwd-CTE1-SP-1, Fwd-CTE1-SP-2, Fwd-CTE1-SP-3, and CTE1-Rev-NoStp primers with the PstCTE1 DNA. First, Fwd-CTE1-SP-1, Fwd-CTE1-SP-2 primers were incorporated, then this product was incorporated with Fwd-CTE1-SP-3 and signal peptide was produced. Finally, this signal peptide product was used together with PstCTE1 DNA as a template and amplified with the primers Fwd-CACC-SP-CTE1 and CTE1-Rev-NoStp to add CACC sequence at the N-terminus of the gene. SP-PstCTE1 was the final PCR product and it was cloned in

pENTR/D-TOPO entry vector (Section 2.3). Next, the gene was transferred into pK7FWG2 destination vector which adds the effector C-terminus GFP and transformed into *Agrobacterium* (Section 2.5). After expression in *N. benthamiana*, it was observed under confocal microscope that SP-PstCTE1 was target to apoplast as expected since signal peptide directs the proteins to apoplast but interestingly it targets chloroplasts, too. The GFP signals from SP-PstCTE1 was overlayed with chloroplast auto-fluorescence and also, SP-PstCTE1 shows apoplast localization (Figure 3.7). So, it can be hypothesized that this effector was secreted to apoplast before it re-enters to chloroplast; but the exact entry mechanism of SP-PstCTE1 cannot be explained because of the lacking information about this mechanism in effector biology.

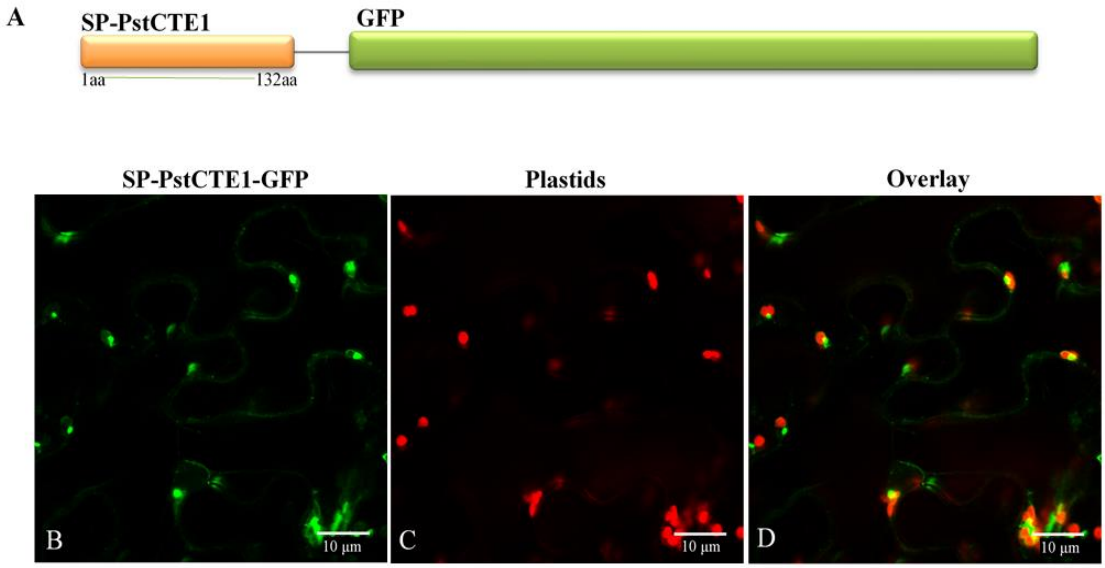


Figure 3.7. Subcellular localization of PstCTE1 with its signal peptide. **A)** SP-PstCTE1 fused to GFP targets the chloroplast. **B)** SP-PstCTE1 fused to GFP targets the chloroplast. **C)** Image of chloroplast autofluorescence. **D)** Overlay image of b and c. Images were obtained at 2 days after infiltration of N. benthamiana leaves. The GFP probe was excited using 488 and 561 nm laser diodes, and fluorescent emissions were collected at 495-550 nm. For chloroplast autofluorescence, far infrared (>800 nm) excitation and emission were used (Leica SP40).

Moreover, another chloroplast targeting effector, Pst651 was used for comparison which is found from our secretome data (unpublished data from our laboratory) with a predicted transit peptide region. From Figure 3.8A, it is observed that Pst651 targets chloroplasts since GFP signals from Pst651 merge with RFP signals from chloroplast auto-fluorescence. However, it is observed in Figure 3.8B, SP-Pst651 targets to apoplast and cytoplasm but no longer localized to chloroplasts. It is hypothesized that, Pst651 needs free N-terminal transit peptide for chloroplast targeting and its signal peptide blocks the transit peptide functionality.

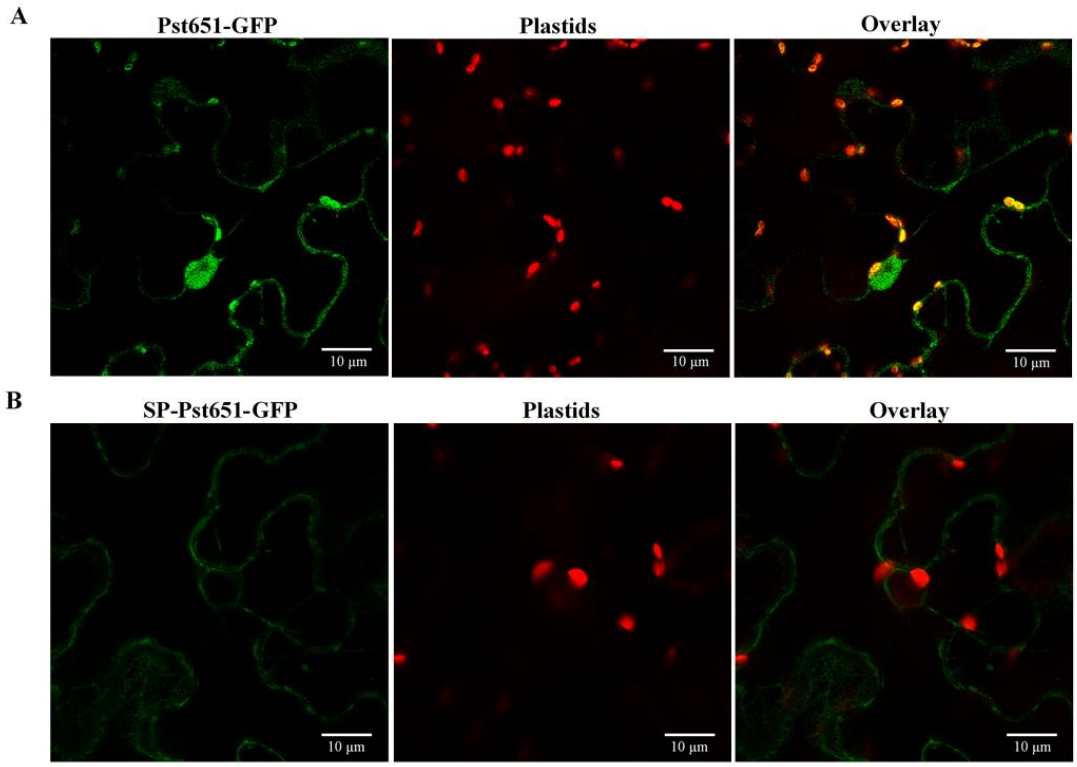


Figure 3.8. Subcellular localization of Pst651 and SP-Pst651. **A)** Image of Pst651 fused to C-terminal GFP targeting chloroplasts. **B)** Image of SP-Pst651 fused to C-terminal GFP targeting the cytoplasm. Images were obtained at 2 days after infiltration of *N. benthamiana* leaves. The GFP probe was excited using 488 and 561 nm laser diodes, and fluorescent emissions were collected at 495-550 nm. For chloroplast autofluorescence, far infrared (>800 nm) excitation and emission were used (Leica SP40).

For controlling whether signal peptide has an effect on chloroplast localization, signal peptide was constructed with the C-terminal part of the PstCTE1 which is named as PstCTE1-SGF$_{84\text{-}132}$. PstCTE1-SGF PCR product which was prepared in truncation of PstCTE1 (Section 3.3) was used as template and amplified with Fwd-CTE1-SP-1, Fwd-CTE1-SP-2, and Fwd-SP-SGF primers, and CTE1-Rev-NoStp. The same procedure which was composed of three PCR was applied as in the signal peptide incorporation to the PstCTE1. First, Fwd-CTE1-SP-1 and Fwd-CTE1-SP-2 primers were amplified, then this product was used as a template and amplified with Fwd-CTE1-SP-1 and Fwd-SP-SGF primers. Lastly, the final product was used with PstCTE1 DNA together as a template and amplified with Fwd-CACC-SP-CTE1 and CTE1-Rev-NoStp to add the CACC sequence at the N-terminus of the gene. Then, this SP-SGF product was cloned in in pENTR/D-TOPO entry vector (Section 2.3). After, the gene was transferred into pK7FWG2 destination vector which leads C-terminus GFP to the gene and *Agrobacterium* transformation was performed with this construct (Section 2.5). Two days after *N. benthamiana* expression, the results were examined under confocal microscope. According to Figure 3.9, pK7FWG2/ SP-CTE1-SGF was localized to nucleus and cytoplasm as same in the control pTRBO/GFP alone sample.

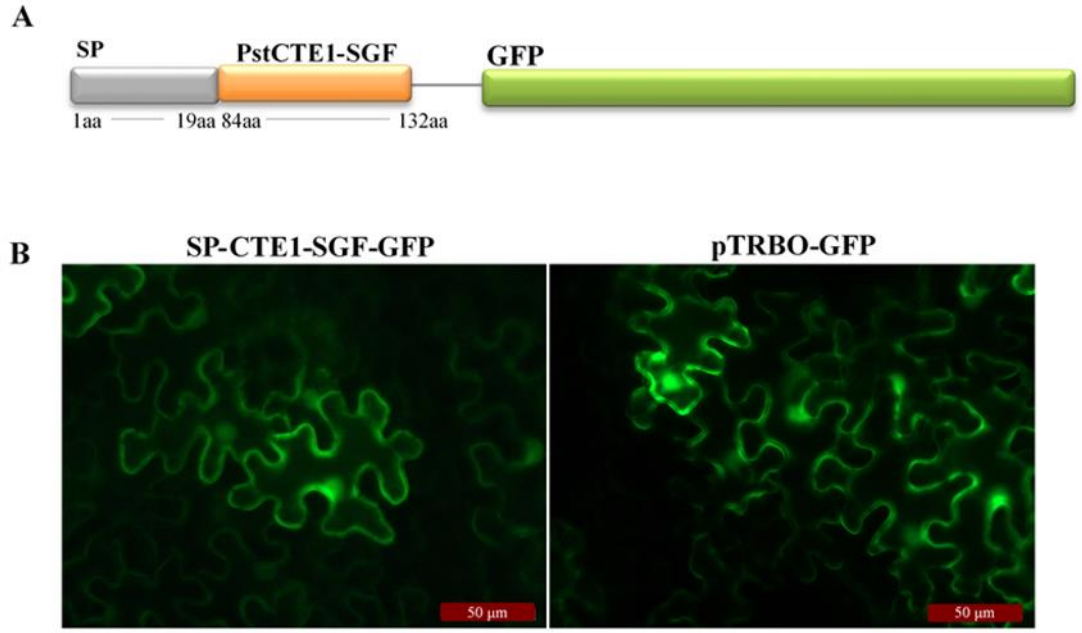


Figure 3.9. Subcellular localization of SP-CTE1-SGF. **A)** Schematic view of SP-PstCTE1-SGF. **B)** Imaging of SP-CTE1-SGF with C-terminal GFP fusion shows localization to the cytoplasm and nucleus, with pTRBO-GFP as a control. Images were obtained at 2 days after infiltration of N. benthamiana leaves. Visualization was conducted under a light microscope (Leica DM4000B microscope/DFC 280 camera, 40X magnification).

In the literature, cell re-entry assay was performed in oomycete effectors and the motifs required for cell entry were identified in oomycete effectors, so far. Many oomycete effectors share RXLR-deeR motif after their signal peptide which is essential for their cell entry. However, in fungal effectors such as *Pst*, the identification of motifs is not advanced like in oomycetes. Nevertheless, in some fungal effectors, RXLR-like motifs were reported but a cell entry motif for fungal proteins which is equivalent to oomycetes common RXLR motif, has not been discovered. Thus, it can be hypothesized that, PstCTPE1 may have an unidentified motif which leads its cell entry.

To sum up, there are three possible explanations for the cell re-entry assay of PstCTE1. The first one is, the effector may be secreted to apoplast first and then enter into chloroplasts. The other explanation is that the effector may escape from the secretory

pathway and turn out to be in host cytoplasm, so the effector has no longer to cross the plasma membrane or the translation may start at alternative site which results in truncated signal peptide. The last explanation is the effector may have unidentified motif which mediates its cell entry.

3.6. Processing of PstCTE1 and SP-PstCTE1

In chloroplast targeting effectors, its transit peptides are cleaved by processing peptidases after their entry into chloroplast (Teixeira and Glaser 2013). For determining the processing of PstCTE1 and SP-PstCTE1, protein isolation was performed from these constructs and they were compared at SDS-PAGE and western blot. pK7FWG2/ PstCTE1 and pK7FWG2/ SP-PstCTE1 transformed in *Agrobacterium* were expressed in *N. benthamiana* and the leaves were collected after 3 days post infiltration. The protein isolation procedure in Section 2.9 was followed and immunoprecipitation was performed with GFP_Trap_A beads, since the constructs have C-terminal GFP. The proteins were trapped in GFP beads at the end of immunoprecipitation and they were eluted from the beads by boiling at 95 °C for 10 min. So, the proteins were ready for separation in 12% SDS-PAGE. Western blot analysis was performed after separation in SDS-PAGE and the samples were detected by anti-GFP antibody to confirm the protein expression.

After western blot analysis, PstCTE1$_{20\text{-}132}$-GFP resulted in three bands which were suspected as PstCTE1$_{20\text{-}132}$-GFP (39.67 kDA); PstCTE1$_{84\text{-}132}$-GFP (32.47 kDA) and GFP (26.9 kDA) which displays the cellular processing event (Figure 3.10). The sample SP-PstCTE1$_{1\text{-}132}$-GFP resulted in four bands which were suspected as the same bands in PstCTE1$_{20\text{-}132}$-GFP and in addition to them, a band near 40 kDA was observed which may corresponds to partially unprocessed SP-PstCTE1$_{1\text{-}132}$-GFP (41.9 kDA) (Figure 3.9). This western blot result strengths the suggestion of SP-PstCTE1$_{1\text{-}132}$-GFP localization as presented in Figure 3.6 which shows that the effector was localized to both chloroplast and apoplast. These results together suggest that signal

peptide leads the effector to apoplast, then either it may re-enter into chloroplast or in the cytoplasm where some of the effector may translocated to the apoplast and some to the chloroplast. The PstCTE1 effector possible secretion route is explained in the drawing Figure 3.11.

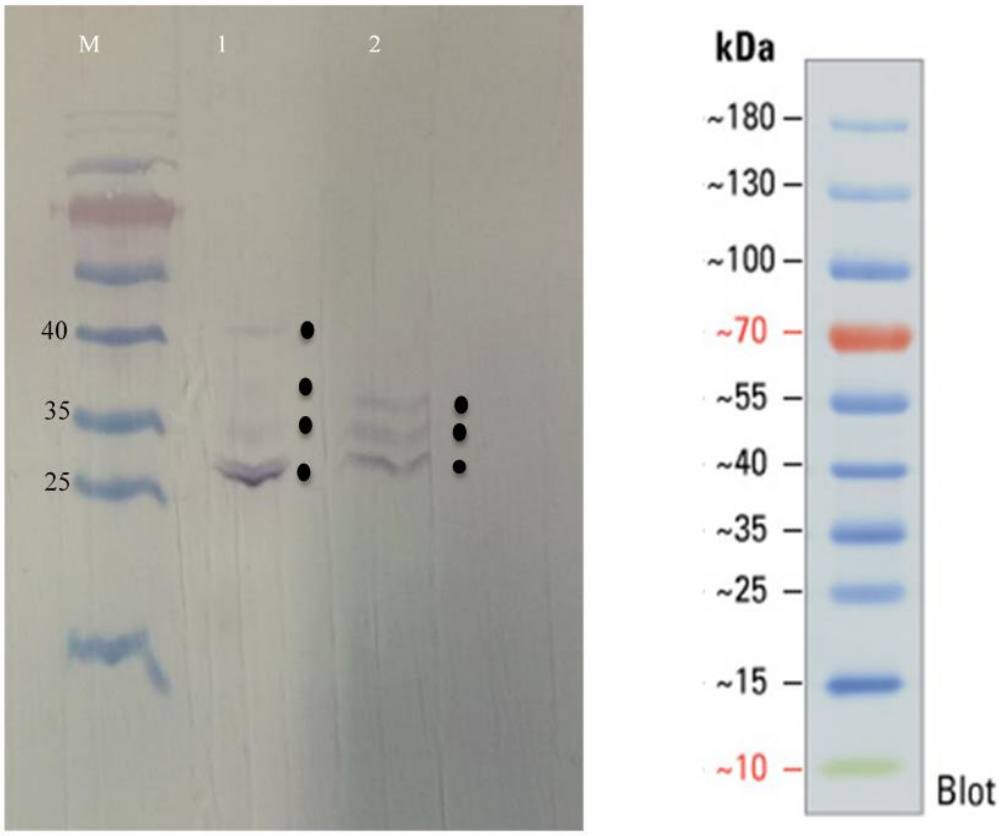


Figure 3.10. Immune detection of SP-PstCTE1-132-GFP and PstCTE120-132-GFP expressed in N. benthamiana. **1)** SP-PstCTE1$_{1\text{-}132}$ produced 4 bands, which were theoretically expected to be 41.9 kDa, 39.67 kDa, 32.47 kDa, and 26.9 kDa. **2)** PstCTE1$_{20\text{-}132}$-GFP produced 3 bands with the same sizes in SP-PstCTE1-GFP. Both samples were detected using an anti-GFP antibody to validate the protein expression. Pre-stained PageRuler (Thermo Scientific) was used as a protein marker, and 12% SDS-PAGE was used for separation. (TE: total extract, IP: immunoprecipitation)

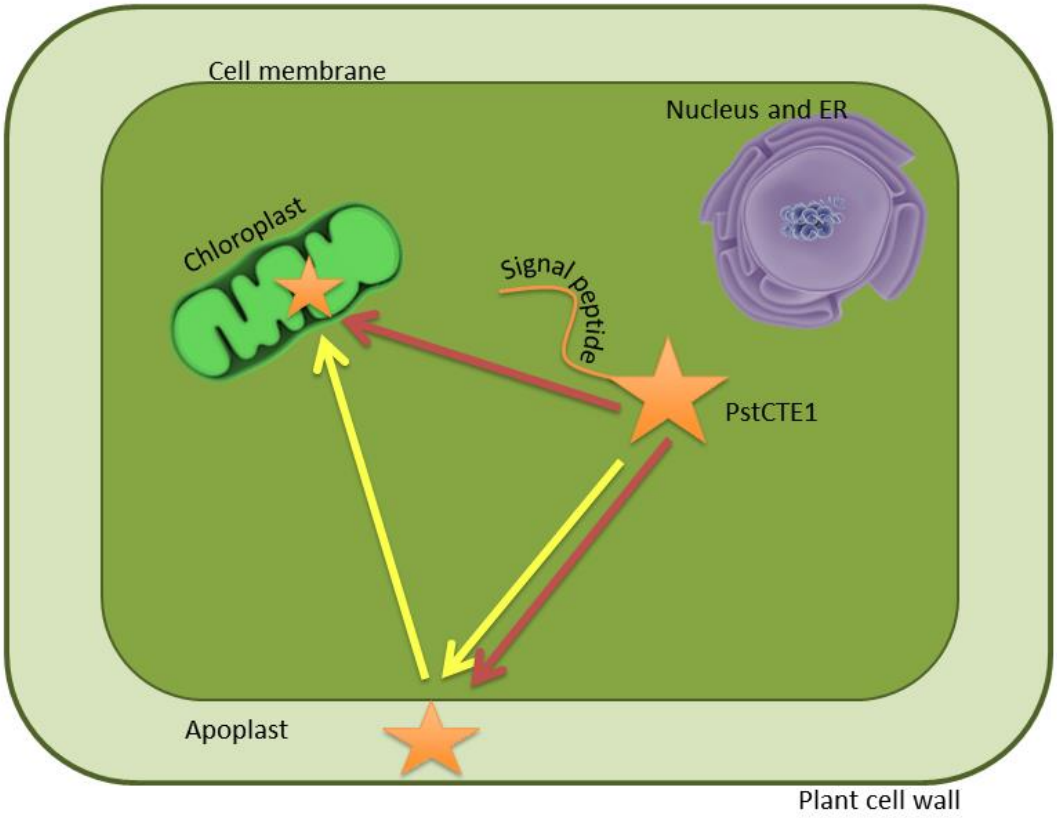


Figure 3.11. SP-PstCTE1 effector secretion route. The yellow path summarizes a possible secretion route: upon translation, PstCTE1 is secreted to the apoplast using the classical secretion pathway and translocates back into the host chloroplast. The red path shows that PstCTE1 is localized to both the apoplast and chloroplast; however, the direct chloroplast targeting of the effector may cause it to escape from the secretion pathway.

The whole eluted protein samples from PstCTE1$_{20\text{-}132}$-GFP and SP-PstCTE1$_{1\text{-}132}$-GFP were separated on 12% SDS-PAGE to send the bands LC-MS/MS analysis. The gel was visualized by staining SYPRO Ruby. Each sample were divided to four and loaded on four lanes in SDS-PAGE in order to easily separate and cut each band from the same lane (Figure 3.12). The bands labelled as 'i' and 'ii' from the SP-PstCTE1$_{1\text{-}132}$-GFP gel (Figure 3.12a) and the band labelled 'ii' from the PstCTE1$_{20\text{-}132}$-GFP gel (Figure 3.12b) were cut and subjected to LC-MS/MS analysis. When the results were compared, it was observed that the 'i' band from SP-PstCTE1 revealed sequences

from the transit peptide region; however, the ‘ii’ bands from SP-PstCTE1 and PstCTE1 contained sequences from the C-terminal region of the effector and showed no sequences from the N-terminal region (Appendix D). This difference possibly arises from the processing of the transit peptide. The reason that ‘i’ band shows sequences from transit peptide in SP-PstCTE1 sample may be the apoplast localization of this effector protein. Since the transit peptide is cleaved after entry of the protein, SP-PstCTE1 which localize to apoplast have intact transit peptide and thus, it can be observed in the LC-MS/MS analysis.

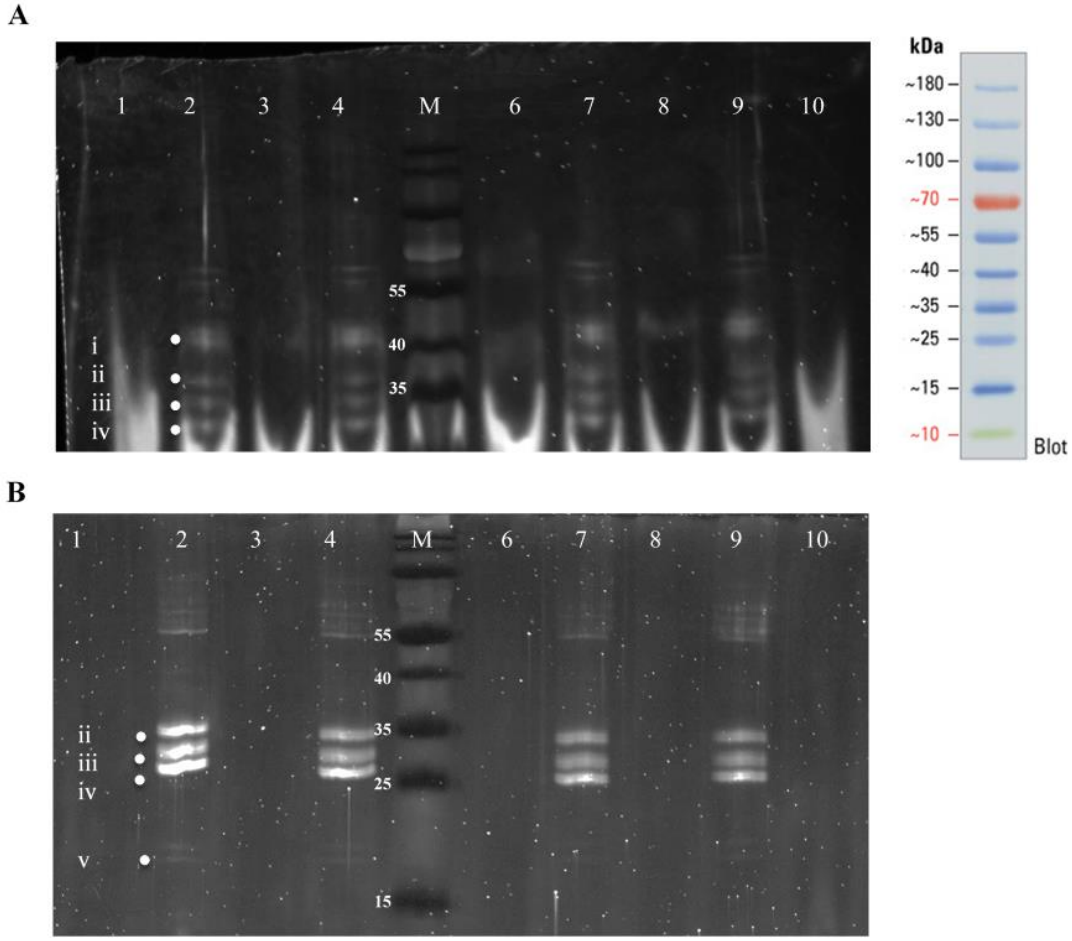


Figure 3.12. SDS-PAGE separation of (A) SP-PstCTE11-132-GFP and (B) PstCTE120-132-GFP expressed in N. benthamiana. The protein mixtures were obtained by anti-GFP antibody immunoprecipitation, separated by 12% SDS-PAGE, and visualized by staining with SYPRO Ruby. The protein bands (**i** and **ii)** in lanes 2, 4, 7, and 9 (A) and the **ii** bands from lanes 2, 4, 7, and 9 (B) were utilized for LC-MS/MS analysis. M: protein marker (Pierce #PI-26614). In both gels, lanes 1, 3, 6, 8, and 10 were kept empty.

3.7. Expression of PstCTE1 with Flag-tag

For expression of PstCTE1 in *N. benthamiana* with Flag-tag, PstCTE1 has already synthesized with N-terminus Flag-tag, also *Pac*I and *Not*I restriction enzyme sites. So that, PstCTE1 gene can be expressed in *N. benthamiana* for further protein isolation with Flag-tag. This gene construct was amplified with PstCTE1-PacI-Fwd and

PstCTE1-NotI-Rev and first cloned into pGEM-T-Easy vector for easily digested with restriction enzymes. Then, the gene of interest was double digested with *Pac*I and *Not*I enzymes. Also, pJL48-TRBO vector was double digested too, with the same enzymes and in the same conditions. This vector was used because it is agro-compatible and high expression vector. Then, double digested insert and the vector was ligated and transformed into *E. coli* TOP10. After choosing the positive clone, the gene of interest was transferred into Agrobacterium for infiltration and expression the gene in *N. benthamiana.* 2-3 days after infiltration, the leaves were collected and stocked at -80 °C. Then, the leaves were used in protein extraction which was mentioned in Section 2.9 and immunoprecipitation was performed with Flag-tag antibody. The eluted proteins were separated on 12% SDS-PAGE. Next, western blot was performed with anti-FLAG antibody to indicate the protein expression of PstCTE1. Expression levels of the effectors PstCTE1 (labeled as 12j12) and 15N21 were very low (Figure 3.13), so it was decided to continue protein expression experiments with pK7FWG2 vector which was mentioned in Section 3.6.

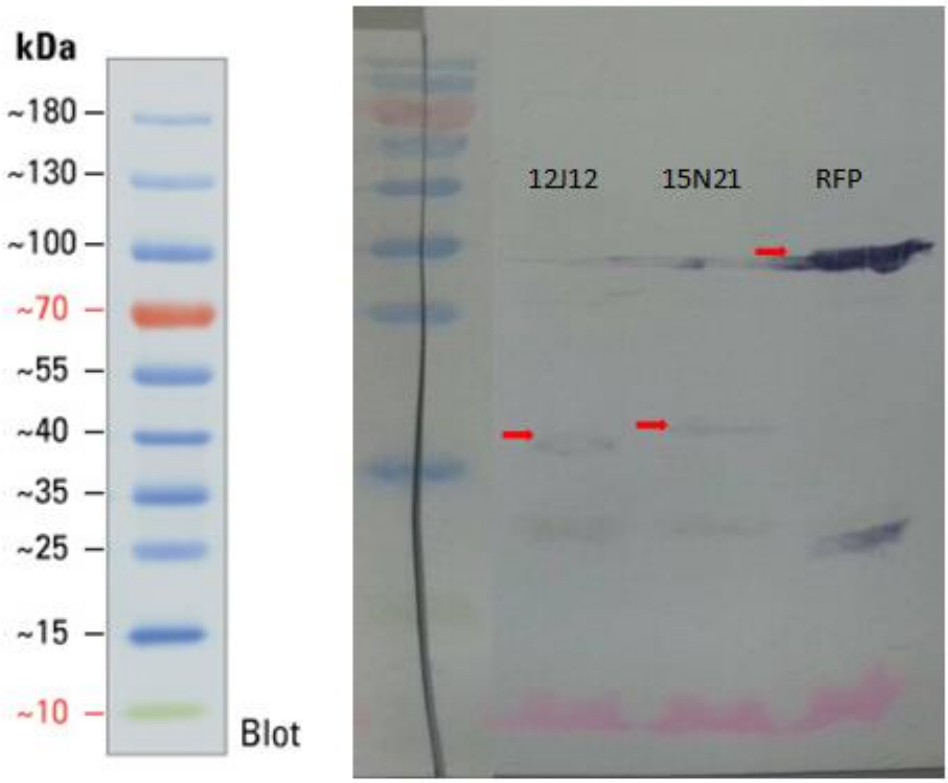


Figure 3.13. Western blot of PstCTE1 with FLAG-tag. The effectors and RFP have N-term FLAG-tag, and total protein extracts of the leaves were blotted using Anti-flag antibody to validate effector expression. The red arrows show PstCTE1 as 12J12, the other effector 15N21 and RFP (control).

3.8. Suppression assay

In suppression assay, effectors are expressed and then they were challenged with cell death inducers. Normally, pathogens deliver their effectors into host cell to suppress the host immunity and if they succeed, they cause disease formation and prevent the host cell death which is hypersensitive response. Thus, pK7FWG2/PstCTE1 and pK7FWG2/SP-PstCTE1 was challenged with cell death inducers which are Inf1 and PstSCR1 to test whether they can suppress or delay the cell death. pTRBO/GFP and pTRBO/SP-GFP were used as controls. First, the effectors pK7FWG2/PstCTE1, pK7FWG2/SP-PstCTE1 and the controls pTRBO/GFP and pTRBO/SP-GFP were infiltrated in *N. benthamiana* and after 2 days, pGR106-INF1 and pTRBO/Pst-SCR

were infiltrated in the same areas, separately. After 4th days, the cell deaths become visible and their rates were examined under UV light. Both Inf1 and PstSCR1 challenged effectors showed the nearly the same rate of cell death when compared the controls (Figure 3.14). Thus, it is seen that PstCTE1 effector is unable to delay or inhibit the cell death.

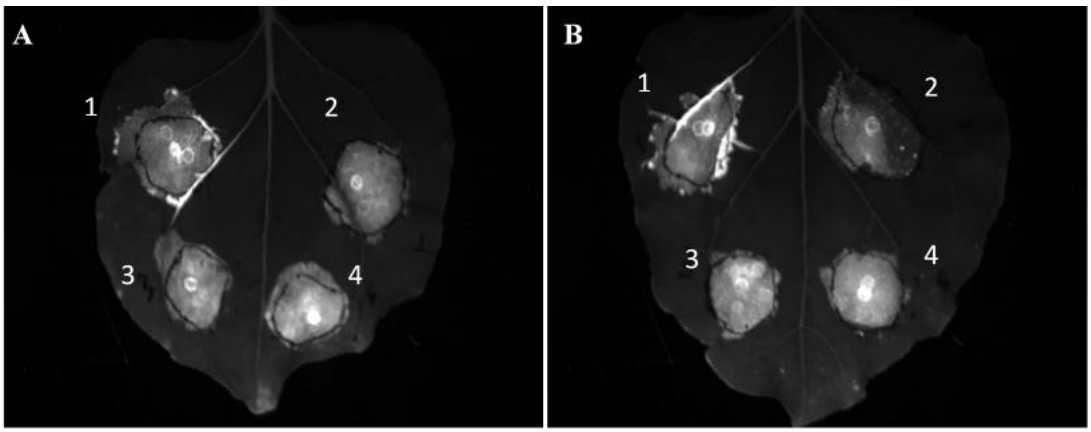


Figure 3.14. Suppression assay with Inf1 and PstSCR1. 1- pTRBO/GFP 2- pTRBO/SP-GFP 3- pK7FWG2/PstCTE1 4-pK7FWG2/SP-PstCTE1. In **A**, the effectors were challenged with Inf1 and in **B**, the effectors were challenged with SCR1 after 2 days post inoculation of effectors. The leaves were imaged under UV light after 4 days post inoculation.

CHAPTER 4

CONCLUSION

The plants are continuously subjected to biotic and abiotic stresses. Plants have developed different defense mechanism in order to protect themselves from these stresses and infections. The first defense mechanism is their basal immunity named PTI for non-host pathogens and ETI for host pathogens in which effectors are delivered for virulence into the host cell. For discovering the mechanisms of these defense systems, effector biology is one of the challenging and highly investigated area in plant-pathology studies. They are used as molecular probes to discover the unknown pieces of plant immunity and biology. Despite all the attempts to discover effector biology from a multitude of pathogens and symbionts, how these effectors manipulate their targets and their biological mechanisms are still not fully discovered.

The effectors of stripe rust caused by *Puccinia striiformis* f. sp. *tritici* have been extensively searched in recent years. Many stripe rust candidate effectors have been reported by genome and transcriptome sequencing but a few of them were confirmed experimentally. Subcellular localization of effectors performed by transiently expression with fluorescent proteins in model plant systems such as *Nicotiana benthamiana via Agrobacterium*-mediated gene transfer is the most widely used method to detect effector targets in the host cell which can reveal its function in plant-pathogen interactions. The model plant *N. benthamiana* was used because i) transiently expression systems like *Agrobacterium* infiltration is not suitable in *Pst* host plant like wheat, ii) *Pst* which is an obligate biotrophic fungus cannot easily be cultured and is not appropriate for genetic manipulations.

In this study, PstCTE1 effector candidate subcellular localization was determined by C-terminal fused green fluorescent protein (GFP). After realizing its chloroplast

localization, its transit peptide was searched by widely used bioinformatic tools but none of them predicted a transit peptide in PstCTE1 effector. This may happen because transit peptides differ in length, composition and structure; also, the targeting information relies on their physicochemical properties of them rather than their sequence information. Thus, transit peptide location was found by experimentally in which the gene was truncated and it was found that N-terminal of the gene is sufficient to target chloroplast. Thus, PstCTE1 gene has a cleavable N-terminal signal; otherwise, it would be unable to target chloroplast because the need of the mature protein.

In the literature, it was shown that when a reporter tag was added to N-terminus of the chloroplast targeting effector, the transit peptide lost its functionality and the effector can no longer target chloroplast. However, PstCTE1 effector still target chloroplast even if tagged with N-terminus RFP. It was observed that N-terminus RFP signal from PstCTE1 was overlapped with the C-terminus GFP signal from PstCTE1 in the same localization which is chloroplasts. Also, PstCTE1 transit peptide remain functional when the signal peptide was added to N-terminus of the gene. Moreover, this result may suggest the cell re-entry of PstCTE1 to chloroplasts since signal peptide directs the effector to apoplast of the cell. It can be concluded from this assay, either SP-PstCTE1 was secreted to apoplast first and then the effector re-enters into the cell with an intact chloroplast targeting signal thereby targeting the chloroplast or the effector escaped from the secretory pathway and end up in the cytoplasm where some of the effector may translocate in apoplast and some to the chloroplast. Additionally, SP-PstCTE1 effector may have an unidentified motif which leads its cell re-entry like some of the oomycete effectors which have RXLR-dEER motif after their signal peptide and responsible for their cell entry.

To sum up, PstCTE1 targets chloroplast which is one of the major ROS producing organelle, thereby main players in plant immunity. This effector has a unique and robust transit peptide and it shows cell re-entry for the site of function in the plant cell. So far, few examples of fungal effectors were reported which shows the cell re-entry

(Petre and Kamoun 2014), and PstCTE1 became one of them. For further, the function of this effector in the chloroplast and how it is related with disease formation can be studied.

REFERENCES

Alfano, James R. 2009. Micro-Review Roadmap for Future Research on Plant Pathogen Effectors. 10:805–13.

Barb Ziesman. 2016; Crop Production News 2016 Issue 3 | Crop Production News - 2016 Issues | Government of Saskatchewan. Retrieved December 10, 2018 (https://www.saskatchewan.ca/business/agriculture-natural-resources-and-industry/agribusiness-farmers-and-ranchers/sask-ag-now/crops/crop-production-news/cpn-archives/crop-production-news---2016-issues/crop-production-news-2016-issue-3).

Bonifacino, Juan S., Esteban C. Dell'Angelica, and Timothy A. Springer. 2001. Immunoprecipitation. *Current Protocols in Immunology* 41(1):8.3.1-8.3.28.

Borem, Aluizio, Roberto Fritsche-Neto, Francisco Murilo Zerbini, Fábio Nascimento da Silva, Gloria Patricia Castillo Urquiza, and Marcos Fernando Basso. 2014. Transgenic Plants. *Biotechnology and Plant Breeding* 179–99.

Bos, Jorunn I. B., Thirumala Devi Kanneganti, Carolyn Young, Cahid Cakir, Edgar Huitema, Joe Win, Miles R. Armstrong, Paul R. J. Birch, and Sophien Kamoun. 2006. The C-Terminal Half of Phytophthora Infestans RXLR Effector AVR3a Is Sufficient to Trigger R3a-Mediated Hypersensitivity and Suppress INF1-Induced Cell Death in Nicotiana Benthamiana. *Plant Journal* 48(2):165–76.

Bozkurt, Tolga O., Sebastian Schornack, Mark J. Banfield, and Sophien Kamoun. 2012. Oomycetes, Effectors, and All That Jazz. *Current Opinion in Plant Biology* 15(4):483–92.

Bruce, Barry D. 2000. Chlorplast Transit Peptides: Structure, Function and Evolution. *Trends in Cell Biology* 10(10):440–47.

Buchan, Daniel W. A., Federico Minneci, Tim C. O. Nugent, Kevin Bryson, and David T. Jones. 2013. Scalable Web Services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Research* 41(Web Server issue).

Bux, Hadi, Awais Rasheed, Mahboob Ali Siyal, Alvina G. Kazi, Abdul Aziz Napar, and A. Mujeeb-Kazi. 2012. An Overview of Stripe Rust of Wheat (Puccinia Striiformis f. Sp. Tritici) in Pakistan. *Archives of Phytopathology and Plant Protection* 45(19):2278–89.

Caillaud, Marie Cécile, Sophie J. M. Piquerez, Georgina Fabro, Jens Steinbrenner, Naveed Ishaque, Jim Beynon, and Jonathan D. G. Jones. 2012. Subcellular Localization of the Hpa RxLR Effector Repertoire Identifies a Tonoplast-Associated Protein HaRxL17 That Confers Enhanced Plant Susceptibility. *Plant Journal* 69(2):252–65.

Caplan, Jeffrey L., Padmavathi Mamillapalli, Tessa M. Burch-smith, and Kirk Czymmek. 2008. Chloroplastic Protein NRIP1 Mediates Innate Immune Receptor Recognition of a Viral Effector. 449–62.

Carrie, Chris, Kristina Kühn, Monika W. Murcha, Owen Duncan, Ian D. Small, Nicholas O'Toole, and James Whelan. 2009. Approaches to Defining Dual-Targeted Proteins in Arabidopsis. *Plant Journal* 57(6):1128–39.

Chen, Wanquan, Colin Wellings, Xianming Chen, Zhengsheng Kang, and Taiguo Liu. 2014. Wheat Stripe (Yellow) Rust Caused by Puccinia Striiformis f. Sp. Tritici. *Molecular Plant Pathology* 15(5):433–46.

Chen, X. M. 2005. Review / Synthèse Epidemiology and Control of Stripe Rust [Puccinia Striiformis f . Sp . Tritici] on Wheat. *Canadian Journal of Botany* 27:314–37.

Chotewutmontri, P., L. E. Reddick, D. R. McWilliams, I. M. Campbell, and B. D. Bruce. 2012. Differential Transit Peptide Recognition during Preprotein Binding and Translocation into Flowering Plant Plastids. *The Plant Cell* 24(7):3040–59.

Cohn, Jonathan, Guido Sessa, and Gregory B. Martin. n.d. Innate Immunity in Plants. 55–62.

Coll, N. S., P. Epple, and J. L. Dangl. 2011. Programmed Cell Death in the Plant Immune System. *Cell Death and Differentiation* 18(8):1247–56.

Cooper, Geoffrey M. and Robert E. Hausman. 2007. *The Cell: A Molecular Approach 2nd Edition*.

Curtis. 2002. Wheat in the World - B.C. CurtisIn: Curtis, B.C., Rajaram, S. and Macpherson, H.G., Eds., Bread Wheat Improvement and Production, Plant Production and Protection Series 30, FAO, Roma, 1-18.

Dangl, J. L. and J. D. Jones. 2001. Plant Pathogens and Integrated Defence Responses to Infection. *Nature* 411(6839):826–33.

Dinu, Monica, Anne Whittaker, Giuditta Pagliai, Stefano Benedettelli, and Francesco Sofi. 2018. Ancient Wheat Species and Human Health: Biochemical and Clinical Implications. *Journal of Nutritional Biochemistry* 52:1–9.

Dou, D., S. D. Kale, X. Wang, R. H. Y. Jiang, N. A. Bruce, F. D. Arredondo, X. Zhang, and B. M. Tyler. 2008. RXLR-Mediated Entry of Phytophthora Sojae Effector Avr1b into Soybean Cells Does Not Require Pathogen-Encoded Machinery. *The Plant Cell Online* 20(7):1930–47.

Dowen, Robert H., James L. Engel, Feng Shao, Joseph R. Ecker, and Jack E. Dixon. 2009. A Family of Bacterial Cysteine Protease Type III Effectors Utilizes Acylation-Dependent and -Independent Strategies Tolocalize to Plasma Membranes. *Journal of Biological Chemistry* 284(23):15867–79.

Gelvin, Stanton B. 2003. Agrobacterium -Mediated Plant Transformation : The Biology behind the Agrobacterium -Mediated Plant Transformation : The Biology behind the " Gene-Jockeying " Tool. *Microbiology and Molecular Biology Reviews* 67(1):16–37.

Göhre, Vera. 2015. Immune Responses: Photosynthetic Defence. *Nature Plants* 1(6):15079.

Guo, Minliang, Xiaowei Bian, Xiao Wu, and Meixia Wu. 2011. Agrobacterium-Mediated Genetic Transformation: History and Progress. in *Genetic Transformation*. InTech.

ten Have, Sara, Séverine Boulon, Yasmeen Ahmad, and Angus I. Lamond. 2011. Mass Spectrometry-Based Immuno-Precipitation Proteomics - The User's Guide. *PROTEOMICS* 11(6):1153–59.

Hovmøller, M. S., Stephanie Walter, and Annemarie Fejer Justesen. 2010. Escalating Threat of Wheat Rusts. *Science* 329(5990):369.

Jelenska, Joanna, Nan Yao, Boris A. Vinatzer, Christine M. Wright, Jeffrey L. Brodsky, and Jean T. Greenberg. 2007. A J Domain Virulence Effector of Pseudomonas Syringae Remodels Host Chloroplasts and Suppresses Defenses. *Current Biology* 17(6):499–508.

Jones, D. T. 1999. Protein Secondary Structure Prediction Based on Position-Specific Scoring Matrices. *Journal of Molecular Biology* 292(2):195–202.

Karimi, M., A. Depicker, and P. Hilson. 2007. Recombinational Cloning with Plant Gateway Vectors. *Plant Physiology* 145(4):1144–54.

Karimi, Mansour, Dirk Inzé, and Ann Depicker. 2002. GATEWAY^{TM} Vectors for Agrobacterium-Mediated Plant Transformation. *Trends in Plant Science* 7(5):193–95.

de la Riva, Gustavo A., Joel Gonzalez-Cabrera, Roberto Vazquez-Padron, and Camilo Ayra-Pardo. 1998. *Agrobacterium Tumefaciens* : A Natural Tool for Plant Transformation. *Electronic Journal of Biotechnology* 1(2):118–113.

Lee, L. Y. and S. B. Gelvin. 2007. T-DNA Binary Vectors and Systems. *Plant Physiology* 146(2):325–32.

Li, Guangyong, John E. Froehlich, Christian Elowsky, Joseph Msanne, Andrew C. Ostosh, Chi Zhang, Tala Awada, and James R. Alfano. 2014. Distinct Pseudomonas Type-III Effectors Use a Cleavable Transit Peptide to Target Chloroplasts. *Plant Journal* 77(2):310–21.

Lindbo, J. A. 2007. TRBO: A High-Efficiency Tobacco Mosaic Virus RNA-Based Overexpression Vector. *Plant Physiology* 145(4):1232–40.

Liu, Peng, Thwin Myo, Wei Ma, Dingyun Lan, Tuo Qi, Jia Guo, Ping Song, Jun Guo,

and Zhensheng Kang. 2016. TaTypA, a Ribosome-Binding GTPase Protein, Positively Regulates Wheat Resistance to the Stripe Rust Fungus. *Frontiers in Plant Science* 7(June).

Van Loon, L. C. and E. A. Van Strien. 1999. The Families of Pathogenesis-Related Proteins, Their Activities, and Comparative Analysis of PR-1 Type Proteins. *Physiological and Molecular Plant Pathology* 55(2):85–97.

Lu, Shunwen and Michael C. Edwards. 2015. Genome-Wide Analysis of Small Secreted Cysteine-Rich Proteins Identifies Candidate Effector Proteins Potentially Involved in Fusarium Graminearum _ Wheat Interactions.

Maddison, A. C. and J. G. Manners. 1972. Sunlight and Viability of Cereal Rust Uredospores. *Transactions of the British Mycological Society* 59(3):429–43.

Mboup, M., M. Leconte, A. Gautier, A. M. Wan, W. Chen, C. de Vallavieille-Pope, and J. Enjalbert. 2009. Evidence of Genetic Recombination in Wheat Yellow Rust Populations of a Chinese Oversummering Area. *Fungal Genetics and Biology*.

McHale, Leah, Xiaoping Tan, Patrice Koehl, and Richard W. Michelmore. 2006. Plant NBS-LRR Proteins: Adaptable Guards. *Genome Biology* 7(4).

Miras, Stéphane, Daniel Salvi, Myriam Ferro, Didier Grunwald, Jérôme Garin, Jacques Joyard, and Norbert Rolland. 2002. Non-Canonical Transit Peptide for Import into the Chloroplast. *Journal of Biological Chemistry* 277(49):47770–78.

Moffett, Peter, Garry Farnham, Jack Peart, and David C. Baulcombe. 2002. Interaction between Domains of a Plant NBS ± LRR Protein in Disease Resistance-Related Cell Death. 21(17).

Murray, G., C. Wellings, and C. Cole. 2005. RECOGNISING STRIPE RUST Causes of Wheat Rusts. *Stripe Rust: Understanding the Disease in Wheat* 1–12.

Nomura, Hironari, Teiko Komori, Shuhei Uemura, Yui Kanda, Koji Shimotani, Kana Nakai, Takuya Furuichi, Kohsuke Takebayashi, Takanori Sugimoto, Satoshi Sano, I. Nengah Suwastika, Eiichiro Fukusaki, Hirofumi Yoshioka, Yoichi Nakahira, and Takashi Shiina. 2012. Chloroplast-Mediated Activation of Plant Immune Signalling in Arabidopsis. *Nature Communications* 3(May):910–26.

Ozyigit, Ibrahim Ilker. 2012. *Crop Production for Agricultural Improvement*.

Padmanabhan, Meenu S. 2010. All Hands on Deck — The Role of Chloroplasts , Endoplasmic Reticulum , and the Nucleus in Driving Plant Innate Immunity. 23(11):1368–80.

Peralta, E. G. and L. W. Ream. 1985. T-DNA Border Sequences Required for Crown Gall Tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 82(15):5112–16.

Petre, Benjamin and Sophien Kamoun. 2014. How Do Filamentous Pathogens Deliver Effector Proteins into Plant Cells? *PLoS Biology* 12(2).

Petre, Benjamin, Michaela Kopischke, Alexandre Evrard, Silke Robatzek, and Sophien Kamoun. 2016. Cell Re-Entry Assays Do Not Support Models of Pathogen- Independent Translocation of AvrM and AVR3a Effectors into Plant Cells. *BioRxiv*.

Petre, Benjamin, Cécile Lorrain, Diane G. O. Saunders, Joe Win, Jan Sklenar, Sébastien Duplessis, and Sophien Kamoun. 2016. Rust Fungal Effectors Mimic Host Transit Peptides to Translocate into Chloroplasts. *Cellular Microbiology* 18(4):453–65.

Petre, Benjamin, Diane G. O. Saunders, and Jan Sklenar. 2015. Candidate Effector Proteins of the Rust Pathogen Melampsora Larici-Populina Target Diverse Plant Cell Compartments. 28(6):689–700.

Przetakiewicz, Anna, Agnieszka Karaś, Wacław Orczyk, and Anna Nadolska-orczyk. 2004. Volume 9, (2004) Pp 903 – 917. *October* 9(May):903–17.

Rafiqi, Maryam, Pamela H. P. Gan, Michael Ravensdale, Gregory J. Lawrence, Jeffrey G. Ellis, David A. Jones, Adrienne R. Hardham, and Peter N. Dodds. 2010. Internalization of Flax Rust Avirulence Proteins into Flax and Tobacco Cells Can Occur in the Absence of the Pathogen. *The Plant Cell* 22(6):2017–32.

Richter, Stefan and Gayle K. Lamppa. 1999. Stromal Processing Peptidase Binds Transit Peptides and Initiates Their ATP-Dependent Turnover in Chloroplasts. *Journal of Cell Biology* 147(1):33–43.

Roelfs, A. P., R. P. Singh, and E. E. Saari. 1992. Rust Diseases of Wheat. 81.

Scheel, Dierk. 1996. Resistance Response Physiology and Signal Transduction.

Schrammeijer, B. 2000. Sequence Analysis of the Vir-Region from Agrobacterium Tumefaciens Octopine Ti Plasmid PTi15955. *Journal of Experimental Botany* 51(347):1167–69.

Serrano, Irene, Corinne Audran, and Susana Rivas. 2016. Chloroplasts at Work during Plant Innate Immunity. *Journal of Experimental Botany* 67(13):3845–54.

Sharma-Poudyal, D. and X. M. Chen. 2011. Models for Predicting Potential Yield Loss of Wheat Caused by Stripe Rust in the U.S. Pacific Northwest. *Phytopathology* 101(5):544–54.

Shewry, Peter R. and Sandra J. Hey. 2015. The Contribution of Wheat to Human Diet and Health. *Food and Energy Security* 4(3):178–202.

Song, Jiangning, Hao Tan, Andrew J. Perry, Tatsuya Akutsu, Geoffrey I. Webb, James C. Whisstock, and Robert N. Pike. 2012. PROSPER: An Integrated Feature-Based Tool for Predicting Protease Substrate Cleavage Sites. *PLoS ONE* 7(11).

Sowden, Robert G., Samuel J. Watson, and Paul Jarvis. 2018. The Role of Chloroplasts in Plant Pathology. *Essays in Biochemistry* 62(1):21–39.

Stael, Simon, Przemyslaw Kmiecik, Patrick Willems, Katrien Van Der Kelen, Nuria S. Coll, Markus Teige, and Frank Van Breusegem. 2015. Plant Innate Immunity - Sunny Side Up? *Trends in Plant Science* 20(1):3–11.

Swarbrick, Philip J., Paul Schulze-Lefert, and Julie D. Scholes. 2006. Metabolic Consequences of Susceptibility and Resistance (Race-Specific and Broad-Spectrum) in Barley Leaves Challenged with Powdery Mildew. *Plant, Cell and Environment* 29(6):1061–76.

Tameling, Wladimir I. L., Sandra D. J. Elzinga, Patricia S. Darmin, Jack H. Vossen, Frank L. W. Takken, Michel A. Haring, and Ben J. C. Cornelissen. 2002. The Tomato *R* Gene Products I-2 and Mi-1 Are Functional ATP Binding Proteins with ATPase Activity. *The Plant Cell* 14(11):2929–39.

Teixeira, Pedro Filipe and Elzbieta Glaser. 2013. Processing Peptidases in Mitochondria and Chloroplasts. *Biochimica et Biophysica Acta - Molecular Cell Research* 1833(2):360–70.

Tonkin, Christopher J., Ming Kalanon, and Geoffrey I. McFadden. 2008. Protein Targeting to the Malaria Parasite Plastid. *Traffic* 9(2):166–75.

Torres, Miguel Angel, Jonathan D. G. Jones, and Jeffery L. Dangl. 2006. Reactive Oxygen Species Signaling in Response to Pathogens. *Plant Physiology* 141(2):373–78.

de Torres Zabala, Marta, George Littlejohn, Siddharth Jayaraman, David Studholme, Trevor Bailey, Tracy Lawson, Michael Tillich, Dirk Licht, Bettina Bölter, Laura Delfino, William Truman, John Mansfield, Nicholas Smirnoff, and Murray Grant. 2015. Chloroplasts Play a Central Role in Plant Defence and Are Targeted by Pathogen Effectors. *Nature Plants* 1(6):15074.

Underwood, William. 2012. The Plant Cell Wall: A Dynamic Barrier Against Pathogen Invasion. *Frontiers in Plant Science* 3(May):1–6.

Ve, T., S. J. Williams, A. M. Catanzariti, M. Rafiqi, M. Rahman, J. G. Ellis, A. R. Hardham, D. A. Jones, P. A. Anderson, P. N. Dodds, and B. Kobe. 2013. Structures of the Flax-Rust Effector AvrM Reveal Insights into the Molecular Basis of Plant-Cell Entry and Effector-Triggered Immunity. *Proceedings of the National Academy of Sciences* 110(43):17594–99.

Wang, Shumei, Petra C. Boevink, Lydia Welsh, Ruofang Zhang, Stephen C. Whisson, and Paul R. J. Birch. 2017. Delivery of Cytoplasmic and Apoplastic Effectors from Phytophthora Infestans Haustoria by Distinct Secretion Pathways. *New Phytologist* 205–15.

Warren, Randall F., Adam Henk, Patricia Mowery, Eric Holub, and Roger W. Innes.

1998. A Mutation within the Leucine-Rich Repeat Domain of the Arabidopsis Disease Resistance Gene RPS5 Partially Suppresses Multiple Bacterial and Downy Mildew Resistance Genes. 10(September):1439–52.

Wellings, Colin R. 2011. Global Status of Stripe Rust: A Review of Historical and Current Threats. *Euphytica* 179(1):129–41.

Whisson, S. C., P. C. Boevink, L. Moleleki, A. O. Avrova, J. G. Morales, E. M. Gilroy, M. R. Armstrong, S. Grouffaud, P. van West, S. Chapman, I. Hein, I. K. Toth, L. Pritchard, and P. R. Birch. 2007. A Translocation Signal for Delivery of Oomycete Effector Proteins into Host Plant Cells. *Nature* 450.

Wroblewski, Tadeusz, Anna Tomczak, and Richard Michelmore. 2005. Optimization of Agrobacterium-Mediated Transient Assays of Gene Expression in Lettuce, Tomato and Arabidopsis. *Plant Biotechnology Journal* 3(2):259–73.

Wu, H., C. Sparks, B. Amoah, and H. D. Jones. n.d. Factors Influencing Successful Agrobacterium-Mediated Genetic Transformation of Wheat. *Plant Cell Reports* 21(7):659–68.

Xia, Chongjing, Meinan Wang, Chuntao Yin, Omar E. Cornejo, Scot H. Hulbert, and Xianming Chen. 2018. Genomic Insights into Host Adaptation between the Wheat Stripe Rust Pathogen (Puccinia Striiformis f. Sp. Tritici) and the Barley Stripe Rust Pathogen (Puccinia Striiformis f. Sp. Hordei). *BMC Genomics* 19(1):1–21.

Yan, Yan, Haihong Jia, Fang Wang, Chen Wang, Shuchang Liu, and Xingqi Guo. 2015. Overexpression of GhWRKY27a Reduces Tolerance to Drought Stress and Resistance to Rhizoctonia Solani Infection in Transgenic Nicotiana Benthamiana. 6(September):1–16.

Yin, Chuntao, Xianming Chen, Xiaojie Wang, Qingmei Han, Zhensheng Kang, and Scot H. Hulbert. 2009. Generation and Analysis of Expression Sequence Tags from Haustoria of the Wheat Stripe Rust Fungus Puccinia Striiformis f. Sp. Tritici. *BMC Genomics* 10:1–9.

Yoo, Sang Dong, Young Hee Cho, and Jen Sheen. 2007. Arabidopsis Mesophyll Protoplasts: A Versatile Cell System for Transient Gene Expression Analysis. *Nature Protocols* 2(7):1565–72.

Zhang, Xiaoxiao, Neal Nguyen, Susan Breen, Megan A. Outram, Peter N. Dodds, Bostjan Kobe, Peter S. Solomon, and Simon J. Williams. 2017. Production of Small Cysteine-Rich Effector Proteins in Escherichia Coli for Structural and Functional Studies. *Molecular Plant Pathology* 18(1):141–51.

APPENDICES

A. Sequence information of PstCTE1 (PstHa12j12)

LOCUS GH737467 568 bp mRNA linear EST 05-JAN-2010

DEFINITION PSTha12j12 Puccinia striiformis f. sp. tritici haustoria cDNA library Puccinia striiformis f. sp. tritici cDNA clone 12j12 5', mRNA sequence.

ACCESSION GH737467

VERSION GH737467.1

DBLINK BioSample: SAMN00167260

KEYWORDS EST.

SOURCE Puccinia striiformis f. sp. tritici

ORGANISM Puccinia striiformis f. sp. tritici

Eukaryota; Fungi; Dikarya; Basidiomycota; Pucciniomycotina; Pucciniomycetes; Pucciniales; Pucciniaceae; Puccinia.

REFERENCE 1 (bases 1 to 568)

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.

FEATURES Location/Qualifiers

source 1..568

/organism="Puccinia striiformis f. sp. tritici"

/mol_type="mRNA"

/strain="PST-78"

/db_xref="taxon:168172"

/clone="12j12"

/clone_lib="SAMN00167260 Puccinia striiformis f. sp.

tritici haustoria cDNA library"

/dev_stage="haustoria"

/note="Organ: haustoria; Vector: pDNR-LIB; Site_1: Sfi I;

Site_2: Sfi I;

forma_specialis: tritici"

ORIGIN

1 gggggacgat ttcaaaaacc acatattccg acgtttgaaa ttttgaataa cctacctacc

61 tctcaagatt ttttccagtt caatccccaa caattattat cgactgttca ccttagtcgg

121 caatgcagct ctacatatca attggattag tactgcttat agcaacgaca gtgaaaaccc

181 taactaagac aggggcattt cttgagccat tgtttacaaa agtaacaaaa cgtgaagaca

241 agttttgtac aaacgggggg gctcattact gcttcaagaa aatcgcgaaa gagggtggct

301 tatttgtaca tattgctata ccaacatcac caaatgaaaa aacggatgat tctttatgca

361 acaagcttcg ttcgggattc aaagcaacta agtcctgctg tgaatataaa aaatttgatc

421 aaattcctca ggaaaaggac attgatagag atatctttat tgttacaaat aagggcttcg

481 aaactgcctg ttcccaagat gttgggagaa gcacatctta atcagctccc atctgcttat

541 caagggctgc ggtctctctc caattcgt

//

B. pK7FWG2 Vector Sequence

(Karimi et al.,2002)

>pK7FWG2 ;11880 bp

CGACGTCGCATGCCTGCAGGTCACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAG
AAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGAAA
CCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACACATTATTCTGGAGAAAAATA
GAGAGAGATAGATTTGTAGAGAGAGACTGGTGATTTTTGCGGACTCTAGCATGGCCGCGGTT
ACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACC
ATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTT
GTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCT
GCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGC
TTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGAT
GTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCT
CGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCC
TGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTA
GCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCA
GCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCC
TCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCAC
CACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTGATATCACCACTTTGTACAAGAAAG
CTGAACGAGAAACGTAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAAAAA
CAGACTACATAATACTGTAAAACACAACATATCCAGTCACTATGGTCGACCTGCAGACTGGC
TGTGTATAAGGGAGCCTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATG
TCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACACTGGC
CATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGGGTAAAGTTCAC
GGGAGACTTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCGGGC
GTGTCAATAATATCACTCTGTACATCCACAAACAGACGATAACGGCTCTCTCTTTTATAGGT
GTAAACCTTAAACTGCATTTCACCAGTCCCTGTTCTCGTCAGCAAAAGAGCCGTTCATTTCA
ATAAACCGGGCGACCTCAGCCATCCCTTCCTGATTTTCCGCTTTCCAGCGTTCGGCACGCAG
ACGACGGGCTTCATTCTGCATGGTTGTGCTTACCAGACCGGAGATATTGACATCATATATGC
CTTGAGCAACTGATAGCTGTCGCTGTCAACTGTCACTGTAATACGCTGCTTCATAGCACACC
TCTTTTTGACATACTTCGGGTATACATATCAGTATATATTCTTATACCGCAAAAATCAGCGC
GCAAATACGCATACTGTTATCTGGCTTTTAGTAAGCCGGATCCACGCGTTTACGCCCCGCCC
TGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACA
GACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATT
TGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAAACTG
GTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGAA
ATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGA
AATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTG
TAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATACGGAATTC
CGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACTTGTGCTTAT
TTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACAT
TGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGT
GGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGCCGGAT
CCTAACTCAAAATCCACACATTATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCT

AATGCGGCCGCCATAGTGACTGGATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTTA
TGCAAAATCTAATTTAATATATTGATATTTATATCATTTTACGTTTCTCGTTCAGCTTTTTT
GTACAAACTTGTGATATCACTAGTGCGGCCGCCTGCAGGTCGACTAGAATAGTAAATTGTAA
TGTTGTTTGTTGTTTGTTTTGTTGTGGTATTGTTGTAAAAATACCGGAGTCCTCTCCAAATG
AAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCC
TTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCT
TTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACCACTGTCGGCAGAGGCATC
TTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTAGGTGCCACCTTCCTTTTCTA
CTGTCCTTTTGATGAAGTGACAGATAGCTGGGCAATGGAATCCGAGGAGGTTTCCCGATATT
ACCCTTTGTTGAAAAGTCTCAATAGCCCTTTGGTCTTCTGAGACTGTATCTTTGATATTCTT
GGAGTAGACGAGAGTGTCGTGCTCCACCATGTTGACGAAGATTTTCTTCTTGTCATTGAGTC
GTAAAAGACTCTGTATGAACTGTTCGCCAGTCTTCACGGCGAGTTCTGTTAGATCCTCGATC
TGAATTTTTGACTCCATGGCCTTTGATTCAGTAGGAACTACTTTCTTAGAGACTCCAATCTC
TATTACTTGCCTGGTTTATGAAGCAAGCCTTGAATCGTCCATACTGGAATAGTACTTCTGAT
CTTGAGAAATATATCTTTCTCTGTGTTCTTGATGCAGTTAGTCCTGAATCTTTTGACTGCAT
CTTTAACCTTCTTGGGAAGGTATTTGATCTCCTGGAGATTATTACTCGGGTAGATCGTCTTG
ATGAGACCTGCCGCGTAGGCCTCTCTAACCATCTGTGGGTCAGCATTCTTTCTGAAATTGAA
GAGGCTAATCTTCTCATTATCGGTGGTGAACATGGTATCGTCACCTTCTCCGTCGAACTTTC
TTCCTAGATCGTAGAGATAGAGAAAGTCGTCCATGGTGATCTCCGGGGCAAAGGAGATCAGC
TTGGCTCTAGTCGACCATATGGGAGAGCTCAAGCTTAGCTTGAGCTTGGATCAGATTGTCGT
TTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGGCGGGTAAACCTAAGAG
AAAAGAGCGTTTATTAGAATAACGGATATTTAAAAGGGCGTGAAAAGGTTTATCCGTTCGTC
CATTTGTATGTGCATGCCAACCACAGGGTTCCCCTCGGGATCAAAGTACTTTGATCCAACCC
CTCCGCTGCTATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGAAAACGACAT
GTCGCACAAGTCCTAAGTTACGCGACAGGCTGCCGCCCTGCCCTTTTCCTGGCGTTTTCTTG
TCGCGTGTTTTAGTCGCATAAAGTAGAATACTTGCGACTAGAACCGGAGACATTACGCCATG
AACAAGAGCGCCGCCGCTGGCCTGCTGGGCTATGCCCGCGTCAGCACCGACGACCAGGACTT
GACCAACCAACGGGCCGAACTGCACGCGGCCGGCTGCACCAAGCTGTTTTCCGAGAAGATCA
CCGGCACCAGGCGCGACCGCCCGGAGCTGGCCAGGATGCTTGACCACCTACGCCCTGGCGAC
GTTGTGACAGTGACCAGGCTAGACCGCCTGGCCCGCAGCACCCGCGACCTACTGGACATTGC
CGAGCGCATCCAGGAGGCCGGCGCGGGCCTGCGTAGCCTGGCAGAGCCGTGGGCCGACACCA
CCACGCCGGCCGGCCGCATGGTGTTGACCGTGTTCGCCGGCATTGCCGAGTTCGAGCGTTCC
CTAATCATCGACCGCACCCGGAGCGGGCGCGAGGCCGCCAAGGCCCGAGGCGTGAAGTTTGG
CCCCCGCCCTACCCTCACCCCGGCACAGATCCGCACGCCCGCGAGCTGATCGACCAGGAAGG
CCGCACCGTGAAAGAGGCGGCTGCACTGCTTGGCGTGCACGCTCGACCCTGTACCGCGCACT
TGAGCGCAGCGAGGAAGTGACGCCCACCGAGGCCAGGCGGCGCGGTGCCTTCCGTGAGGACG
CATTGACCGAGGCCGACGCCCTGGCGGCCGCCGAGAATGAACGCCAAGAGGAACAAGCATGA
AACCGCACCAGGACGGCCAGGACGAACCGTTTTTCATTACCGAAGAGATCGAGGCGGAGATG
ATCGCGGCCGGGTACGTGTTCGAGCCGCCCGCGCACGTCTCAACCGTGCGGCTGCATGAAAT
CCTGGCCGGTTTGTCTGATGCCAAGCTGGCGGCCTGGCCGGCCAGCTTGGCCGCTGAAGAAA
CCGAGCGCCGCCGTCTAAAAAGGTGATGTGTATTTGAGTAAAACAGCTTGCGTCATGCGGTC
GCTGCGTATATGATGCGATGAGTAAATAAACAAATACGCAAGGGGAACGCATGAAGGTTATC
GCTGTACTTAACCAGAAAGGCGGGTCAGGCAAGACGACCATCGCAACCCATCTAGCCCGCGC
CCTGCAACTCGCCGGGGCCGATGTTCTGTTAGTCGATTCCGATCCCCAGGGCAGTGCCCGCG
ATTGGGCGGCCGTGCGGGAAGATCAACCGCTAACCGTTGTCGGCATCGACCGCCCGACGATT
GACCGCGACGTGAAGGCCATCGGCCGGCGCGACTTCGTAGTGATCGACGGAGCGCCCCAGGC
GGCGGACTTGGCTGTGTCCGCGATCAAGGCAGCCGACTTCGTGCTGATTCCGGTGCAGCCAA
GCCCTTACGACATATGGGCCACCGCCGACCTGGTGGAGCTGGTTAAGCAGCGCATTGAGGTC
ACGGATGGAAGGCTACAAGCGGCCTTTGTCGTGTCGCGGGCGATCAAAGGCACGCGCATCGG

CGGTGAGGTTGCCGAGGCGCTGGCCGGGTACGAGCTGCCCATTCTTGAGTCCCGTATCACGC
AGCGCGTGAGCTACCCAGGCACTGCCGCCGCCGGCACAACCGTTCTTGAATCAGAACCCGAG
GGCGACGCTGCCCGCGAGGTCCAGGCGCTGGCCGCTGAAATTAAATCAAAACTCATTTGAGT
TAATGAGGTAAAGAGAAAATGAGCAAAAGCACAAACACGCTAAGTGCCGGCCGTCCGAGCGC
ACGCAGCAGCAAGGCTGCAACGTTGGCCAGCCTGGCAGACACGCCAGCCATGAAGCGGGTCA
ACTTTCAGTTGCCGGCGGAGGATCACACCAAGCTGAAGATGTACGCGGTACGCCAAGGCAAG
ACCATTACCGAGCTGCTATCTGAATACATCGCGCAGCTACCAGAGTAAATGAGCAAATGAAT
AAATGAGTAGATGAATTTTAGCGGCTAAAGGAGGCGGCATGGAAAATCAAGAACAACCAGGC
ACCGACGCCGTGGAATGCCCCATGTGTGGAGGAACGGGCGGTTGGCCAGGCGTAAGCGGCTG
GGTTGTCTGCCGGCCCTGCAATGGCACTGGAACCCCCAAGCCCGAGGAATCGGCGTGACGGT
CGCAAACCATCCGGCCCGGTACAAATCGGCGCGGCGCTGGGTGATGACCTGGTGGAGAAGTT
GAAGGCCGCGCAGGCCGCCCAGCGGCAACGCATCGAGGCAGAAGCACGCCCCGGTGAATCGT
GGCAAGCGGCCGCTGATCGAATCCGCAAAGAATCCCGGCAACCGCCGGCAGCCGGTGCGCCG
TCGATTAGGAAGCCGCCCAAGGGCGACGAGCAACCAGATTTTTTCGTTCCGATGCTCTATGA
CGTGGGCACCCGCGATAGTCGCAGCATCATGGACGTGGCCGTTTTCCGTCTGTCGAAGCGTG
ACCGACGAGCTGGCGAGGTGATCCGCTACGAGCTTCCAGACGGGCACGTAGAGGTTTCCGCA
GGGCCGGCCGGCATGGCCAGTGTGTGGGATTACGACCTGGTACTGATGGCGGTTTCCCATCT
AACCGAATCCATGAACCGATACCGGGAAGGGAAGGGAGACAAGCCCGGCCGCGTGTTCCGTC
CACACGTTGCGGACGTACTCAAGTTCTGCCGGCGAGCCGATGGCGGAAAGCAGAAAGACGAC
CTGGTAGAAACCTGCATTCGGTTAAACACCACGCACGTTGCCATGCAGCGTACGAAGAAGGC
CAAGAACGGCCGCCTGGTGACGGTATCCGAGGGTGAAGCCTTGATTAGCCGCTACAAGATCG
TAAAGAGCGAAACCGGGCGGCCGGAGTACATCGAGATCGAGCTAGCTGATTGGATGTACCGC
GAGATCACAGAAGGCAAGAACCCGGACGTGCTGACGGTTCACCCCGATTACTTTTTGATCGA
TCCCGGCATCGGCCGTTTTCTCTACCGCCTGGCACGCCGCGCCGCAGGCAAGGCAGAAGCCA
GATGGTTGTTCAAGACGATCTACGAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAGTTCTGT
TTCACCGTGCGCAAGCTGATCGGGTCAAATGACCTGCCGGAGTACGATTTGAAGGAGGAGGC
GGGGCAGGCTGGCCCGATCCTAGTCATGCGCTACCGCAACCTGATCGAGGGCGAAGCATCCG
CCGGTTCCTAATGTACGGAGCAGATGCTAGGGCAAATTGCCCTAGCAGGGGAAAAAGGTCGA
AAAGGTCTCTTTCCTGTGGATAGCACGTACATTGGGAACCCAAAGCCGTACATTGGGAACCG
GAACCCGTACATTGGGAACCCAAAGCCGTACATTGGGAACCGGTCACACATGTAAGTGACTG
ATATAAAAGAGAAAAAAGGCGATTTTTCCGCCTAAAACTCTTTAAAACTTATTAAAACTCTT
AAAACCCGCCTGGCCTGTGCATAACTGTCTGGCCAGCGCACAGCCGAAGAGCTGCAAAAAGC
GCCTACCCTTCGGTCGCTGCGCTCCCTACGCCCCGCCGCTTCGCGTCGGCCTATCGCGGCCG
CTGGCCGCTCAAAAATGGCTGGCCTACGGCCAGGCAATCTACCAGGGCGCGGACAAGCCGCG
CCGTCGCCACTCGACCGCCGGCGCCCACATCAAGGCACCCTGCCTCGCGCGTTTCGGTGATG
ACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGC
CATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCA
GATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAT
ACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTG
CGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAA
CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGT
TGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGT
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCT
CGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGG
GAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGC
TCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA
CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTA
ACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAAC

TACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGG
AAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTG
TTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCT
ACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCATGATAT
ATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCT
GATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAATCGCTTGAGTTAACGCCGGCG
AAGCGGCGTCGGCTTGAACGAATTTCTAGCTAGACATTATTTGCCGACTACCTTGGTGATCT
CGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCT
TCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCG
CTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACC
AAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTC
CATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAG
TTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAG
CCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCAT
TCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAAC
AATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAA
GGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAA
TCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAG
CAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTT
CGGCGATCACCGCTTCCCCCATGATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTA
ACATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGA
TGCCCGAGGCATAGACTGTACCCCAAAAAAACATGTCATAACAAGAAGCCATGAAAACCGCC
ACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGACGGCAGTTA
CGCTACTTGCATTACAGCTTACGAACCGAACGAGGCTTATGTCCACTGGGTTCGTGCCCGAA
TTGATCACAGGCAGCAACGCTCTGTCATCGTTACAATCAACATGCTACCCTCCGCGAGATCA
TCCGTGTTTCAAACCCGGCAGCTTAGTTGCCGTTCTTCCGAATAGCATCGGTAACATGAGCA
AAGTCTGCCGCCTTACAACGGCTCTCCCGCTGACGCCGTCCCGGACTGATGGGCTGCCTGTA
TCGAGTGGTGATTTTGTGCCGAGCTGCCGGTCGGGGAGCTGTTGGCTGGCTGGTGGCAGGAT
ATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAA
TGTACTGAATTAACGCCGAATTGAATTATCAGCTTGCATGCCGGTCGATCTAGTAACATAGA
TGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATT
AAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTAC
ATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACC
GGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGATCTGCTTGACTCT
AGCTAGAGTCCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCG
ATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCC
GCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACAC
CCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAG
CAGGCATCGCCGTGGGTCACGACGAGATCCTCGCCGTCGGGCATCCGCGCCTTGAGCCTGGC
GAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGAC
CGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAG
GTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGC
AGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCC
TTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCAC
GATAGCCGCGCTGCCTCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAA
AAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCT
GTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAAT
CCATCTTGTTCAATCATGCCTCGATCGAGTTGAGAGTGAATATGAGACTCTAATTGGATACC
GAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTGACAAGAAATATTTGCTAGCTGATAGT

GACCTTAGGCGACTTTTGAACGCGCAATAATGGTTTCTGACGTATGTGCTTAGCTCATTAAA
CTCCAGAAACCCGCGGCTGAGTGGCTCCTTCAACGTTGCGGTTCTGTCAGTTCCAAACGTAA
AACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCCCTTAATTCTCATGTATGA
TAATTCGAGGGTACCCGGGGATCCTCTAGAGGGCC
//

C. pjl48-TRBO Vector Sequence

(Lindbo, 2007)

>pJL48vectorsequence(10606bp):(nucleotide1=firstnucleotideofTMV)

GTATTTTTACAACAATTACCAACAACAACAAACAACAGACAACATTACAATTACTATTTACA
ATTACAATGGCATACACACAGACAGCTACCACATCAGCTTTGCTGGACACTGTCCGAGGAAA
CAACTCCTTGGTCAATGATCTAGCAAAGCGTCGTCTTTACGACACAGCGGTTGAAGAGTTTA
ACGCTCGTGACCGCAGGCCCAAGGTGAACTTTTCAAAAGTAATAAGCGAGGAGCAGACGCTT
ATTGCTACCCGGGCGTATCCAGAATTCCAAATTACATTTTATAACACGCAAAATGCCGTGCA
TTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATATCTGATGATGCAAATTCCCTACG
GATCATTGACTTATGACATAGGCGGGAATTTTGCATCGCATCTGTTCAAGGGACGAGCATAT
GTACACTGCTGCATGCCCAACCTGGACGTTCGAGACATCATGCGGCACGAAGGCCAGAAAGA
CAGTATTGAACTATACCTTTCTAGGCTAGAGAGAGGGGGGAAAACAGTCCCCAACTTCCAAA
AGGAAGCATTTGACAGATACGCAGAAATTCCTGAAGACGCTGTCTGTCACAATACTTTCCAG
ACATGCGAACATCAGCCGATGCAGCAATCAGGCAGAGTGTATGCCATTGCGCTACACAGCAT
ATATGACATACCAGCCGATGAGTTCGGGGCGGCACTCTTGAGGAAAAATGTCCATACGTGCT
ATGCCGCTTTCCACTTCTCCGAGAACCTGCTTCTTGAAGATTCATGCGTCAATTTGGACGAA
ATCAACGCGTGTTTTTCGCGCGATGGAGACAAGTTGACCTTTTCTTTTGCATCAGAGAGTAC
TCTTAATTACTGTCATAGTTATTCTAATATTCTTAAGTATGTGTGCAAAACTTACTTCCCGG
CCTCTAATAGAGAGGTTTACATGAAGGAGTTTTTAGTCACCAGAGTTAATACCTGGTTTTGT
AAGTTTTCTAGAATAGATACTTTTCTTTTGTACAAAGGTGTGGCCCATAAAAGTGTAGATAG
TGAGCAGTTTTATACTGCAATGGAAGACGCATGGCATTACAAAAAGACTCTTGCAATGTGCA
ACAGCGAGAGAATCCTCCTTGAGGATTCATCATCAGTCAATTACTGGTTTCCCAAAATGAGG
GATATGGTCATCGTACCATTATTCGACATTTCTTTGGAGACTAGTAAGAGGACGCGCAAGGA
AGTCTTAGTGTCCAAGGATTTCGTGTTTACAGTGCTTAACCACATTCGAACATACCAGGCGA
AAGCTCTTACATACGCAAATGTTTTGTCCTTCGTCGAATCGATTCGATCGAGGGTAATCATT
AACGGTGTGACAGCGAGGTCCGAATGGGATGTGGACAAATCTTTGTTACAATCCTTGTCCAT
GACGTTTTACCTGCATACTAAGCTTGCCGTTCTAAAGGATGACTTACTGATTAGCAAGTTTA
GTCTCGGTTCGAAAACGGTGTGCCAGCATGTGTGGGATGAGATTTCGCTGGCGTTTGGGAAC
GCATTTCCCTCCGTGAAAGAGAGGCTCTTGAACAGGAAACTTATCAGAGTGGCAGGCGACGC
ATTAGAGATCAGGGTGCCTGATCTATATGTGACCTTCCACGACAGATTAGTGACTGAGTACA
AGGCCTCTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAAGAAACGGAAGTGATG
TACAATGCACTTTCAGAATTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTT
TTCCCAGATGTGCCAATCTTTGGAAGTTGACCCAATGACGGCAGCGAAGGTTATAGTCGCGG
TCATGAGCAATGAGAGCGGTCTGACTCTCACATTTGAACGACCTACTGAGGCGAATGTTGCG
CTAGCTTTACAGGATCAAGAGAAGGCTTCAGAAGGTGCATTGGTAGTTACCTCAAGAGAAGT
TGAAGAACCGTCCATGAAGGGTTCGATGGCCAGAGGAGAGTTACAATTAGCTGGTCTTGCTG
GAGATCATCCGGAATCGTCCTATTCTAAGAACGAGGAGATAGAGTCTTTAGAGCAGTTTCAT
ATGGCGACGGCAGATTCGTTAATTCGTAAGCAGATGAGCTCGATTGTGTACACGGGTCCGAT
TAAAGTTCAGCAAATGAAAAACTTTATCGATAGCCTGGTAGCATCACTATCTGCTGCGGTGT
CGAATCTCGTCAAGATCCTCAAAGATACAGCTGCTATTGACCTTGAAACCCGTCAAAAGTTT
GGAGTCTTGGATGTTGCATCTAGGAAGTGGTTAATCAAACCAACGGCCAAGAGTCATGCATG
GGGTGTTGTTGAAACCCACGCGAGGAAGTATCATGTGGCGCTTTTGGAATATGATGAGCAGG
GTGTGGTGACATGCGATGATTGGAGAAGAGTAGCTGTTAGCTCTGAGTCTGTTGTTTATTCC
GACATGGCGAAACTCAGAACTCTGCGCAGACTGCTTCGAAACGGAGAACCGCATGTCAGTAG

CGCAAAGGTTGTTCTTGTGGACGGAGTTCCGGGCTGTGGAAAAACCAAAGAAATTCTTTCCA
GGGTTAATTTTGATGAAGATCTAATTTTAGTACCTGGGAAGCAAGCCGCGGAAATGATCAGA
AGACGTGCGAATTCCTCAGGGATTATTGTGGCCACGAAGGACAACGTTAAAACCGTTGATTC
TTTCATGATGAATTTTGGGAAAAGCACACGCTGTCAGTTCAAGAGGTTATTCATTGATGAAG
GGTTGATGTTGCATACTGGTTGTGTTAATTTTCTTGTGGCGATGTCATTGTGCGAAATTGCA
TATGTTTACGGAGACACACAGCAGATTCCATACATCAATAGAGTTTCAGGATTCCCGTACCC
CGCCCATTTTGCCAAATTGGAAGTTGACGAGGTGGAGACACGCAGAACTACTCTCCGTTGTC
CAGCCGATGTCACACATTATCTGAACAGGAGATATGAGGGCTTTGTCATGAGCACTTCTTCG
GTTAAAAAGTCTGTTTCGCAGGAGATGGTCGGCGGAGCCGCCGTGATCAATCCGATCTCAAA
ACCCTTGCATGGCAAGATCTTGACTTTTACCCAATCGGATAAAGAAGCTCTGCTTTCAAGAG
GGTATTCAGATGTTCACACTGTGCATGAAGTGCAAGGCGAGACATACTCTGATGTTTCACTA
GTTAGGTTAACCCCTACACCGGTCTCCATCATTGCAGGAGACAGCCCACATGTTTTGGTCGC
ATTGTCAAGGCACACCTGTTCGCTCAAGTACTACACTGTTGTTATGGATCCTTTAGTTAGTA
TCATTAGAGATCTAGAGAAACTTAGCTCGTACTTGTTAGATATGTATAAGGTCGATGCAGGA
ACACAATAGCAATTACAGATTGACTCGGTGTTCAAAGGTTCCAATCTTTTTGTTGCAGCGCC
AAAGACTGGTGATATTTCTGATATGCAGTTTTACTATGATAAGTGTCTCCCAGGCAACAGCA
CCATGATGAATAATTTTGATGCTGTTACCATGAGGTTGACTGACATTTCATTGAATGTCAAA
GATTGCATATTGGATATGTCTAAGTCTGTTGCTGCGCCTAAGGATCAAATCAAACCACTAAT
ACCTATGGTACGAACGGCGGCAGAAATGCCACGCCAGACTGGACTATTGGAAAATTTAGTGG
CGATGATTAAAAGAAACTTTAACGCACCCGAGTTGTCTGGCATCATTGATATTGAAAATACT
GCATCTTTGGTTGTAGATAAGTTTTTTGATAGTTATTTGCTTAAAGAAAAAAGAAAACCAAA
TAAAAATGTTTCTTTGTTCAGTAGAGAGTCTCTCAATAGATGGTTAGAAAAGCAGGAACAGG
TAACAATAGGCCAGCTCGCAGATTTTGATTTTGTGGATTTGCCAGCAGTTGATCAGTACAGA
CACATGATTAAAGCACAACCCAAACAAAAGTTGGACACTTCAATCCAAACGGAGTACCCGGC
TTTGCAGACGATTGTGTACCATTCAAAAAAGATCAATGCAATATTCGGCCCGTTGTTTAGTG
AGCTTACTAGGCAATTACTGGACAGTGTTGATTCGAGCAGATTTTTGTTTTTCACAAGAAAG
ACACCAGCGCAGATTGAGGATTTCTTCGGAGATCTCGACAGTCATGTGCCGATGGATGTCTT
GGAGCTGGATATATCAAAATACGACAAATCTCAGAATGAATTCCACTGTGCAGTAGAATACG
AGATCTGGCGAAGATTGGGTTTCGAAGACTTCTTGGGAGAAGTTTGGAAACAAGGGCATAGA
AAGACCACCCTCAAGGATTATACCGCAGGTATAAAAACTTGCATCTGGTATCAAAGAAAGAG
CGGGGACGTCACGACGTTCATTGGAAACACTGTGATCATTGCTGCATGTTTGGCCTCGATGC
TTCCGATGGAGAAAATAATCAAAGGAGCCTTTTGCGGTGACGATAGTCTGCTGTACTTTCCA
AAGGGTTGTGAGTTTCCGGATGTGCAACACTCCGCGAATCTTATGTGGAATTTTGAAGCAAA
ACTGTTTAAAAAACAGTATGGATACTTTTGCGGAAGATATGTAATACATCACGACAGAGGAT
GCATTGTGTATTACGATCCCCTAAAGTTGATCTCGAAACTTGGTGCTAAACACATCAAGGAT
TGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTGATGTTGCTGTTTCGTTGAACAATTG
TGCGTATTACACACAGTTGGACGACGCTGTATGGGAGGTTCATAAGACCGCCCCTCCAGGTT
CGTTTGTTTATAAAAGTCTGGTGAAGTATTTGTCTGATAAAGTTCTTTTTAGAAGTTTGTTT
ATAGATGGCTCTAGTTGTTAAAGGAAAAGTGAATATCAATGAGTTTATCGACCTGACAAAAA
TGGAGAAGATCTTACCGTCGATGTTTACCCCTGTAAAGAGTGTTATGTGTTCCAAAGTTGAT
AAAATAATGGTTCATGAGAATGAGTCATTGTCAGGGGTGAACCTTCTTAAAGGAGTTAAGCT
TATTGATAGTGGATACGTCTGTTTAGCCGGTTTGGTCGTCACGGGCGAGTGGAACTTGCCTG
ACAATTGCAGAGGAGGTGTGAGCGTGTGTCTGGTGGACGATTTCAGTTCAAGGTCGTTCCCA
ATTATGCTATAACCACCCAGGACGCGATGAAAAACGTCTGGCAAGTTTTAGTTAATATTAGA
AATGTGAAGATGTCAGCGGGTTTCTGTCCGCTTTCTCTGGAGTTTGTGTCGGTGTGTATTGT
TTATAGAAATAATATAAAATTAGGTTTGAGAGAGAAGATTACAAACGTGAGAGACGGAGGGC
CCATGGAACTTACAGAAGAAGTCGTTGATGAGTTCATGGAAGATGTCCCTATGTCGATCAGG
CTTGCAAAGTTTCGATCTCGAACCGGAAAAAAGAGTGATGTCCGCAAAGGGAAAAATAGTAG
TAGTGATCGGTCAGTGCCGAACAAGAACTATAGAAATGTTAAGGATTTTGGAGGAATGAGTT

TTAAAAAGAATAATTTAATCGATGATGATTCGGAGGCTACTGTCGCCGAATCGGATTCGTTT
TAAATAGATCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATTAATTAACGGC
CTAGGGCGGCCGCGGTCCTGCAACTTGAGGTAGTCAAGATGCATAATAAATAACGGATTGTG
TCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTTTTTCCCTCCACTTAAATCGAAG
GGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATATACATCCGCAGGCACGTAA
TAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCAGGTACCCGGATGTGTT
TTCCGGGCTGATGAGTCCGTGAGGACGAAACCCTGCAGGCATGCAAGCTTGGCGTAATCATG
GTCATAGCCTAGCTAGAGTCCGCAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTAT
TTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTC
GCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTAT
CAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAGCCCGGCCGGGGGATCCA
CTAGCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGGC
GGGTAAACCTAAGAGAAAAGAGCGTTTATTAGAATAATCGGATATTTAAAAGGGCGTGAAAA
GGTTTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGAGATCTCAGTAAAGCGCTG
GCTGAACCCCCAGCCGGAACTGACCCCACAAGGCCCTAGCGTTTGCAATGCACCAGGTCATC
ATTGACCCAGGCGTGTTCCACCAGGCCGCTGCCTCGCAACTCTTCGCAGGCTTCGCCGACCT
GCTCGCGCCACTTCTTCACGCGGGTGGAATCCGATCCGCACATGAGGCGGAAGGTTTCCAGC
TTGAGCGGGTACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGGCCGTCGGCGA
CAGCTTGCGGTACTTCTCCCATATGAATTTCGTGTAGTGGTCGCCAGCAAACAGCACGACGA
TTTCCTCGTCGATCAGGACCTGGCAACGGGACGTTTTCTTGCCACGGTCCAGGACGCGGAAG
CGGTGCAGCAGCGACACCGATTCCAGGTGCCCAACGCGGTCGGACGTGAAGCCCATCGCCGT
CGCCTGTAGGCGCGACAGGCATTCCTCGGCCTTCGTGTAATACCGGCCATTGATCGACCAGC
CCAGGTCCTGGCAAAGCTCGTAGAACGTGAAGGTGATCGGCTCGCCGATAGGGGTGCGCTTC
GCGTACTCCAACACCTGCTGCCACACCAGTTCGTCATCGTCGGCCCGCAGCTCGACGCCGGT
GTAGGTGATCTTCACGTCCTTGTTGACGTGGAAAATGACCTTGTTTTGCAGCGCCTCGCGCG
GGATTTTCTTGTTGCGCGTGGTGAACAGGGCAGAGCGGGCCGTGTCGTTTGGCATCGCTCGC
ATCGTGTCCGGCCACGGCGCAATATCGAACAAGGAAAGCTGCATTTCCTTGATCTGCTGCTT
CGTGTGTTTCAGCAACGCGGCCTGCTTGGCCTCGCTGACCTGTTTTGCCAGGTCCTCGCCGG
CGGTTTTTCGCTTCTTGGTCGTCATAGTTCCTCGCGTGTCGATGGTCATCGACTTCGCCAAA
CCTGCCGCCTCCTGTTCGAGACGACGCGAACGCTCCACGGCGGCCGATGGCGCGGGCAGGGC
AGGGGGAGCCAGTTGCACGCTGTCGCGCTCGATCTTGGCCGTAGCTTGCTGGACCATCGAGC
CGACGGACTGGAAGGTTTCGCGGGGCGCACGCATGACGGTGCGGCTTGCGATGGTTTCGGCA
TCCTCGGCGGAAAACCCCGCGTCGATCAGTTCTTGCCTGTATGCCTTCCGGTCAAACGTCCG
ATTCATTCACCCTCCTTGCGGGATTGCCCCGACTCACGCCGGGGCAATGTGCCCTTATTCCT
GATTTGACCCGCCTGGTGCCTTGGTGTCCAGATAATCCACCTTATCGGCAATGAAGTCGGTC
CCGTAGACCGTCTGGCCGTCCTTCTCGTACTTGGTATTCCGAATCTTGCCCTGCACGAATAC
CAGCGACCCCTTGCCCAAATACTTGCCGTGGGCCTCGGCCTGAGAGCCAAAACACTTGATGC
GGAAGAAGTCGGTGCGCTCCTGCTTGTCGCCGGCATCGTTGCGCCACATCTAGGTACTAAAA
CAATTCATCCAGTAAAATATAATATTTTATTTTCTCCCAATCAGGCTTGATCCCCAGTAAGT
CAAAAAATAGCTCGACATACTGTTCTTCCCCGATATCCTCCCTGATCGACCGGACGCAGAAG
GCAATGTCATACCACTTGTCCGCCCTGCCGCTTCTCCCAAGATCAATAAAGCCACTTACTTT
GCCATCTTTCACAAAGATGTTGCTGTCTCCCAGGTCGCCGTGGGAAAAGACAAGTTCCTCTT
CGGGCTTTTCCGTCTTTAAAAAATCATACAGCTCGCGCGGATCTTTAAATGGAGTGTCTTCT
TCCCAGTTTTCGCAATCCACATCGGCCAGATCGTTATTCAGTAAGTAATCCAATTCGGCTAA
GCGGCTGTCTAAGCTATTCGTATAGGGACAATCCGATATGTCGATGGAGTGAAAGAGCCTGA
TGCACTCCGCATACAGCTCGATAATCTTTTCAGGGCTTTGTTCATCTTCATACTCTTCCGAG
CAAAGGACGCCATCGGCCTCACTCATGAGCAGATTGCTCCAGCCATCATGCCGTTCAAAGTG
CAGGACCTTTGGAACAGGCAGCTTTCCTTCCAGCCATAGCATCATGTCCTTTTCCCGTTCCA
CATCATAGGTGGTCCCTTTATACCGGCTGTCCGTCATTTTTAAATATAGGTTTTCATTTTCT

CCCACCAGCTTATATACCTTAGCAGGAGACATTCCTTCCGTATCTTTTACGCAGCGGTATTT
TTCGATCAGTTTTTTCAATTCCGGTGATATTCTCATTTTAGCCATTTATTATTTCCTTCCTC
TTTTCTACAGTATTTAAAGATACCCCAAGAAGCTAATTATAACAAGACGAACTCCAATTCAC
TGTTCCTTGCATTCTAAAACCTTAAATACCAGAAAACAGCTTTTTCAAAGTTGTTTTCAAAG
TTGGCGTATAACATAGTATCGACGGAGCCGATTTTGAAACCACAATTATGGGTGATGCTGCC
AACTCGAGAGCGGGCCGGGAGGGTTCGAGAAGGGGGGGCACCCCCCTTCGGCGTGCGCGGTC
ACGCGCACAGGGCGCAGCCCTGGTTAAAAACAAGGTTTATAAATATTGGTTTAAAAGCAGGT
TAAAAGACAGGTTAGCGGTGGCCGAAAAACGGGCGGAAACCCTTGCAAATGCTGGATTTTCT
GCCTGTGGACAGCCCCTCAAATGTCAATAGGTGCGCCCCTCATCTGTCAGCACTCTGCCCCT
CAAGTGTCAAGGATCGCGCCCCTCATCTGTCAGTAGTCGCGCCCCTCAAGTGTCAATACCGC
AGGGCACTTATCCCCAGGCTTGTCCACATCATCTGTGGGAAACTCGCGTAAAATCAGGCGTT
TTCGCCGATTTGCGAGGCTGGCCAGCTCCACGTCGCCGGCCGAAATCGAGCCTGCCCCTCAT
CTGTCAACGCCGCGCCGGGTGAGTCGGCCCCTCAAGTGTCAACGTCCGCCCCTCATCTGTCA
GTGAGGGCCAAGTTTTCCGCGAGGTATCCACAACGCCGGCGGCCGGCCGCGGTGTCTCGCAC
ACGGCTTCGACGGCGTTTCTGGCGCGTTTGCAGGGCCATAGACGGCCGCCAGCCCAGCGGCG
AGGGCAACCAGCCCGGTGAGCTCTAGTGGACTGATGGGCTGCCTGTATCGAGTGGTGATTTT
GTGCCGAGCTGCCGGTCGGGGAGCTGTTGGCTGGCTGGTGGCAGGATATATTGTGGTGTAAA
CAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGGGGTGGTT
TTGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAG
GTCAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGA
AGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAACCTCCTCGGATTCC
ATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAAGGTGGCACCTACAAA
TGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAA
AGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAA
AGCAAGTGGATTGATGTGATAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATC
AAAGATACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGG
AAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGG
AAGGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT
GCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGT
TCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG
CACAATCCCACTATCCTTCGCAAGACCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGA
GG

//

D. Mascot result of mass spectroscopy analysis

a

Search parameters

MS data file:	LTQ_18A010_Akkaya_SP-12J12-1.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 42%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

b

Search parameters

MS data file:	LTQ_18A010_Akkaya_SP-12J12-2.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 21%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

c

Search parameters

MS data file:	LTQ_18A010_Akkaya_12J12-1.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 28%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

a)SP-PstCTE1-1 named as band ‘i’ b) SP-PstCTE1-2 named as band ‘ii’ c)PstCTE1-1 named as band ‘ii’ in SDS-gel in Figure 3.12.

E. Detailed mascot result of mass spectroscopy

Protein View: Akkaya_01

Akkaya 01

Database: UB_target
Score: 238
Monoisotopic mass (M_r): 13101
Calculated pI: 8.74

Sequence similarity is available as an NCBI BLAST search of Akkaya_01 against nr.

Search parameters

MS data file: LTQ_18A010_Akkaya_SP-12J12-1.mgf
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 42%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

Unformatted sequence string: 114 residues (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
644	39 – 55	603.6567	1807.9483	1807.9468	0.81	0	20	0.0099	1	U	K.EGGLFVHIAIPTSPNEK.T
452	80 – 87	502.7569	1003.4992	1003.4975	1.75	0	22	0.0061	1	U	K.FDQIPQEK.D
608	88 – 99	483.5955	1447.7645	1447.7671	-1.77	1	36	0.00028	1	U	K.DIDRDIFIVTNK.G
609	88 – 99	483.5958	1447.7654	1447.7671	-1.14	1	29	0.0012	1	U	K.DIDRDIFIVTNK.G
610	88 – 99	483.5960	1447.7661	1447.7671	-0.70	1	35	0.00031	1	U	K.DIDRDIFIVTNK.G
611	88 – 99	724.8908	1447.7671	1447.7671	0.021	1	33	0.00049	1	U	K.DIDRDIFIVTNK.G
612	88 – 99	724.8910	1447.7675	1447.7671	0.26	1	39	0.00013	1	U	K.DIDRDIFIVTNK.G

Calculated pI: 8.74

Sequence similarity is available as an NCBI BLAST search of Akkaya_01 against nr.

Search parameters

MS data file: LTQ_18A010_Akkaya_12J12-1.mgf
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 28%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

Unformatted sequence string: 114 residues (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
821	79 – 87	566.8033	1131.5921	1131.5924	-0.24	1	47	2e-005	1	U	K.KFDQIPQEK.D
822	79 – 87	566.8034	1131.5922	1131.5924	-0.18	1	52	6.3e-006	1	U	K.KFDQIPQEK.D
823	79 – 87	566.8037	1131.5928	1131.5924	0.35	1	41	8e-005	1	U	K.KFDQIPQEK.D
640	80 – 87	502.7563	1003.4981	1003.4975	0.61	0	30	0.0009	1	U	K.FDQIPQEK.D
641	80 – 87	502.7563	1003.4981	1003.4975	0.66	0	30	0.0009	1	U	K.FDQIPQEK.D
642	80 – 87	502.7567	1003.4989	1003.4975	1.41	0	27	0.002	1	U	K.FDQIPQEK.D
1140	88 – 99	724.8911	1447.7677	1447.7671	0.44	1	46	2.7e-005	1	U	K.DIDRDIFIVTNK.G
1141	88 – 99	724.8911	1447.7677	1447.7671	0.45	1	34	0.00044	1	U	K.DIDRDIFIVTNK.G
1142	88 – 99	483.5966	1447.7679	1447.7671	0.59	1	33	0.00045	1	U	K.DIDRDIFIVTNK.G
1143	88 – 99	483.5966	1447.7680	1447.7671	0.63	1	35	0.00033	1	U	K.DIDRDIFIVTNK.G
1144	88 – 99	724.8913	1447.7680	1447.7671	0.65	1	42	6.8e-005	1	U	K.DIDRDIFIVTNK.G
1145	88 – 99	483.5967	1447.7684	1447.7671	0.88	1	26	0.0027	1	U	K.DIDRDIFIVTNK.G
589	92 – 99	475.2711	948.5276	948.5280	-0.46	0	52	6.6e-006	1	U	R.DIFIVTNK.G
590	92 – 99	475.2716	948.5287	948.5280	0.74	0	37	0.00019	1	U	R.DIFIVTNK.G
591	92 – 99	475.2717	948.5288	948.5280	0.84	0	52	6.7e-006	1	U	R.DIFIVTNK.G
1021	100 – 111	663.7910	1325.5675	1325.5670	0.37	0	80	1.1e-008	1	U	K.GFETACSQDVGR.S
1022	100 – 111	663.7912	1325.5678	1325.5670	0.55	0	74	4.1e-008	1	U	K.GFETACSQDVGR.S
1023	100 – 111	663.7913	1325.5680	1325.5670	0.69	0	63	5.3e-007	1	U	K.GFETACSQDVGR.S

Protein View: Akkaya_01

Akkaya 01

Database:	UB_target
Score:	131
Monoisotopic mass (M_r):	13101
Calculated pI:	8.74

Sequence similarity is available as an NCBI BLAST search of Akkaya_01 against nr.

Search parameters

MS data file:	LTQ_18A010_Akkaya_SP-12J12-2.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 21%

Matched peptides shown in ***bold red***.

```
  1 MLTKTGAFLE PLFTKVTKRE DKFCTNGGAH YCFKKIAKEG GLFVHIAIPT
 51 SPNEKTDDSL CNKLRSGFKA TKSCCEYKKF DQIPQEKDID RDIFIVTNKG
101 FETACSQDVG RSTS
```

Unformatted sequence string: 114 residues (for pasting into other applications).

Sort by residue number | increasing mass | decreasing mass
Show matched peptides only | predicted peptides also

Query	Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
q688	88 - 99	483.5954	1447.7645	1447.7671	-1.80	1	21	0.0086	1	U	K.DIDRDIFIVTNK.G
q689	88 - 99	724.8901	1447.7656	1447.7671	-1.05	1	42	6.7e-005	1	U	K.DIDRDIFIVTNK.G
q690	88 - 99	483.5961	1447.7664	1447.7671	-0.48	1	22	0.0064	1	U	K.DIDRDIFIVTNK.G
q691	88 - 99	724.8905	1447.7665	1447.7671	-0.41	1	27	0.002	1	U	K.DIDRDIFIVTNK.G
q692	88 - 99	724.8908	1447.7671	1447.7671	0.0041	1	30	0.0011	1	U	K.DIDRDIFIVTNK.G
q693	88 - 99	483.5964	1447.7674	1447.7671	0.22	1	29	0.0011	1	U	K.DIDRDIFIVTNK.G
q652	100 - 111	663.7908	1325.5671	1325.5670	0.032	0	39	0.00014	1	U	K.GFETACSQDVGR.S

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name	: Andaç, Ayşe
Nationality	: Turkish (TC)
Date and Place of Birth	: 5 April 1989, Güzelyurt/Kıbrıs
Phone	: +90 312 210 3197
E-mail	: e157301@metu.edu.tr

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biotechnology	2013
BS	METU Biology	2011
High School	Güzelyurt Türk Maarif Koleji, Ankara	2006

WORK EXPERIENCE

Year	Place	Enrollment
2010- Summer	Atatürk Araştırma Hastanesi, Patoloji Laboratuarı	Intern student

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Dagvadorj, B., Ozketen, A. C., Andac, A., Duggan, C., Bozkurt, T. O., & Akkaya, M. S. (2017). A Puccinia striiformis f. sp. Tritici secreted protein activates plant immunity at the cell surface. *Scientific Reports, 7*(1). https://doi.org/10.1038/s41598-017-01100-z

2. Andac, A., Ozketen, A.C., Dagvadorj B., Akkaya, M. S. (Submitted), An effector of Puccinia striiformis f. sp. tritici targets chloroplasts with a novel and robust targeting signal.

HOBBIES

Computer Technologies, Movies, Sports