CLONING, TRANSFORMATION AND WHEAT INFILTRATION ASSAY OF A *PUCCINIA STRIIFORMIS* F. SP. *TRITICI* EFFECTOR CANDIDATE

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ABSTRACT

CLONING, TRANSFORMATION AND WHEAT INFILTRATION ASSAY OF A *PUCCINIA STRIIFORMIS* F. SP. *TRITICI* EFFECTOR CANDIDATE

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The stripe rust pathogen, *Puccinia striiformis* f. sp. *tritici* is the causative agent of the yellow (stripe) rust which is globally one of the most devastating and economically significant diseases in wheat. Although using fungicide can present a practical solution against the disease, an efficient, better and environmentally safer approach is needed to grow disease free wheat. To achieve this, better comprehension of the plant-pathogen interaction at the molecular and cellular level is required.

The pathogen effectors are the key to discover this molecular maze of the plant-microbe interactions. With the advances in *Puccinia* genome sequencing projects and development of new strategies, researchers now have a chance to predict and test the candidate effectors on various biological assays.

In this thesis, one of the predicted effector candidates from *Puccinia striiformis* f. sp. *tritici* is cloned into three different vectors to test for avirulence, to determine subcellular localization and to pull-down the interacting host factors. PSTha15N21 pathogen gene is predicted as an effector candidate using bioinformatic tools and expression data (Yin *et al*., 2009a).

The pathogen effector gene was cloned into a gateway destination vector, pK7FWG2 to study subcellular localization, using agroinfiltration method. The gene construct was also cloned into a pEDV6 vector for avirulence testing by searching the presence of hypersensitive (HR) response using Type III secretion system of *P. fluorescens* (EtHAn).

Lastly, the gene construct with FLAG-tag fusion in its N-terminus site was cloned into pJL48-TRBO expression vector for expressing FLAG-fused protein to perform tagged protein co-immunoprecipitation experiment toward identification of host interacting factors in future studies.

Keywords: Wheat, *Puccinia striiformis* f. sp. *trtici*, yellow rust, wheat infiltration assay, co-immunoprecitation, *P.fluorescens* (EtHAn), agroinfiltration, Gateway cloning, subcellular localization

ÖZ

BİR *PUCCINIA STRIIFORMIS* F. SP. *TRITICI* EFEKTÖR ADAYININ KLONLANMASI, TRANSFORMASYONU VE BUĞDAYDA İNFİLTRASYON ANALİZİ

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Puccinia striiformis f. sp. *tritici* küresel ölçekte en çok zarar veren ve ekonomik önemi olan buğday hastalıklarından birisinin, sarı pas hastalığının nedeni olan patojendir. Mantar ilaçlarının kullanımı hastalığa karşı pratik bir çözüm sunsa da daha verimli, etkili ve çevre problemlerine duyarlı yaklaşımlara gereksinim duyulmaktadır. Bunu başarmak için bitki ve patojen ilişkisi moleküler ve hücresel düzeyde anlaşılmalıdır.

Patojen efektörleri bitki-mikrop etkileşiminde moleküler labirentleri keşfetmek için bir anahtardır. *Puccinia* genomu için sürdürülen sekanslama projelerindeki ilerlemeler ve yeni stratejilerin geliştirilmesiyle beraber araştırmacılar efektör adaylarını bulabilmek ve biyolojik analizlerde test edebilmek için şimdi bir şans yakaladılar.

Bu tezde, *Puccinia striiformis* f. sp. *tritici* patojenine ait bir efektör adayı, hücre içi konumu saptayabilmek, efektör adayının etkileşime geçtiği konukçul faktörleri belirleyebilmek ve de avirülens testinin yapılabilmesi için ilgili üç ayrı vektöre klonlandı. Biyoinformatik programlarının ve anlatım düzeyi sonuçlarının yardımıyla PstHa15N21 patojen geni (Yin *et al*., 2009a) bu çalışmanın efektör adayı olarak belirlendi.

Efektör geni bir gateway varış vektörü olan pK7FWG2'a hücre içi konumunu agroinfiltrasyon yöntemiyle saptayabilmek için klonlandı. Tip III Salgı Sistemi protein geni içeren vector, pEDV6, ise *P. fluorescens* (EtHAn) bakterisi yoluyla efektörün buğdaya aktarılmasını sağlamak için kullanıldı, Bu sayede bitkide hiper duyarlı yanıta dayalı avirülanslığın sınanması için çalışılabildi. Son olarak, ilgili gen N-terminus ucunda FLAG başlığı takılı olarak pJL48-TRBO anlatım vektörüne klonlandı. Klonlanan bu yapı daha

sonra agroinfiltrasyon yöntemiyle bitkiye aktarılacak ve ko-immunopresipitasyon analizleri ile etkileşen bitki faktörlerinin sonraki çalışmalarda saptanması mümkün olacaktır.

Anahtar Kelimeler: Buğday, *Puccinia striiformis* f. sp. *tritici*, sarı pas, buğday infiltrasyon analizi, ko-immünopresipitasyon, *P. fluorescens* (EtHAn), agroinfiltrasyon, Gateway klonlaması, hücre içi konumlandırma

Dedicated to my family…

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LIST OF ABBREVIATIONS

AOS: Active Oxygen Species

Avr: Avirulence

bp: Base pair

CWD: Cell wall degradation

DNA: Deoxyribonucleic acid

dNTP: Deooxy-nucleotidetriphosphate

dpi: Days post-inoculation

EtHAn: Effector to host analyzer

EST: Expression sequence tags

ETI: Effector-triggered im munity

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

MAMPS: Microbe associated molecular patterns

mg: Miligram

mL: Mililiter

NB: Nucleotide binding

ng: Nanogram

NT: No-template

PAMP: Pathogen-associated molecular patterns

PCD: Programmed cell death

PCR: Polymerase chain reaction

pmol: Picomole

PR: Pathogenesis related

PRR: Pattern recognition receptor

Pst: *Puccinia striiformis* f. sp. *tritici*

PTI: PAMP-triggered immunity

R: Resistance

Rif: Rifampicin

Sm: Spectinomycin

Sp: Streptomycin

T-DNA: Transfer DNA

Taq: *Thermus aquaticus*

Ti: Tumor-inducing

TMV: Tobacco mosaic virus

TRBO: TMV RNA-based overexpression

TTSS: Type-III secretion system

u: Unit

Vir: Virulence

CHAPTER 1

INTRODUCTION

1.1 Wheat (Triticum aestivum)

Wheat is one of the cereal grains which is used as human food and livestock feed. It is the leading crop in the many countries based on production and consumption rate. Wheat is one of the earliest domesticated crop species in the world. The origin of the wheat species goes back 10000 years ago. The very first cultivated wheat had diploid genome AA (einkorn) and tetraploid genome AABB (emmer). The genetic studies reveal that these earliest domesticated wheat forms originated from the south-eastern part of Turkey (Heun, 1997; Shewry, 2009). Since then, wheat takes a starring role in human nutrition. Because of its partnership with human species, wheat has spread to all continents except Antarctica and it is the major crop in cultivated areas with a total area of 240 million hectares (Curtis *et al*., 2002). Moreover, it gains the title of 'big three' cereal crops (along with its rivals rice and maize) with a production of over 600 million tons, annually (Shewry, 2009). Wheat dominates world cereal market as a major player and it has the biggest world trade ratio that competes with all other crops combined (Curtis *et al*., 2002). According to FAO (Food and Agriculture Organization)'s 2013 Food Outlook report, wheat served as a dominant crop with 701.5 million tons whereas world cereal market including wheat has 2354.2 million tonnes production in 2011/12. Wheat has a major impact in Turkey's economy, also. It is the main human nutrition source and livestock feed which gives the crop vital importance. Hence, Turkey is one of the top producers and placed at the 9^{th} place in 2012 wheat production statistics (Table 1.1).

The success of the wheat domination lies in its genetic diversity, adaptive nature and its relation with human. Although wheat has a relatively recent origin, it possesses sufficient amount of genetic diversity of over 25 000 types which allows the plant to adapt a broad range of environments (Feldman *et al*., 1995). Wheat can grow successfully between the latitudes of 30° and 60°N and 27° and 40°S (Nuttonson 1955) and this range can widen outside of this latitudes and attitude. It requires 25 °C optimum growth temperature, but the plant can also grow between minimum 3 °C and maximum 32 °C. Wheat also needs moisture; it can endure precipitation ranges between 250-1750 mm (annual). However, too much moisture can lead to disease and rot formation (Curtis *et al*., 2002). Since its first domestication, wheat becomes indispensable for humans. It has gluten protein and selenium which makes wheat a good dietary source. Wheat cultivation is relatively favorable because the yield may exceed 10 tons ha^{-1} (Shewry, 2009). Wheat is quite adaptive, storable and technologically feasible. Due to this reasons, wheat is a crucial grain for human life. Hence,

humans spread wheat all over the world except Antarctica. This makes wheat the dominant crop species in the cultivated areas. Moreover, this gives wheat a vital spot in world market.

For understanding the importance of wheat, research on wheat breeding and wheat diseases become valuable. Various countries found organizations and institutes such as the International Maize and Wheat Improvement Center (CIMMYT). Hence, Turkey also has to take necessary measures to eliminate any threat to this crucial plant.

Table 1.1 Wheat production: Leading producers

Country	2012(Estim.) *million tonnes*	2013(f'cast) *million tonnes*	Change(%): 2013 over 2012
European Union	131.3	139.0	5.9
China (Mainland)	120.6	121.8	1.0
India	94.9	93.6	-1.4
United States	61.8	56.0	-9.4
Russian Federation	37.7	55.0	45.9
Canada	27.2	29.4	8.1
Australia	22.1	24.0	8.6
Pakistan	24.0	26.3	9.6
Turkey	20.1	21.0	4.5
Ukraine	15.8	20.2	27.8
Kazakhstan	9.8	14.1	43.9
Iran Islamic Rep. of	13.8	14.5	5.1
Argentina	9.0	11.0	22.2
Egypt	8.8	9.4	6.8
Uzbekistan	6.7	6.7	0.0
Other Countries	55.5	60.0	8.1
World	659.1	702.0	6.5

* Countries listed according to their position in global production (average 2011-2013)

Source: Food and Agriculture Organization, Food Outlook Report, June 2013 (http://www.fao.org/docrep/018/al999e/al999e.pdf)

1.2 Wheat Yellow (Stripe) Rust

Wheat yellow rust which is also known as the stripe rust is one of the most notorious and devastating wheat disease worldwide. Although it is believed that the disease was present in Roman times, first scientific documentations of the stripe rust was in 1777 (Bushnell & Roelfs, 1984) (Eriksson and Henning, 1896). The causative agent of the stripe rust disease is *Puccinia striiformis* f. sp. *tritici* (*Pst*).

Puccinia striiformis f. sp. *tritici* (*Pst*) is a filamentous fungus and an obligate biotrophic parasite (Huang, Chen, Coram, Wang, & Kang, 2011). The disease starts with the infection of the green tissues of cereal crops and develops through utilization of water and nutrients from host plants. Infection does not require a specific time point, it can happen after the first leaf appears. Sporulation stage occurs nearly 2 weeks after infection with formation of yellow to orange colored rust urediospores. These urediospores can travel long distances with the help of wind like other airborne fungal pathogens (Chen, 2005). Wellings and

McIntosh (1990) stated that the stripe rust might spread from Australia from New Zealand by wind dispersal. Another example for long distance travelling of the stripe rust is stated about 2400 km in a time interval of 6 months in United States, gradually (Zadoks, 1961). In addition to its long distance migration ability, the disease can cause severe yield losses. In wheat cultivated areas, yield losses can array between %10 and % 70 depending on many reasons including the specific susceptibility of the wheat cultivar, infection conditions, the severity and life span of the disease (Chen, 2005). If the infection begins at the early leaf stage of the wheat and the disease is able to advance through the growing season of the plant, yield losses can be disastrous for the plant with 100% yield damage (Chen, 2005).

Figure 1.1 Wheat yellow (stripe) rust
Source: (https://extension.usu.edu/newsletters/images/uploads/images/wheat%20stripe1.jpg)

The damage rate of the disease depends on various environmental factors. The three main environmental factors are moisture, temperature and wind. The moisture is required for favorable germination and infection rate. The urediospores of the rust fungi germinated and penetrate through leaf tissues with the help of humidity(Chen, 2005). It is well stated that high moist environment favors excellent germination rate. Moreover, rain also may help dispersal of urediospores through splashes (Rapilly, 1979). The temperature is another factor that directly has influence on the disease severity. The optimum temperature for infection stage of the yellow rust pathogen is between 9-13 ^{o}C for night temperature (Brown and Sharp, 1969). The daytime temperature is a lesser limiting factor compared to night temperatures for the disease development. However, it is also important for the spore germination, infection, durability of the disease, longevity of the spore and host resistance (Chen, 2005). The last factor is the wind. The wind dries the urediospores of the fungi and

decrease the germination and infection rate of the spores (Chen, 2005). In this aspect, it seems like the wind negatively affects the spores. However, it also helps to extend the viability period of urediospores. The migration of the wheat stripe rust pathogen mainly depends on wind dispersal as we expect from an airborne fungal pathogen (Chen, 2005). Another factor that affects the infection is light intensity. It stated that susceptible reactions were happened under low light intensities, and vice versa (Manners, 1950; Stubbs, 1967).

To summarize, wheat stripe is one of the most destructive disease on cereal crops with yield losses up to 70%. With the optimum conditions of moisture, temperature, wind and light, the yield losses can reach to %100 (Chen, 2005). Although fungicides are useful against disease protection, they are expensive cause environmental problems. The long lasting solution against the disease to generate new resistant cultivars because the disease severity mainly depends on the cultivar of wheat (Chen, 2005) (Rapilly, 1979). However, there is an arm race between wheat and the pathogen, *Puccinia striiformis* f. sp. *tritici* . Thus, understanding the interaction between plant and its pathogen is a key aspect to overcome the disease.

1.3 Plant innate immunity

Plants have encountered with countless number of microbial threats throughout ages and they are still present in modern world (Boller & He, 2009). They survive with the help of their immune system. Plant immune system is deprived of adaptive immunity and thereby mobile protector cells. However, they compensate it with their innate immune system which has larger recognition repertoire than animals (Jones & Dangl, 2006) (Dangl & Jones, 2001) (Dodds & Rathjen, 2010).

To understand the plant immune system better, we must take a closer look on the plant disease resistance in the gene-for-gene manner. The gene-for-gene concept was discovered after the experiments on the inheritance of rust resistance in flax and of pathogenicity in the flax rust fungus (Jeff Ellis, Dodds, & Pryor, 2000). The concept states that for every rust resistance in the host, there is a corresponding gene in the pathogen which is called avirulence gene (J. G. Ellis, Lawrence, Luck, & Dodds, 1999). When avirulence gene (Avr) interacts with the resistance gene (R), immune response occurs and the development of the pathogen halts through hypersensitive response (HR). The hypersensitive response (HR) is a plant defense mechanism to prevent pathogen infection by inducing the death of attacked host cells. This response includes the activation of an oxidative burst at the plasma membrane which result in generation of active oxygen species (AOS) as in the swift dismutation of superoxide to hydrogen peroxide (Vanacker, Carver, & Foyer, 2000).

Plant innate immunity consists of two branches of defense layers which protect plant from external threats such as pathogens. The first layer of the plant innate immunity gives response to conserved microbial elicitors. These microbial elicitors are known as microbe associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs) (Jones & Dangl, 2006) (Dodds & Rathjen, 2010). Therefore, the stimulation of an immune response by these pathogen-associated molecular patterns is called PAMP-triggered immunity (PTI). The recognition of PAMPs is achieved by pathogen recognition receptors

(PRR) which are found in transmembrane. These PRRs can recognize specific epitopes such as the flagellin structure of a pathogen. After recognition, plant cell awakes the PAMP-triggered immunity (PTI) to cease colonization and further development of the threat (Jones & Dangl, 2006). However, successful pathogens may avoid from the PAMP triggered immune response. They achieve this by releasing various pathogen effectors into the host cell to suppress or interfere with PTI. The plant may overcome hampering of PTI by the pathogen effectors if the specific NB-LRR proteins are present in the cell. These NB-LRR proteins can directly or indirectly identify the pathogen effectors. Subsequently, second layer of the plant immune system is activated through specific recognition of the effectors and therefore, it is called effector triggered immunity (ETI) (Figure 1.2) (Jones & Dangl, 2006) (Dodds & Rathjen, 2010).

PTI and ETI are the 2 main defense strategy of the plants to combat wide variety of plant pathogens. They both induce overlapping sets of genes. Moreover, they activate similar groups of immune responses such as oxidative burst, hormonal changes and transcriptional reprogramming after the recognition of the pathogen proteins (Tsuda & Katagiri, 2010). Upon the pathogen attack, specific immune response mechanisms are triggered through the utilization of the downstream processes at different levels. These specific immune mechanisms generally start with the production of reactive oxygen species. ROS generation such as H_2O_2 and superoxide ($\cdot O_2$) radicals, is one of the earliest response of plants. It leads to cellular destruction of both host and pathogen alike to form swift, localized cell death associated with the HR. Moreover, it is very probable that the production of H_2O_2 may contribute to cell wall strengthening through cell wall lignification to halt microbial development. After the pathogen attack, more robust systemic resistance can be triggered against secondary threat of a wide spectrum of pathogens (Cohn, Sessa, & Martin, 2001). This resistance is known as systemic-acquired resistance and involves stimulation of defense-related proteins such as pathogenesis related (PR) proteins. A broad range of physical changes are documented after a pathogen attacks. These physiological changes involve the generation of reactive oxygen species, transient ion-flux resulting in change of intracellular pH, cell wall lignification near infection area, production of secondary signals like nitric oxide and formation of antimicrobial compounds such as phytoalexins and pathogenesis-related (PR) proteins(Cohn *et al*., 2001). Both PTI and ETI are capable of inducing this downstream processes or mechanism at different rate. ETI is more prolonged and robust than PTI. However, low pathogen recognition specificity allows PTI not to exhaust the plant through induction of excessive immune response (Tsuda & Katagiri, 2010). ETI response generally results in programmed cell death (PCD) or hypersensitive response (HR) and systemic acquired resistance. On the other hand, PTI leads to early and transient immune response to ensure survival of the plant while ceasing disease development. ETI is race specific and follows the gene-for-gene relationship whereas PTI recognized conserved pathogen elicitors (Göhre & Robatzek, 2008).

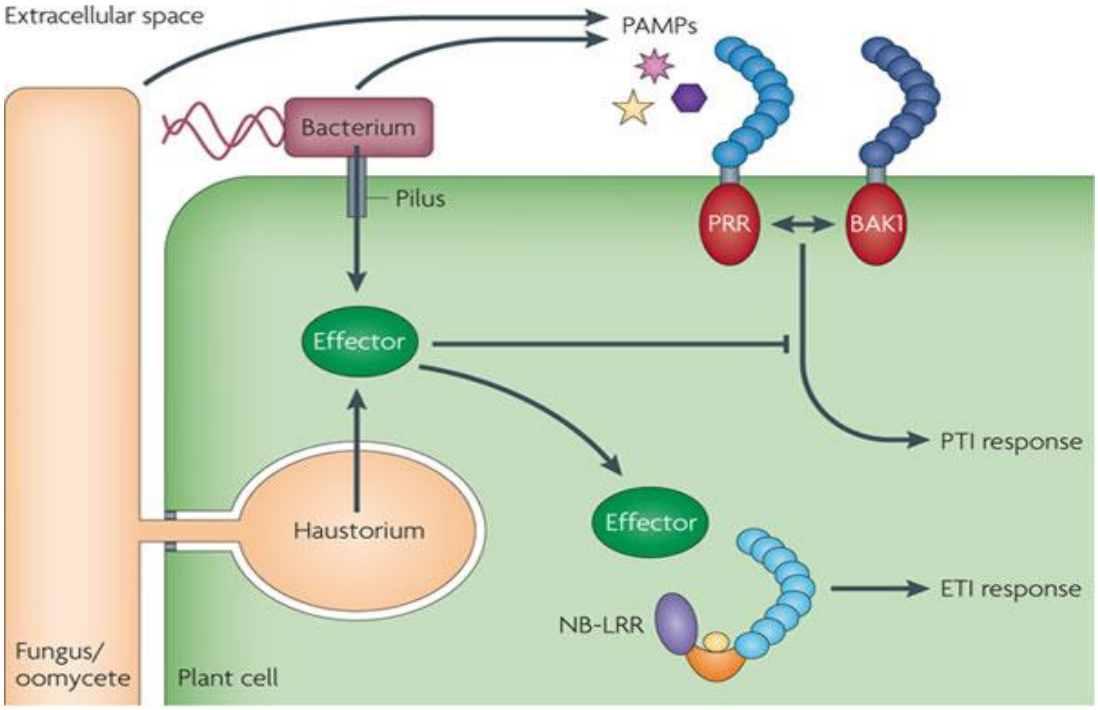


Figure 1.2 Overview of plant innate immune system.

Both bacteria and fungi invade host' extracellular space. Bacteria directly proliferate in apoplastic fluid. Fungi and oomycetes exploit nutrients of plant cell *via* feeding complex called haustoria. Haustoria, or invasive hyphae in some fungi, penetrate the cell forming a chamber inside the host without disturbing plasma membrane. Some molecules such as flagellin, chitin or lipopolysaccharides serve as elicitors that trigger PTI by interacting with PRRs. Pathogens release effectors to evade PTI. Delivery mechanism of effectors is not yet fully discovered for fungi and oomycetes. However, bacterial pathogens release their effectors into the host cell *via* type-3 secretion system. Successful plants recognize these effectors and stimulate ETI response (Dodds & Rathjen, 2010).

To summarize, plants protect themselves by means of 2 defense measure. First defense measure encompasses specific recognition of PAMPs/MAMPs with PRRs and downstream immune responses of the host. This defense layer acts as a primary weapon in a plant's arsenal against microbial threats. It induces specific immune response mechanisms in a very swift and transient manner while ensuring host cell survival in most cases (Göhre & Robatzek, 2008). Successful pathogens may evade this defense trap by suppressing PTI with the help of pathogen effectors. However, plants may counteract by detecting and interacting with these pathogen effectors through specific R proteins. This second layer of immunity is known as effector triggered immunity. It recognizes effectors in race specific or gene-for-gene manner. It is robust and prolonged and it stimulates hypersensitive response and systemic acquired resistance upon activation (Göhre & Robatzek, 2008) (Tsuda & Katagiri, 2010).

Although there are many researchers studying plant immune system, the knowledge about PTI, ETI and immune suppression by the effectors is limited. Thus, discovering underlying molecular mechanisms is critical to overcome plant associated disease.

1.4 The plant pathogen effectors

The plant pathogen effectors are the small secreted proteins of various pathogens to overcome plant defense layers. These molecules can assist disease formation by inducing invasion of host tissues, durability inside the host, repression of immune signals, gathering nutrients, propagation and growth (Göhre & Robatzek, 2008). Different effectors suppress downstream processes of PTI response in various ways. However, these effectors may serve as a ligand to wake ETI. For successful ETI response, the plant should have corresponding R gene product as in the gene-for-gene relationship to halt further proliferation of the pathogen. R proteins interact with Avr directly or indirectly by monitoring effectors action on host targets (guard hypothesis) (Jones & Dangl, 2006). Effectors may evade ETI by either altering effector gene or producing supplementary effectors that can withhold ETI (Jones & Dangl, 2006). Plants also feel the driving force of evolution to diversify their R proteins to counteract against effectors. R proteins classify as CC-NB-LRR or TIR-NB-LRR depending on their structure (Göhre & Robatzek, 2008). These NB-LRR genes are subjected to change through recombination to keep up with alternating effectors (J Ellis, Dodds, & Pryor, 2000). Hence, there is a continuous struggle between plants and their pathogens.

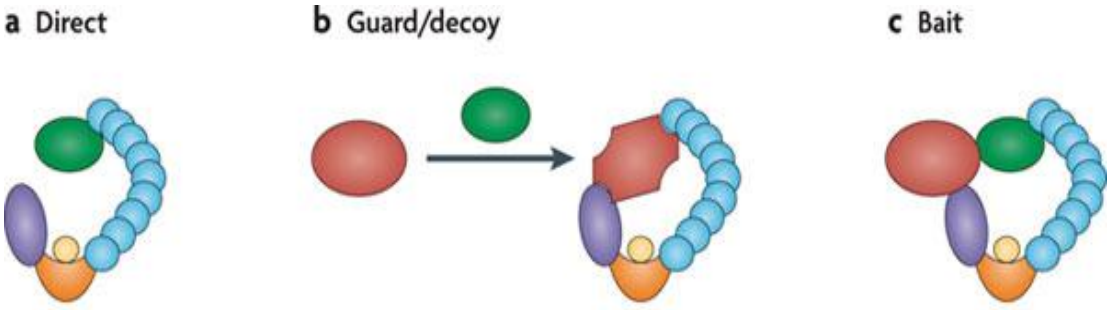


Figure 1.3 Illustration of direct and indirect recognition of plant immune system. NB-LRR receptors of plant immune system can recognize directly or indirectly in 3 different models: (a) effector (green) can interact with NB-LRR directly. (b) Effector induces conformational changes on an accessory protein or virulence target and NB-LRR recognize the resulting compound. (c) NB-LRR recognizes accessory protein-effector complex (Dodds & Rathjen, 2010).

The microbial effectors' genes are induced upon host colonization. The delivery of the pathogen effectors into the host cells is achieved in various ways depending on the type of the pathogen. For instance, bacteria are noted to transport their effectors *via* Type II or Type III-Secretion System into the host cell to access nutrients or to suppress PTI (Göhre & Robatzek, 2008). Type III secretion system' core components are derived from the flagellar machine. *Agrobacterium tumefaciens*, on the other hand, uses Type IV secretion system which has conjugation machines' components (Christie & Vogel, 2000). Fungi and oomycetes secrete their effectors *via* exocytosis into the apoplastic fluid surrounded by extracellular matrix (Göhre & Robatzek, 2008). Release of the effectors is occurred at the hyphal tips with the exception of some filamentous fungi including rusts, downy and powdery mildew. Interestingly, the delivery site of the effectors in rusts and mildews is happened to be haustorium. Haustoria are known as a feeding structure which is formed to penetrate the plant's plasma membrane (Jonge, Bolton, & Thomma, 2011). Another, intriguing form of the effector distribution is reported in the rice pathogen, *M. oryzae*. The delivery is achieved by a structure named as the biotrophic interfacial complex and effectors are accumulated in the complex. Moreover, the complex does not change its location even the invasive hyphae moves to other host cells (Jonge *et al*., 2011). The location of the effectors is depending on the function. Although all effectors are secreted into apoplastic fluid they may travel into the host cell to perform their cytoplasmic function. The ones that remain in the apoplastic fluid may have roles including cell wall degradation (CWDEs), necrosis and ethylene induction (NLPs). However, there are still some effectors with unknown function (Jonge *et al*., 2011). Probably, the most interesting and fascinating ones are called as small cysteine-rich secreted proteins. Only few of the cysteine-rich secreted effectors' function have been revealed. For instance, effectors belonged to *Cladosporium fulvum* and *Phytophthora infestans* are reported for their role in inhibition of extracellular host proteases. Some of the effectors from same organisms are also defend the pathogen from chitin-triggered host defenses. These host defenses have role in basal defense of the host against outside threat. Although their effect to fungal proliferation is not destructive, the chitin oligosaccharides parts which are torn apart from the pathogen's cell wall can serve as PAMP to activate bigger immune response on the host. The fungi avoid from detection with the help of specific effectors such as *Avr*4 and *Ecp*6 (Burg *et al.*, 2006). Both *Avr*4 and *Ecp*6 strengthen the cell wall by interacting different domains of the pathogen's cell wall to defend it from host enzymes (Jonge *et al*., 2011). We know that some of the effectors are delivered inside the host because of R protein mediated immune response (Jonge *et al*., 2011). However, the uptake of the effectors inside the plant cells is yet to be discovered. It has been proposed that N-terminal RxLR motif or Y/F/WxC motifs of the effectors might be the key for translocation. As in the mechanism of the delivery and translocation of effectors into the host cells, cytoplasmic function of the effectors remains also unclear (Jonge *et al*., 2011).

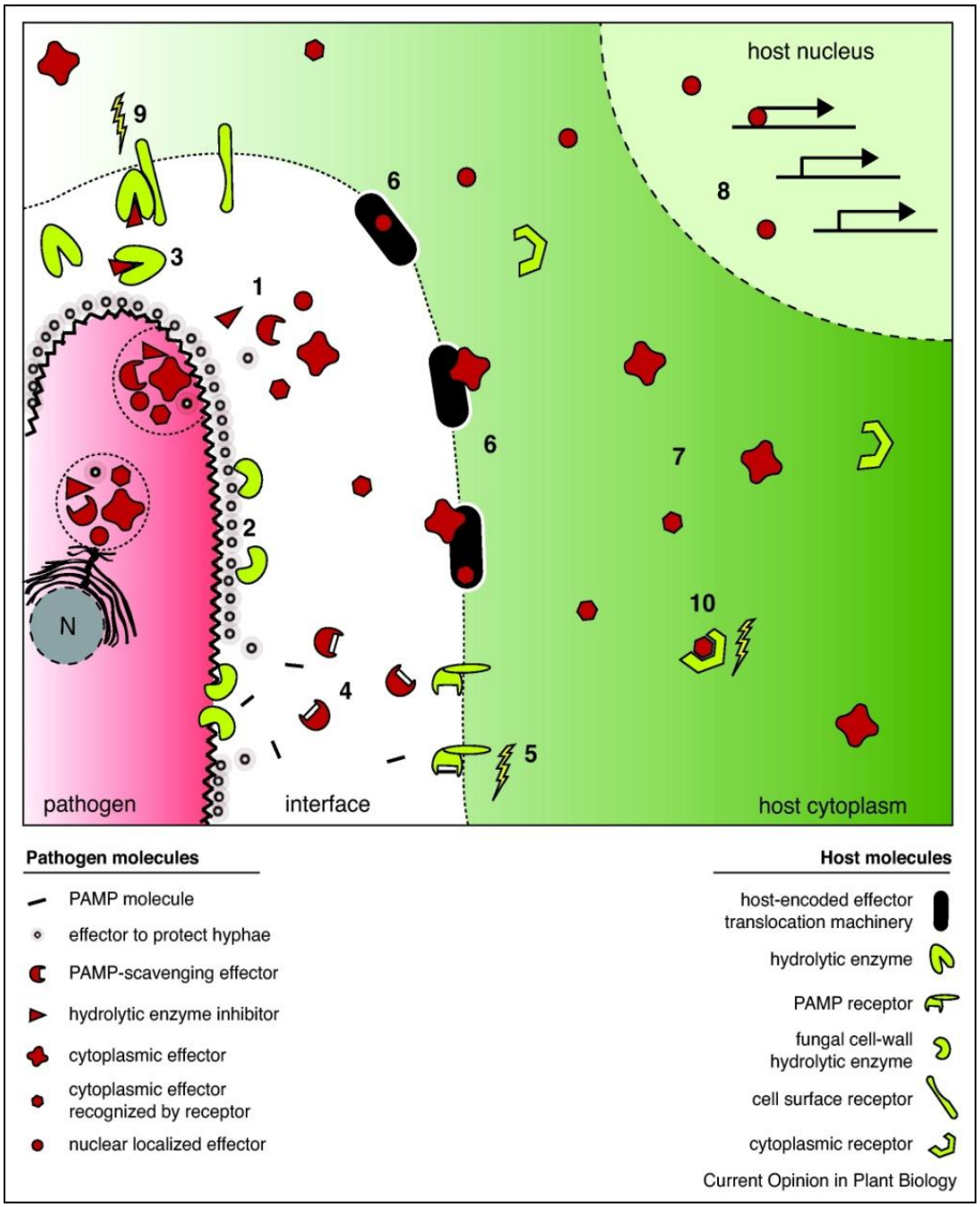


Figure 1.4 General illustration of fungal effector functions. (1) Effectors are secreted in apoplastic fluid. (2) They may provide protection to pathogen from hydrolytic defense enzymes. (3) They can inhibit defense enzymes or (4) Block PAMP molecules. (5) PAMP s may induce host defense. (6) Effectors may translocate inside of the host cytoplasm. (7) They may interfere with cytoplasmic processes of down-stream signals. (8) Effectors may control transcription of target genes in nucleus. (9) Cell surface receptors may recognize effectors to trigger ETI or (10) NB-LRR receptors of plant cytoplasm could recognize them (Jonge *et al*., 2011).

The strategies to identify the effectors are increased dramatically. First of them is to use bioinformatics programs. A good effector protein candidate should be small and secreted; also it should have cysteine rich residues in its structure as well as signal peptide. RxLR and Y/F/WxC motifs are also preferable (Göhre & Robatzek, 2008). With the results of ongoing genome projects and expression data, determination of possible effector candidates is quite practicable using bioinformatic tools. To do their confirmation experiments, marker-reporter gene constructs are useful such as *NHO*1 and luciferase. *NHO*1 is activated upon flagellin recognition. Therefore, effectors that are inhibit recognition can be scanned through this method (Göhre & Robatzek, 2008). Another strategy to observe immune responses such as ROS, callose deposition and HR can be detected *via* special staining techniques (Göhre & Robatzek, 2008). However, all of these strategies have their own drawbacks. First of all, effectors are species-specific or even isolate-specific so using other hosts such as *Arabidopsis thaliana* or *Nicotiana benthamiana* or even protoplasts of same organism might change the defense signaling pathway. Second drawback is the fact that transient expression is not occurred at the same concentration as in a real infection by its own nature. Other drawbacks are the overexpression, incorrect timing or oscillations in expression and lack of proper modifications which is only achieved *via* pathogen (Göhre & Robatzek, 2008). These obstacles may be overcomed using model pathogens to deliver candidates such as *P. Syringae* or *P. fluorescens*. Effector-vector-detector system of methodology is developed for these model organisms which enable us to characterize effectors and even identify new ones (Göhre & Robatzek, 2008).

In *Puccinia* rusts, genome sequencing project is completed for *P. graminis* f. sp *tritici* but not for *P. triticina* and *P. striiformis* f. sp. *tritici* (Yin & Hulbert, 2010). However, methods for purification of haustorial cells enable to identify the haustorial transcripts of *P. striiformis* f. sp. *tritici* (Yin *et al*., 2009a). Therefore, the effector candidate prediction is possible for *P. striiformis* f. sp. *tritici*. Moreover, using new identification and characterization methods, it is now possible to test avirulence on host depending on the presence of the HR response.

1.5 Gateway cloning

The protein expression of a gene is needed to perform the functional analysis including phenotype examination, protein purification, protein-protein interaction and subcellular localization determination (Hartley, 2000). The genes of interest should be subcloned into specific expression vector for each characterization analysis. The number of subcloning reactions may present a troublesome drawback if the gene number increases (Hartley, 2000). Easier and more efficient cloning methodologies are needed to achieve large scale functional genomics analysis. Site-specific recombination cloning based methods are quite useful in this manner. Among them, the Gateway technology of Life Technologies, Inc. (Hartley, 2000), is a good choice because its practical approach allow us to subclone our genes into multiple expression vector efficiently and effortlessly.

The technology is based on site-specific recombination which exploits the DNA integration mechanism of the bacteriophage λ. Bacteriphage has integrase enzyme which enables it to insert its DNA into the host chromosome (Karimi, Depicker, & Hilson, 2007). The gateway cloning method takes advantage of the phage's *att*B x *att*P → *att*L x *att*R recombination site mediated integration mechanism. The direction requires different proteins and sites to react. Hence, the direction of the recombination reaction can be manageable as in the gateway's BP and LR clonases mix. The cloning process starts with the cloning of the gene of interest into a donor vector (pDONR) (Hartley, 2000). This first step reaction can be achieved in various ways including restriction enzyme cutting and ligating, BP cloning reaction and TOPO cloning reaction. TOPO cloning occurs with the help of Topoisomerase I from *Vaccinia* virus and CACC-nucleotide site bearing PCR product. A PCR product with CACC-nucleotide site on its forward end invades the cloning vector on the overhang region of GTGG. Hence, directional cloning is achieved with efficiency greater than 90%. After integration of the PCR product into donor vector, it is named as pENTR clone and it ready to second step (Invitrogen protocol). Second step is to transfer the gene of interest from entry clone to destination vector (pDEST). The gateway technology exploits the *att*L and *att*R sites and their specific recombination reaction. In particular, *att*L1 site of the entry clone is react only with *att*R1 site of the destination vector and so *att*L2 site only with *att*R2 site (Karimi *et al*., 2007). Therefore, directionality of the cloning is not disrupted. At the end of the LR recombination reaction, 4 possible outcomes are present in the reaction environment. Entry vector with gene of interest and *ccdB* gene from the destination vector will die due to negative selection. Entry and destination vectors contain different antibiotics. Unreacted destination vector is selected due to the presence of the lethal *ccdB* gene between the recombination sites of the plasmid. Hence, only the destination vector with the gene of interest will survive and the clone is now named as expression clone (pEXPR) (Hartley, 2000) (Karimi *et al*., 2007).

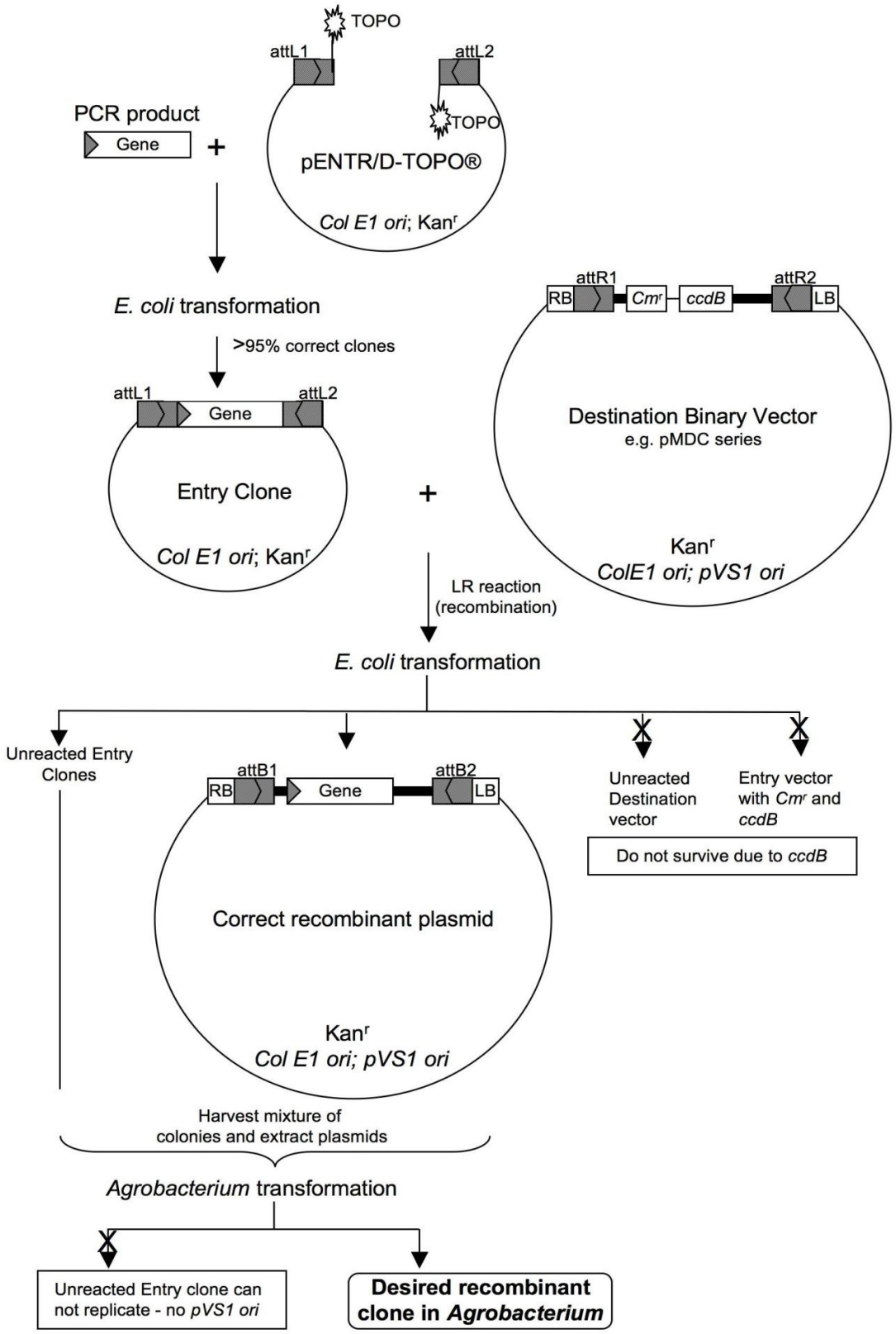


Figure 1.5 Schematic of Gateway cloning strategy (Xu & Li, 2008).

1.5.1 pEDV6 vector

pEDV6 is a modified Gateway destination vector which is useful to study pathogen effectors in plant cell. pEDV6 is an effector detector vector as pEDV3 and both of them are engineered from a broad host range having pBBR 1MCS-5 vector. *Avr*RPS4 is a well-characterized, secreted protein of *Pst* DC3000 which uses type III secretion system. The protein is cleaved at the 136th amino acid site of its N-terminus *in planta*. Moreover, it is reported that 88 amino acids from C-terminus is enough to perform avirulence function (Sohn, Lei, Nemri, & Jones, 2007). K. Sohn and his colleagues have exploited these features of the *Avr*RPS4. They constructed N-terminal fusions of *Avr*RPS4 to various proteins and tested these constructs for avirulence. The study showed that *Avr*RPS4 N-terminal signal bearing fusions regulated by the AvrRPS4 promoter can be delivered into the host cells. Therefore, designed constructs of AvrRPS4 can serve as an effector detector vector and named as pEDV. pEDV3 has AvrRPS4-Effector-HA design whereas pEDV6 owns *Avr*RPS4-HA-Effector pattern.

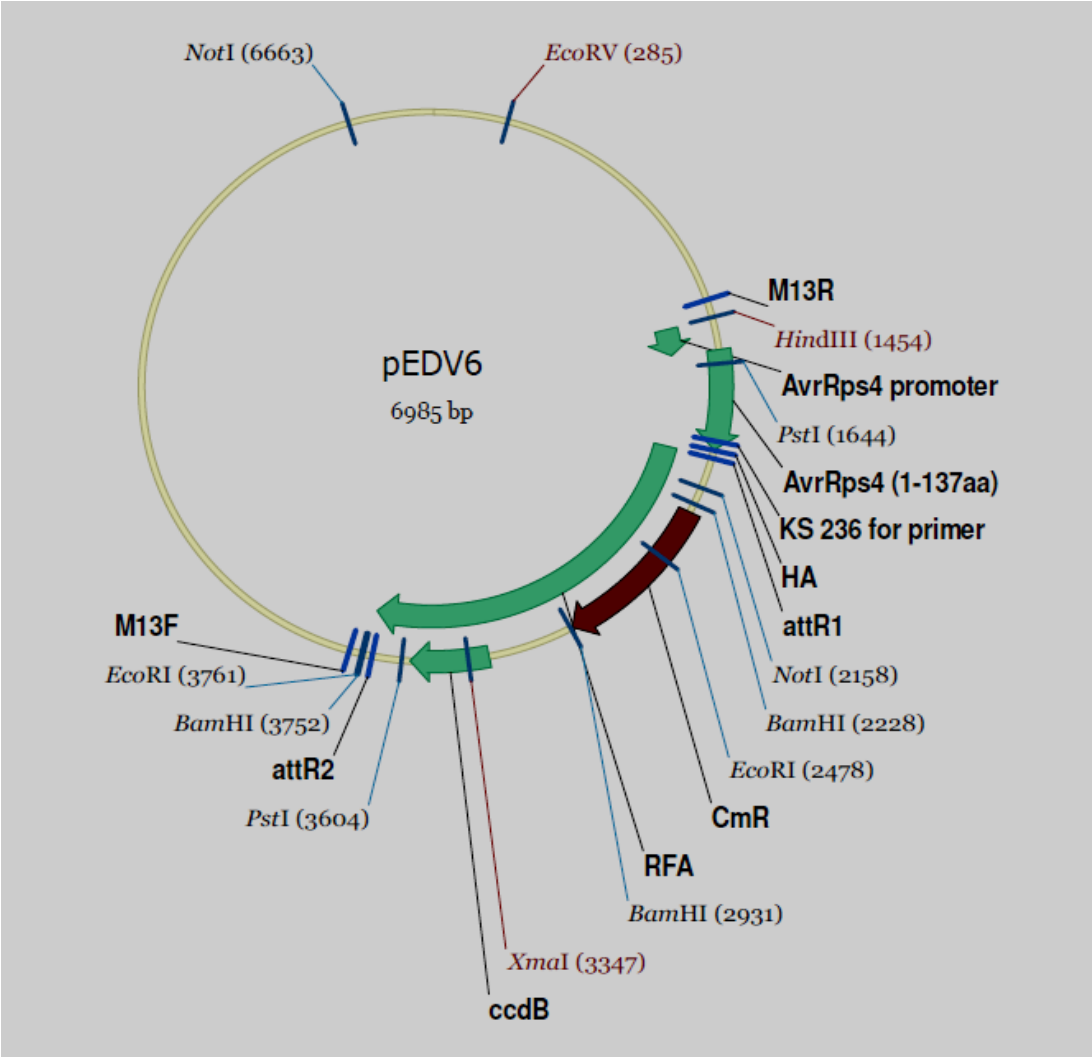


Figure 1.6 Vector map of pEDV6.

1.6 Wheat infiltration assay

We are now able to predict effector proteins of cereal rusts, thanks to advances in *Puccinia* genome sequencing projects and expression data profiles, along with the help of several bioinformatics' tools. However, we need certain biological assays to assess the specific functions of these candidates in a relatively high throughput manner. Transient expression in protoplasts, biolistic delivery of effector candidates and *Agrobacterium* mediated gene transfer are some of the methods to study and assign the functions of the predicted effectors. Each has their own drawbacks. For instance, in transient expression systems, effectors are not always accumulated as in the real infection process. Biolistic delivery requires examination of the individual cells in an effortful manner. *Agrobacterium* mediated gene transfer is applicable to a narrow range of grasses. There is another alternative method to study the specific functions of the pathogen effectors which uses type III delivery system (TTSS). It is called as *Pseudomonas fluorescens* mediated wheat infiltration assay.

Pseudomonas syringae DC3000 is a plant pathogen which can trigger HR upon infiltration to the wheat lines. Also, well-characterized AvrRPS4 protein of the same pathogen is engineered to serve as a vector promoter and N-terminal signal for the effector studies. The predicted effectors can be fused the vector and be delivered *via* TTSS to the host. Hence, this strategy is quite useful for effector researches. *Pseudomonas syringae* can trigger HR response by itself, so there is a need for another strategy to deliver effector in the same manner. For this purpose, W. J. Thomas and his colleagues stably integrate the TTSS-encoding genes of *Pseudomonas syringae* pv. *syringae* 61 into the genome of the soil born bacterium, *Pseudomonas fluorescens* Pf0-1. The transferred TTSS-encoding parts is in the *hrp/hrc* region of the donor *Pseudomonas syringae* and it also worked in its new host *Pseudomonas fluorescens* Pf0-1 making the host deliver its effectors. The engineered host named as *Pseudomonas fluorescens* Effector-to-Host Analyzer (EtHAn). Moreover, the new engineered system does not trigger hypersensitive response in wheat lines. Therefore; the specific functions of the effector will be likely monitored because the usual functions of the effectors, which is delivered by the pathogen's TTSS, will not masked the virulence functions of the effector of interest.

In summary, *P. fluorescens* (EtHAn) mediated wheat infiltration assay is quite promising to study specific effector functions *in planta*. Upon infiltration, hypersensitive (HR) of the host can be monitored visually or *via* staining methods depending on the presence of hydrogen peroxide or callose deposition.

1.7 Agrobacterium mediated gene transfer

Agrobacterium tumefaciens is a soil borne microorganism which is causative agent of the crown gall disease on a broad range of plants. Besides being a pathogen, *Agrobacterium* is important for the studies in various area including genetic transformation and oncogenesis. In nature, *Agrobacterium* uses its tumor-inducing (Ti) plasmid to produce the virulence

factors needed to form tumor and integrate the transfer DNA (T-DNA) into the host genome. Transferred DNA parts trigger the production of plant hormones such as opines and these hormones provide nutrients for *Agrobacterium* and lead to tumor formation.

Agrobacterium is one of the unique pathogen because of its ability to perform inter-kingdom gene transfer in such wide-range of hosts. This ability is exploited by the scientists to use *Agrobacterium* as a genetic manipulation tool. The unnecessary genes such as tumor growth are removed to prevent transgene escape. The disarmed plasmid of T-DNA region with engineered other DNA fragments were constructed as vector. Subsequently, *Agrobacterium* becomes the major genetic manipulation tool in plant researchers' arsenal. *Agrobacterium*-mediated transformation present great opportunity for studying host-pathogen interactions and also release of macromolecules into the host cells. The interaction requires many steps including host cell recognition, virulence (*Vir*) gene expression, attachment to the target cell, releasing *Vir* factors and T-DNA into the target cell and integration of the T-DNA to the host chromosome. After the successful integration of T-DNA, genes of interest in the T-DNA region can be expressed using the host own machinery.

Agrobacterium-mediated transient gene expression system can provide rapid, robust and practical analysis of the target gene expression *in planta*. Transient gene expression can be monitored a few days after agroinfiltration. The expression level reaches its peak at the 2-3 days post-infiltration. With the advances in new agroinfiltration based vector system, advantages of the *Agrobacterium* can be exploited. The rapid and robust expression levels and the effortless genetic manipulation of the method will present great opportunities in the plant-pathogen interaction studies.

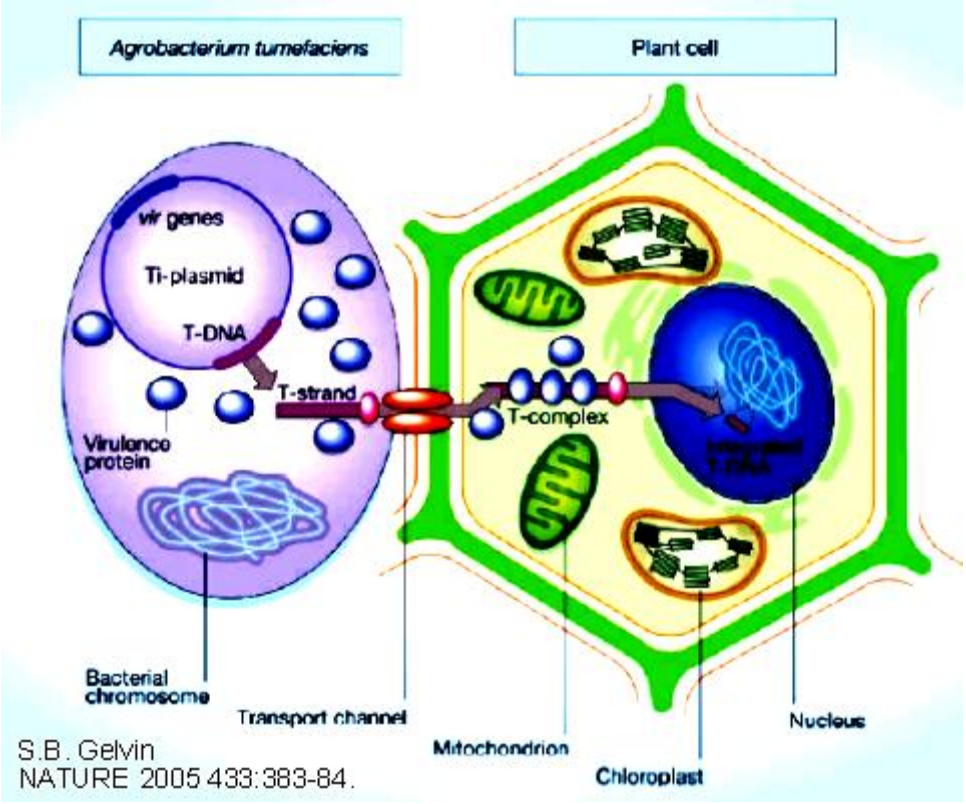


Figure 1.7 *Agrobacterium*-mediated gene transfer (Gelvin, 2005).

1.7.1 pK7FWG2 expression vector

pK7FWG2 is a gateway destination vector for plant expression studies. It has compatible binary T-DNA vector for *agrobacterium* mediated gene transfer. The vector is designed for subcellular localization experiments *in planta* using advantages of *Agrobacterium* which can transform wide-range of host species (Karimi, Inzé, & Depicker, 2002).

Usual *Agrobacterium* vectors work by transferring a gene into a cloning site between the left and the right borders of binary T-DNA vector (Karimi *et al*., 2002). The main problem of traditional vectors is having small multiple cloning site in very large vector construct. The low number of restriction enzyme makes cloning harder and time consuming so there is a trend to use gateway destination vectors in *Agrobacterium* mediated gene transfer studies (Dubin, Bowler, & Benvenuto, 2008)(Karimi *et al*., 2007). pK7FWG2 has *att*R1 and *att*R2 sites to clone the gene of interest into destination vector *via* recombination. It furnishes fast and reliable cloning opportunity because it uses Gateway conversion technology. It has an origin of replication to multiply in both *E. coli* and *Agrobacterium*. It also has enhanced green fluorescent protein (eGFP) in C-terminus for subcellular localization studies (Karimi *et al*., 2002). Because it is a Gateway destination vector, it has *ccdB* gene for negative

selection. Positive selection in bacteria is provided by Spectinomycin (Sm) and Streptomycin (Sp) resistance markers. The vector map is illustrated in Figure 1.8.

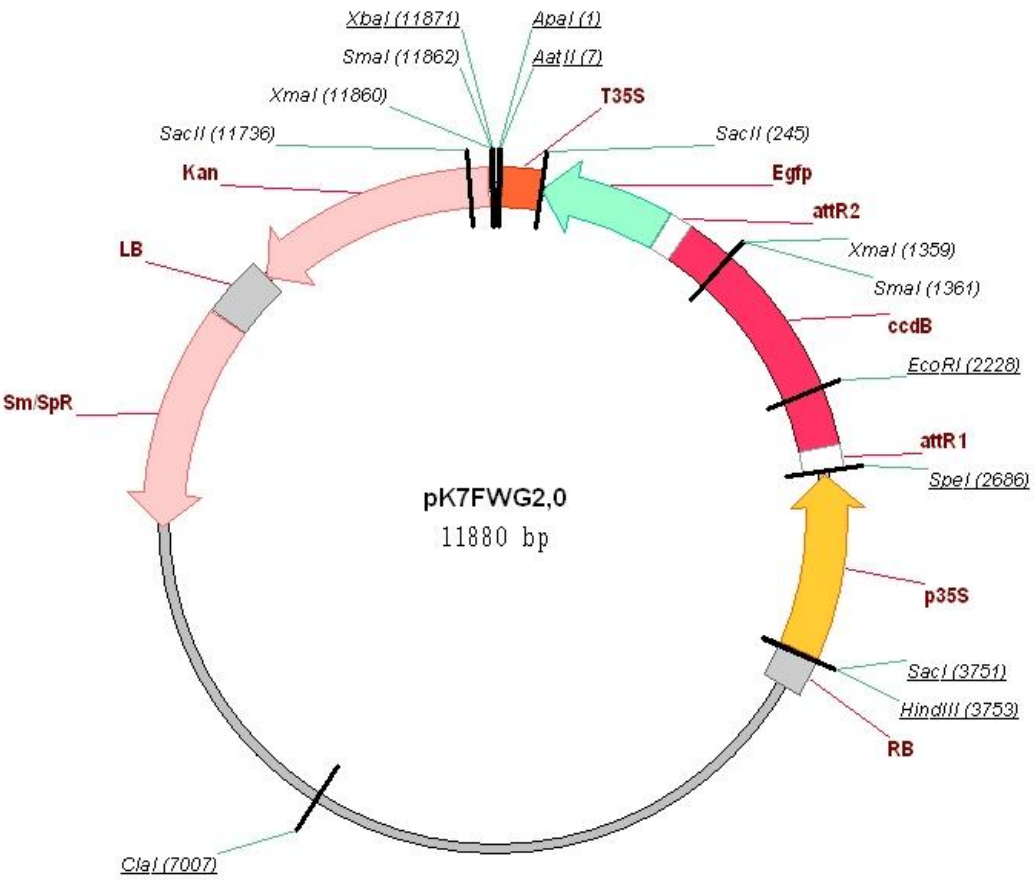


Figure 1.8 Vector map of pK7FWG2 (Karimi *et al*., 2002).

1.7.2 pJL48-TRBO

In plant biotechnology, there is an outstanding need for efficient recombinant protein expression system to conduct researches or to achieve large scale productions. *Agrobacterium* mediated gene transfer is quite practical method for this purpose (Wroblewski, Tomczak, & Michelmore, 2005)(Lindbo, 2007a). However, transient and robust expression couldn't be achieved due to posttranscriptional gene silencing. *Tobacco mosaic virus* (TMV) RNA-based overexpression vector (TRBO) was developed for rapid, efficient and transient expression (Lindbo, 2007b).

pJL48-TRBO is a compatible binary T-DNA vector designed for *Agrobacterium* mediated gene transfer analysis. It is a large vector (~10 kb) containing small multiple cloning sites with unique restriction enzymes (Figure 1.9). However, it is still smaller than other TMV.

The vector has 35S promoter-driven cloning site for expression. TMV CP gene sequence was removed from the vector which leads to higher and transient protein expression levels *in planta*. Moreover, this upgrade provides greater agroinfection efficiency. Therefore, hypersensitive response (HR) due to infiltration of host tissues with large amount of cells is evaded (Lindbo, 2007b).

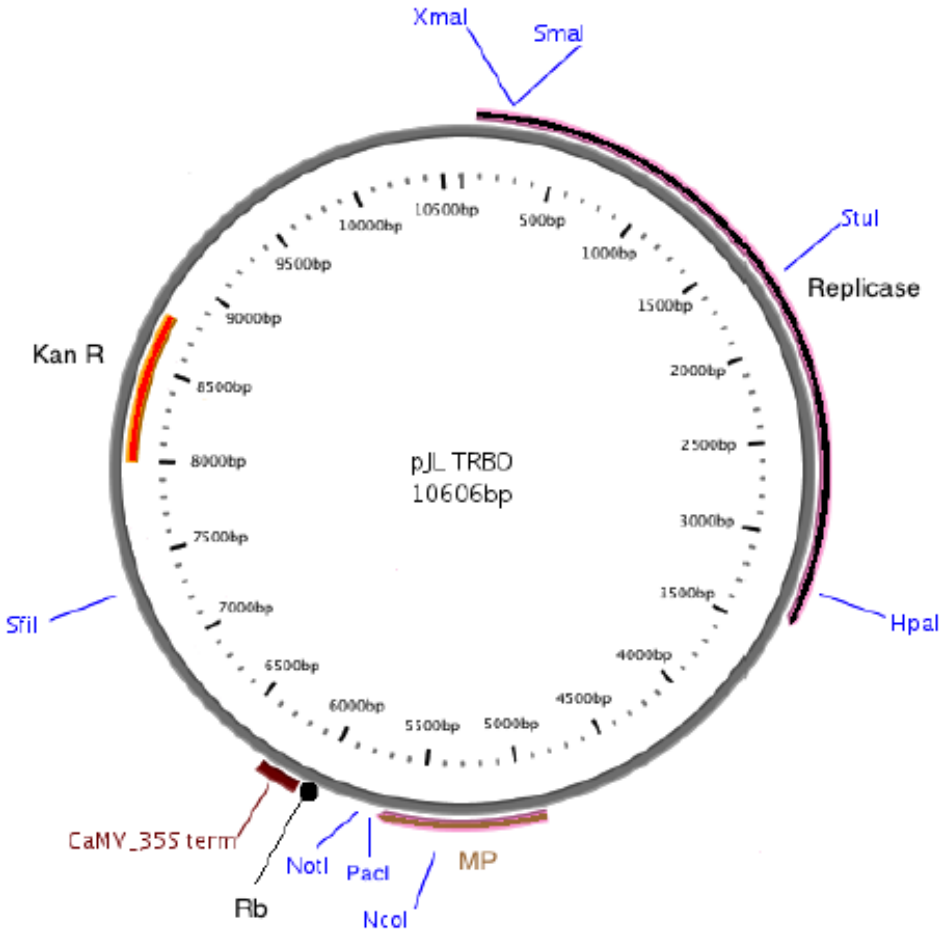


Figure 1.9 Vector map of pJL48TRBO (Xiaoli Dong *et al*., 2004).

1.8 Pstha15N21 gene

The identification of possible *Puccinia striiformis* f. sp. *tritici* effectors were achieved by constructing haustorial cDNA library and using data mining tools on these sequences (Yin *et al*., 2009b). The effector candidates were predicted based on the several criteria which are common to the effectors. First, effector should be small and secreted protein to reach their cellular target *in planta*; apoplastic or inside the host cell. Second, it should have high Cys-

rich residues because previous studies on haustorial transcripts from other cereal rust showed that they have high cysteine rich residues in their sequences. Another feature of the effectors is being unique or showing no significant similarity to known genes (Torto *et al*., 2003)(Göhre & Robatzek, 2008).

PstHa15N21 gene belongs to haustorial cDNA library of *Puccinia striiformis* f. sp. *tritici* (Table 1.2) (Yin *et al*., 2009a). Expression level analysis of the gene is yet to be studied. However, the gene has been predicted as a small, secreted protein and bears 7 cysteine residue in total 97 amino acids. The presence of the signal peptide has been predicted by using several online bioinformatics' tools including 'SignalP' (http://www.cbs.dtu.dk/services/SignalP-4.0/) and 'iPSORT' (http://ipsort.hgc.jp/). PstHa15N21 gene does not have Y/F/WxC motif after its cleavage site and also RxLR motif which is involved in secretion of oomycete effectors is not present in PstHa15N21. However, these motifs may show high level polymorphism due to 'arm race' model between pathogen and its host (Jonge *et al*., 2011).

Table 1.2 Predicted secreted effectors of cDNA library of *Pst* haustoria (Yin *et al*., 2009b).

Unisequence	GenBank accession	Size (aa)	No. of Cysteine residues	Homology in databases	E value
PSTha2a5	GH737102	117	6	predicted protein of *Puccinia graminis*	5.23E-23
PSTha9F18	GH737274	259	14	hypothetical protein of *Puccinia graminis*	8.96E-41
PSTha12a4	GH737444	289	10	predicted protein of *Puccinia graminis*	4.00E-23
PSTha12j12	GH737467	133	6	no homology	-
PSTha15N21	GH737567	98	7	no homology	-
PSTha21O8	GH737139	92	1	putative sulfate transporter	1.42E-38
PSTha5a23	GH737046	108	4	no homology	-
PSTha6i16	GH737950	73	5	predicted protein of *Puccinia graminis*	3.00E-10
PSTha8F13	GH738007	116	6	no homology	-
PSTha2c7	GH737231	204	13	predicted protein of *Puccinia graminis*	1.15E-15
PSTha16B3	GH737129	87	2	no homology	-
PSTha10F24	GH737323	56	3	no homology	-
PSTha16D6	GH737598	66	3	hypothetical protein of *Aspergillus niger*	2.00E-16
PSTha9C13	GH738022	65	1	predicted protein of *Puccinia graminis*	6.00E-10
PSTha12h2	GH737173	70	5	no homology	-

In summary, PstHa15N21 is a good effector candidate due to its high cysteine residues, presence of a signal peptide sequence in its N-terminus. Moreover, it shows no homology to other sequences in public databases. Hence, it makes the candidate important to determine its biological function. Confirmation of avirulence will provide us a model effector to use as bait to discover cellular mechanism of plant immune response.

1.9 Aim of the study

Biotic and abiotic stresses are the main causes of yield and quality loss in cultivated cereals worldwide. Cereal rusts are among the most devastating and damaging diseases on crops. The stripe (yellow) rust is one of these cereals rusts which is notorious for leading huge yield losses up to 70% in wheat cultivated areas. Depending on the susceptibility of the cultivar and environmental conditions, the lost can be increased up to 100%. The causative agent of the disease is *Puccinia striiformis* f. sp. *tritici* (*Pst*) which is an obligate biotrophic pathogen of wheat.

Plant innate immunity is a hot topic among researches in the field of plant biotechnology because overcoming plant diseases is only possible *via* understanding molecular and cellular mechanism of plant immune system. Discovering the aspects of immune system will help us to better engineer resistant cultivars to overcome diseases. One way to achieve this is to understand disease formation and resistance mechanism at cellular level. The identifying pathogen effectors and to determine their role and interactions in PTI and ETI will produce critical information. Although there are many studies to comprehend the molecular complexity of the plant pathogen interaction, there are still much to learn.

In this thesis study, a candidate effector protein of *Pst* was studied for investigating its function. For this purpose, an effector candidate was selected from the literature of which was identified using haustorial cDNA library and data mining tools. The selected effector candidate, PstHa15N21, is a small (97 amino acids) secreted protein which has 7 cysteine residues in its sequence. These features qualify, it as an effector protein of *Pst*. Various cloning experiments were conducted using uniquely engineered vectors for specific purposes. PstHa15N21 was cloned into pEDV6 vector for testing its avirulence by transforming *Pst* differential lines (plant line) each having a particular R-gene. The pEDV6 vector uses Type three secretion system (TTSS) of *P. fluorescens* (EtHAn) for bacterial transformation of the effector. Another vector used for cloning the effector was Gateway recombination cloning vector, pK7FWG2, to detect subcellular localization of PstHa15N21 in *planta* by agro-infiltration method. The effector of interest was also cloned into plant expression vector, pJL48-TRBO, with a FLAG-Tag and linker sequence to determine the interacting host factors in *N. tobaccum* and/or *N. benthamiana*. Aside from the determination of host interactering factors, the virulence tests and subcellular localization analysis of the effector were carried out with important preliminary data.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials and pathogen maintenance

2.1.1 Wheat cultivation, pathogen inoculation and maintenance

For *Pst* pathogen maintenance, Avocet-S wheat line was used due to its susceptibility to yellow rust races, as the ones we maintained such as *Pst*TR09-97 and *Pst*TR-Mix races. About 40-50 seeds per pot, with a volume of 650 cm^3, were planted in soil. The plants were kept under 16-hour light at 18 ^oC and 8-hour dark at 15 ^oC conditions with sufficient humidity in a growth chamber for ten days. At one- to two-leaf stage, the sprouted wheat plants were inoculated with yellow rust spores. Before inoculation, yellow rust spore which were stored at -80 ^oC were incubated at 42 ^oC for 10 minutes to prepare the spores for inoculation. If the storage was performed at +4 ^{o} C in a desiccator, 5 minutes incubation at 42 ^{o} C was enough for successful inoculation. After this step, the spores were treated with mineral oil to increase attachment to leaf surface. Then, the spores were sprayed on the leaves with the help of air pump tank which resembles an atomizer. All the steps were carefully performed in a sterile hood to prevent possible contamination. The hood was sterilized before and in between the inoculations with alcohol wipes and UV exposure to especially when different strains were used. The *Pst* inoculated plants were placed in a sterile glass chamber with humidifier and they kept at dark for overnight. The humidifier was removed after about 16-18 hours and the infected plants were kept under their normal growth conditions, described as above. In 10-15 days of post-inoculation, the new yellow rust spores were observed on leaf blades. The spores were collected under the aseptic conditions and placed at +4 ^{o} C in desiccators for short term storage or – 80 ^oC for long term storage.

Figure 2.1 Yellow rust inoculation. Lab mate holding the sprayer.

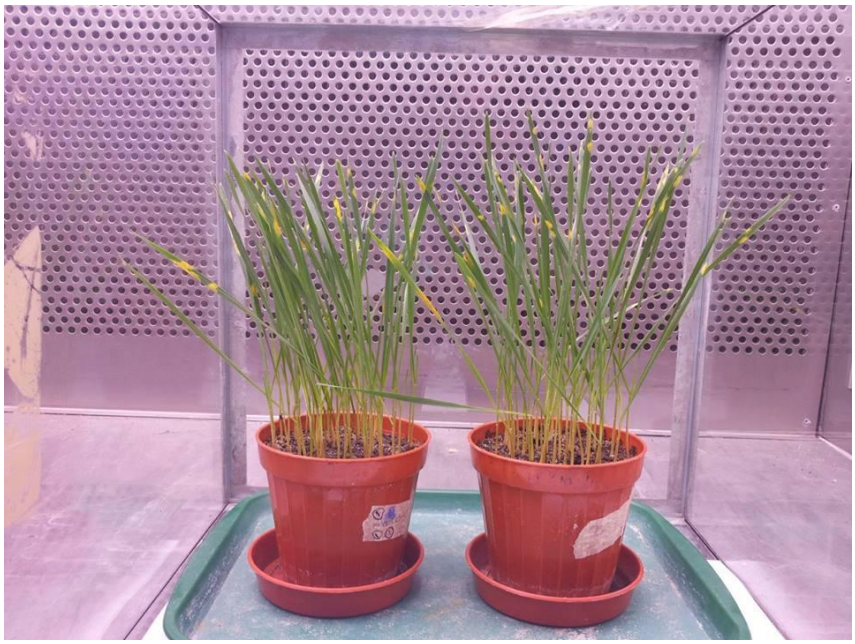


Figure 2.2 Wheat plants after 10 days post inoculation (dpi). Yellow color on the leaves is due to spore accumulations.

2.1.2 The growth of *Nicotiana benthamiana*

Nicotiana benthamiana seeds were planted in a small pot (250 cm^3 volume) under 16-hour light and 8-hour dark conditions at 24 ^oC. The pot was covered with a stretch film with holes for breathing to increase the humidity. After 1 week, new sprouts were sub-cultured into larger pots (650 cm^3 volume). Each pot had one sprout. After 4-6 weeks, the grown *N. benthamiana* plants were selected and used for *Agrobacterium* mediated gene transfer for subcellular localization assays.

2.1.3 Wheat differential lines

The differential lines of the wheat (*Triticum aestivum*) were obtained from ICARDA originally. Table 2.1 shows the names of the planted seeds and their corresponding resistance genes. Total of 27 lines were planted in soil, 6 seeds per each pot (250 cm^3) for single line. After 10-12 days, two-leaf staged wheat lines were used for *Pseudomonas fluorescens* mediated transformation for infiltration assays.

Table 2.1 The list of the wheat differential lines.

13YR-DIFF-SET	Cultivar Name	GROUP	YR genes
1	MOROCCO	YR	**-**
2	AVOCET-YRA	YR	**No YrA**
3	AVOCET+YRA	YR	**YrA**
4	AVOCET+YR1	YR	**Yr1**
5	SIETE CERROS T66	YR	**Yr2**
6	TATARA	YR	**Yr3+Yr29,+**
7	AVOCET+YR5	YR	**Yr5**
8	AVOCET+YR6	YR	**Yr6**
9	AVOCET+YR7	YR	**Yr7**
10	AVOCET+YR9	YR	**Yr9**
11	AVOCET+YR10	YR	**Yr10**
12	AVOCET+YR15	YR	**Yr15**
13	AVOCET+YR18	YR	**Yr18**
14	AVOCET+YR24	YR	**Yr24**
15	AVOCET+YR26	YR	**Yr26**
16	AVOCET+YR27	YR	**Yr27**
17	AVOCET+YRSP	YR	**YrSP**
18	PAVON F 76*	YR	**Yr6, Yr7, Yr29, Yr30, +**
19	SERI M 82*	YR	**Yr2, Yr9, Yr29, Yr30, +**
20	OPATA M 85*	YR	**Yr18, Yr27, Yr30, +**
21	SUPER KAUZ*	YR	**Yr9, Yr18, Yr27, Yr30, +**
22	POLLMER_2.1.1	YR	**?**
23	AVOCET+YRCV*	YR	**YrCv**
24	AVOCET-YR*3/3/ALTAR 84/AE.SQ//OPATA	YR	**Yr28?**
25	AVOCET-YR*3//LALBMONO1*4/PVN	YR	**Yr29**
26	AVOCET-YR*3/PASTOR	YR	**Yr31**
27	AVOCET-YR/ATTILA	YR	**Yr29, Yr18?**

*(Yang *et al.*, 2011). ?: Inconclusive reports.

2.2 Prediction of the effector candidate and design of the gene construct

PstHa15N21 gene was predicted as an effector candidate from the haustorial cDNA library data by Yin *et al.*, 2009. PstHa15N21 sequence with GenBank accession number of GH737567 was gathered from expression tag sequences (EST) of National Center of Biotechnology Information (NCBI) databases (Yin *et al.*, 2009b). The cDNA sequence was placed in ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) of NCBI to determine the full length protein sequence in order to confirm the presence of signal peptide and the number of cysteines.

The PstHa15N21 was tested in two different signal prediction programs to verify the presence of a signal peptide in N-terminus of the effector. 'SignalP 4.0' (http://www.cbs.dtu.dk/services/SignalP-4.0/) tool predicted that PstHa15N21 has a signal peptide region and the possible cleavage site between 17th and 18th amino acid. 'iPSORT' signal prediction tool (http://ipsort.hgc.jp/) supported the result.

We removed the signal peptide encoded part from the gene sequence in our gene construct because the signal peptide is removed upon the delivery in real infections. Moreover, this signal part may lead to transfer of our effector candidate to the outside of the host.

We designed the gene construct with the restriction enzymes; *Pac*I at 5' end and *Not*I at the 3'end since the cloning sites are present in the pJL48-TRBO vector for directional cloning with the correct ORF. The construct was synthesized by GenScript Company and cloned into pUC57 vector as in the following figure (Figure 2.3). The construct also has a FLAG-Tag region on its N-terminus to perform immunoprecipitation assay on the host cells. The vector was transformed into *E.coli* Top10 cells immediately upon arrival and stored at -80 ^{o}C.

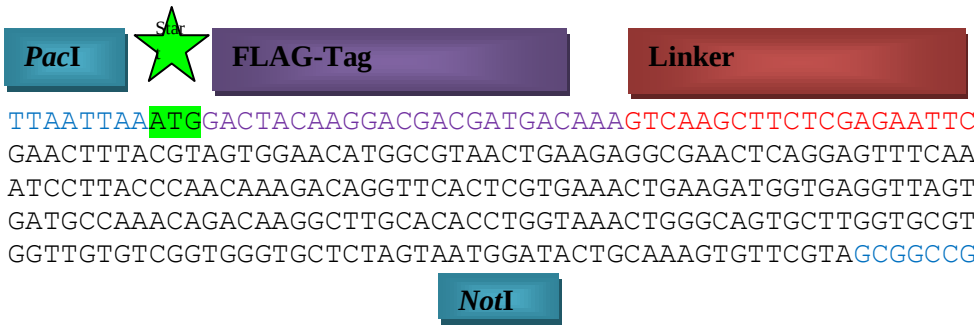


Figure 2.3 PstHa15N21 gene construct. The gene of interest without its stop codon and without the signal peptide region is presented in black. Restriction enzyme cut sites are labeled blue and the start codon with green. Flag-tag and its linker sequences are indicated with purple and red color, respectively.

2.2.1 Primers of the PstHa15N21 gene

Total of 5 primer sets were designed to work with three different vectors each to carry out different assays. The list of the primers is presented in Table 2.2. Forward primer of the Gateway cloning was designed with an additional CACC nucleotide site to achieve directional cloning into Gateway vectors. Two different reverse primers were designed for Gateway cloning with and without a stop codon. Subcellular localization vector pK7FWG2 has a GFP fusion on its C-terminus, while pEDV6 vector needed a stop codon for effector delivery. In pJL48-TRBO vector primers, forward primer has a part of FLAG-Tag region, *Pac*I cut site and additional nucleotides to increase the restriction enzyme efficiency. Reverse primer of the same vector had a stop codon, *Not*I site with additional nucleotides for the cut site.

Table 2.2 The list of PstHa15N21 primers.

Primer Names	Primer sequences (5' end to 3' end)
Pst15N21-CACCFwd	CACCATGACGGAAGAACTTTACG
Pst15N21-NoSTP-Rev	TAC GAA CAC TTT GCA GTA TCC ATT AC
Pst15N21-Stp-Rev	CTATACGAACACTTTGCAGTATCCAT
Pst15N21-Flag-Fwd	GGATTAATTAAATGGACTACAAGGACGACGATGACAAAGTCAAGCTTCT
Pst15N21-Rev	TTTGCGGCCGCCTGCCCAGTTTACCAGGT

2.3 Polymerase chain reaction

The gene of interest was amplified using Q5 High Fidelity *Taq* DNA Polymerase (NEB, Cat #: M0491S, Lot # 0041209) with the corresponding primer sets depending on the vector of choice. Polymerase chain reaction (PCR) was performed following the manufacturer's protocol. The names of the reagents and their amounts used in each reaction were presented at Table 2.3.

Table 2.3 The final concentrations of PCR components.

PCR Components	Final concentration
Template DNA (Variable; 20ng-150ng)	<1000 ng
5X Q5 Reaction Buffer	1X
10 mM dNTPs (NEB, Lot#0711209)	200 µM
10 µM Forward Primer (10pmol/ µL)	0.5 µM
10 µM Reverse Primer (10pmol/ µL)	0.5 µM
Q5 High Fidelity DNA Polymerase (2000U/mL)	0.02 U/µL
5X Q5 High GC Enhancer	1X
Nuclease Free Water	
Total volume: 25 µL	

Upon mixing of the reagents in 200 µL PCR tubes, reaction tubes were placed in thermocycler (Eppendorf-Mastercycler Gradient). The thermocycling conditions of the PCR process were presented in Table 2.4.

Table 2.4 Thermocycling conditions of the polymerase chain reactions.

Steps	Temperature ^{o}C	Time	Cycle number
Initial Denaturation	98	30 sec	1
Denaturation	98	15 sec	35
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	2 min	1

Agarose gels were prepared using 0.5 g of agarose (AppliChem, Lot# 1R002306), 50 mL 10X TAE buffer (AppliChem, Lot# 7B010350) and EtBr (0.5 µg/mL) (Merck, Lot# 0C476872) to a final volume of 50 mL. The PCR products were loaded and separated on 1% agarose gel in 1X TAE buffer under 60 V for ~50 minutes. The results were recorded.

2.4 Plasmid isolations

All plasmid isolation steps were performed using QIAprep® Spin Miniprep Kit (Cat# 27106, Lot# 142349895) following the manufacturer's procedure with slight changes. The fresh cell cultures, grown in their respective growth conditions and media (usually in 4 mL LB medium with corresponding selective antibiotic at 37 ^{o}C) for overnight, were collected through centrifugation (CLP Model 3410 Microcentrifuge) at 4000 rpm for 2 minutes in a sterile 2.0 mL eppendorf tube. Medium was removed from the cell pellet. The harvested cells were treated with 250 µL Buffer P1 with RNAseA (to final concentration of 0.1 mg/mL) and the cells were vortexed (Fisons, Cat# SGP-202-010J) to achieve resuspension without any visible cell clusters. 250 µL of Buffer P2 was immediately added to the samples and the tubes were mixed gently by inversions for 4-6 times until the lysate solution looks like viscous, clear blue color if the LyseBlue agent was mixed in Buffer P1 before usage. 350 µL Buffer N3 was added to the mixture as a neutralization buffer and the tubes were mixed gently *via* inversions for 4-6 times until the blue color of the samples becomes clear. The samples were centrifuged at 13,000 rpm for 10 minutes with bench-top microcentrifuge to remove cell debris. The clear, colorless supernatant of the samples was transferred into a QIAprep spin column without disturbing the white pellet. The column was centrifuged at 13000 rpm for 1 minutes and the flow through was discarded. PB buffer was added to the column and centrifugation was performed at 13,000 rpm for 1 minutes. The flow through was discarded. The PE buffer of 750 µL was added to wash the column. The centrifugation was performed at 13,000 rpm for 1 minutes and the flow through was discarded. An additional centrifugation was performed at 13,000 rpm for 2 minutes to remove all traces of the alcohol. The column was placed in a new, sterile 1.5 mL centrifuge tube and 30-50 µL of nuclease free water was added directly to the membrane of the column. The sample was allowed to stand for 1 minute and centrifugation was performed at 13,000 rpm for 1 minute. The flow through was analyzed using NanoDrop (ND-1000 spectrophotometer). The results were recorded.

2.5 *P. fluorescens* mediated wheat infiltration assay

The PCR product of PstHa15N21 with stop codon and CACC nucleotide sequence was planned to clone into the Gateway destination vector, pEDV6. Then, the cloned vector was transformed into the *P.fluorescens* (EtHAn) by using electroporation method. Transformed bacteria were infiltrated into wheat leaves. The leaves were stained with DAB and visualized if any HR symptom was present. Figure 2.4 represents the flow chart of the assay in summary.

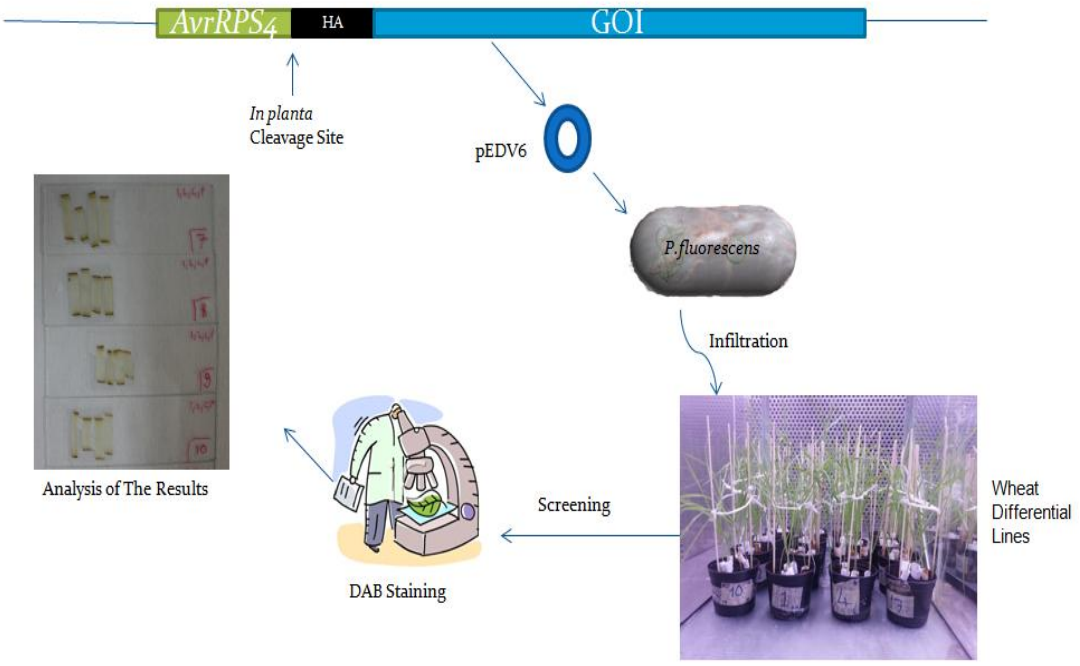


Figure 2.4 Illustration of the general flow of the wheat infiltration assay.

2.5.1 Gateway cloning

PstH15N21 effector candidate was amplified with Pst15N21-CACCFwd and Pst15N21-Stp-Rev for pEDV6 vector. Before subcloning into pEDV6 destination vector, the gene of interest must have cloned into the entry vector for the success of the Gateway cloning. To achieve the first step, we used the entry vector known as pENTR^{TM}/D-TOPO. After cloning our insert into the entry vector, we proceeded with subcloning into destination vectors.

2.5.1.1 pENTR™/D-TOPO vector cloning

Amplified PstHa15N21 gene products having both CACC-nucleotide sequence and stop codon were inserted into the pENTR™/D-TOPO vector by the aid of topo-isomerase. The reaction was performed using pENTR™ Directional TOPO® cloning kit (Invitrogen, Lot# 1300973A) following the manufacturer's protocol with slight changes in the procedure. The concentration of the PCR product was measured using NanoDrop and the vector/PCR product ratio was determined as 1:2. Reagents of the reaction were presented in Table 2.5 with the amounts used for the reactions.

Table 2.5 The reagents and their reaction volumes for pENTR/D-TOPO cloning.

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

In pENTR/D-TOPO, Topo-isomerase enzyme was attached to covalently to the linearized vector. Therefore, the components of the reaction were mixed gently in a sterile 200 µL PCR tube to avoid possible damage to the enzyme. The reaction took placed at 22-23 ^{o}C for 30 minutes in thermocycler. The reaction products were kept at +4 ^{o}C until they were used for transformation.

2.5.1.2 Preparation of *E.coli* TOP10 competent cells

A streak plate was prepared from *E.coli* TOP10 master stock (-80 ^{o}C) to obtain single colony and incubated at 37 ^{o}C for overnight. The single colony was inoculated into 4mL LB (Lysogeny broth was prepared *via* autoclaving 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 1.6 mL NaOH (0.5 M) mixture which was dissolved in 0.5 L ddH_2O) and incubated overnight at 37 ^{o}C at 250 rpm in a shaker without any antibiotic. *E.coli* TOP10 cells of 1 mL used to inoculate 100 mL sterile LB medium. Inoculum was incubated at 37 ^{o}C at 250 rpm for about 2 hours until the absorbance of the cells (A_{600nm}) reached to 0.375. The cells were transferred into two sterile, pre-chilled 50 mL falcon tubes. The culture stood on ice bath for 10 minutes and collected by centrifugation at 5000 rpm, 4 ^{o}C for 5 minutes. The supernatant was removed and to the pellet, ice cold, filter sterilized (MN sterilizer, 0.22 µm cellulose acetate filter) 10 mL 100 mM $CaCl_2$ was added. The cells were suspended gently to avoid any harm, let sit on ice for 10 minutes. The cells were again centrifuged at 5000 rpm for 5 min, at 4 ^{o}C for washing with 10 mL ice cold, sterile 100 mM $CaCl_2$. Cells we washed for twice as above and suspended in ice cold, 2 mL 100 mM $CaCl_2$. The competent cells were stored at +4 ^{o}C. The competency of the cells lasts about 10 days.

2.5.1.3 Transformation of pENTR™/D-TOPO ligates

To the 100 µL of *E.coli* Top10 competent cells 3 µL ligation product added. The mixture was let stand on ice for 10 min. The cells were immediately placed in water bath at 42 °C for 45 seconds. After the heat shock, the cells were swiftly placed back on ice for 5 minutes. 500 µL LB medium was added onto the cells and incubated at 37 °C by shaking at 200 rpm for 1-2 hours. The cells of 10 µL, 50 µL and 200 µL were spread to LB agar (LB agar medium consists of autoclaved mixture of 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g agar, 1.6 mL NaOH (0.5 M) in 0.5 L ddH_2O) containing 50 µg/mL Kanamycin (Applichem, Lot# NR12477). Following day, successful colonies used to inoculate 4 mL LB medium containing Kanamycin (50 µg/mL). The cells were grown at 37 °C at 250 rpm in a shaker for overnight. The grown cells were stored at -80 °C as glycerol stocks.

2.5.1.4 Colony PCR

The transformants were verified by colony PCR to ensure the grown colonies weren't false positive. In colony PCR *Taq* DNA Polymerase with Standard *Taq* Buffer (New England Biolabs, Cat# M0320S, Lot#0141203) was used with the primers used for cloning. Also, one additional PCR was done without template as negative control. Colony PCR components are presented in Table 2.6.

Table 2.6 The final concentrations of the colony PCR components.

Reagents	Final concentration
10X Standard *Taq* Reaction Buffer	1X
25 mM $MgCl_2$	1.5 mM
10 mM dNTPs	200 µM
10 µM forward primer	0.2 µM
10 µM reverse primer	0.2 µM
Template DNA (100-1000 ng/µL)	<1000 ng
Taq Polymerase (5000 U/mL)	1.25 Units
Nuclease free water	
Total volume: 25 µL	

The reaction mixtures were prepared in sterile 200 µL PCR tubes. The thermocycler was preheated to 95 °C and the tubes were immediately placed on thermocycler to avoid non-specific annealing. The thermocycling conditions of the colony PCR are presented in Table 2.7.

Table 2.7 Thermocycling conditions of the colony PCR**.**

PCR steps	Temperature ^{o}C	Time	Number of cycles
Initial denaturation	95	30 sec	1
Denaturation	95	30 sec	
Annealing	55	1 min	33
Extension	68	1 min	
Final extension	68	5 min	1

The colony PCR products were loaded on 1% agarose gel in 1X TAE buffer and separated under 60 V for about 50 min. After confirmation of the transformants by colony PCR, we isolated the plasmids of the positive clones which were stored at -80 ^{o}C. The isolation step was performed following the procedure described in Section 2.4.

2.5.1.5 Subcloning into pEDV6 destination vector

Subcloning into pEDV6 destination vector was another Gateway cloning. In this reaction, Invitrogen Gateway^{R} LR Clonase^{TM} II Enzyme Mix kit (Invitrogen, Cat # 11791-020, Lot # 1296175) was used to transfer the gene of interest from the entry vector into the destination vector. The isolated plasmids of pENTRY-PstHa15N21 were prepared as in the Table 2.8. The reaction was performed following the manufacturer's protocol with both negative and positive controls which were provided by the enzyme kit.

Table 2.8 Reagents of the LR cloning reaction and the amounts.

Reaction components	Sample (µL)	Neg. Control (µL)	Pos. Control (µL)
pENTR-gus (50ng/µL)	-	-	1.0
pENTRY-PstHa15N21h2 (90 ng/µL)	1.0	-	-
pEDV6 vector (76 ng/µL)	1 .0	1.0	1.0
2X TE buffer	2.5	3.5	2.5
LR Clonase enzyme mix	0.5	0.5	0.5
Total volume	5.0	5.0	5.0

Sample: Gene of interest (Pst15N21).

The reaction mixture was prepared by gently pipetting as in Table 2.7 in sterile 200 µL PCR tubes. LR reaction was carried out at 25 ^{o}C for 1 hour and terminated by adding 1 µL of Proteinase K (2 µg/mL) and incubating at 37 ^{o}C for 10 minutes. The half of the reaction product (2.5 µL) was placed on ice bath for transformation while the other half was stored at +4 ^{o}C. The transformation was carried out as described in Section 2.5.1.3. Different volumes of the cells (10, 50 and 200 µL) were spread on LB agar media containing 100 µg/mL Gentamycin (FisherBioReagents, Cat# BP918-1, Lot# 055317) plates. The single colonies

were picked and grown for plasmid isolation in LB containing Gentamycin (100 µg/mL). The colonies were stored in glycerol at -80 ^{o}C.

2.5.1.5 Confirmation of pEDV6 clones

To verify the transformants, we used colony PCR, double digestion of the destination vector and sequencing of the insert.

We repeated the same procedure as in the section 2.5.1.4. The PCR products of the colony PCR were separated by electrophoresis. The single colonies were picked and plasmids were isolated from them in Section 2.4.

The plasmids were double digested using *Pst*I (NEB, Cat# R0140S, Lot# 0411106) and *EcoR*I high fidelity (NEB, Cat# R3101S, Lot# 0031106) restriction enzymes to confirm subcloning. These two enzyme cut sites positioned at the of the recombination site. Hence, if the gene of interest was successfully recombined into cloning site, it would produce bands on its own size. Double digestion reaction mixture was prepared as in Table 2.8 following the manufacturer's protocol.

Table 2.9 Double digestion conditions of pEDV6-PstHa15N21.

Reaction Components	**Volume (µL)**
Plasmid DNA (~ 100 ng)	15.85
*EcoR*I-HF (20.000 U/mL)	1
*Pst*I (20.000 U/mL)	1
NEB buffer#4 (10X)	2
BSA (100X)	0.15
Total volume	20 µL

The reaction was prepared as in Table 2.8. Empty pEDV6 vector was also cut with the enzymes for comparison. The reaction products separated by electrophoresis explained above.

Following the verification, the plasmids were made sequences by MCLAB Company, USA, The primers used were sequencing were the same as the ones used for amplification to clone. Hence, we proceeded with the next step.

2.5.2 Transformation of *P. fluorescens* (EtHAn)

There were two transformation method available in the literature; heat shock transformation and electroporation for transforming the *P. fluorescens* (EtHAn). We have tested both of them; transformation by heat shock (Appendix F) was failed in our hands, but electroporation with various optimization experiments made to work very successfully.

2.5.2.1 Electrocompetent cell preparation and electroporation

Thus, we adapted a protocol used for electrocompetent *P. aeruginosa* cells (Choi, Kumar, & Schweizer, 2006) with various modifications and optimizations.

6 mL of the fresh overnight culture of *P. fluorescens* was divided into 4 microcentrifuge tube. The cells were harvested by centrifugation at 16,000 g at room temperature for 1-2 min. The pelleted cells were washed twice with filter sterilized 1 mL 300 mM sucrose. The cells were combined and used in electroporation step.

5 µL of pEDV6-PstHa15N21 plasmids were mixed with 100 µL of electrocompetent cells. The mixture was poured into pre-cooled pulser cuvette (1 mm gap). 25 µF, 2.2 kV electric pulse was applied to the cells with electroporator (Celljject duo, Therma corporation). 1 mL of SOC was added into the cells. For recovery, the cells were incubated at 28 ^{o}C at 200 rpm in a shaker. The incubated cells were centrifuged at 4000 rpm for 2 min. The medium was removed until 200 µL of medium remains. The remaining cells were spread in a LB agar with Gentamycin (100 µg/mL) and incubated at 29 ^{o}C. The colonies were observed after 2 days and they were confirmed by colony PCR as described in section 2.5.1.4. After confirmation, the cells were stored in 20% glycerol solution at -80 ^{o}C.

2.5.3 Wheat infiltration assay

Wheat infiltration assay (adapted from David Joly, Canada) protocol was obtained from CSIRO-Plant industry (N.Upadhyaya) and all analyses were performed following this protocol with slight changes. Upon the preparation of the cell cultures for the analyses, wheat differential lines (listed in Table 2.1) reached fully expanded two-leaf stage and these plants were used for the infiltration assay.

One big loopful of *P.fluorescens* (EtHAn) was inoculated in 5 mL LB medium with 100 µg/mL Gentamycin. Also, empty *P.fluorescens* (EtHAn) and *P.fluorescens* (EtHAn) with pNR527 were inoculated into 5 mL LB medium without antibiotic and with 15µg/mL, respectively. The cells were incubated at 29 ^{o}C for overnight at 200 rpm in shaker. The overnight cultures were inoculated into 50 mL LB medium with selective antibiotic. The cells were incubated at 29 ^{o}C for 4-6 hours at 200 rpm in shaker until the A_{600nm} value reaches 0.5. The cells were harvested in sterile falcon tubes by centrifugation at 5000 g, 4 ^{o}C

for 12 min. The supernatants were discarded and the pellets were washed with 25 mL of filter sterilized and chilled 10 mM $MgSO_4$ and resuspended. The centrifugation and washing steps were repeated. The cells were resuspended in 15 mL of sterile minimal media with sucrose which was prepared as in the Table 2.9 and antibiotic was added if necessary. The A_{600nm} values of the resuspensions were adjusted to 1.0. The cultures were incubated at 20 ^oC at 200 rpm for overnight. Next day, the cells were collected by centrifugation at 4 ^oC for 12 min. The pellets were resuspended in 10 mM $MgCl_2$. The A_{600nm} was adjusted to 2.0. The *P.fluorescens* (EtHAn) cells were kept at room temperature until the infiltration step.

Table 2.10 Preparation of minimal media with sucrose

Reagents	**Amounts**	**Final concentrations**
1M K_2HPO_4 (Merck, Lot# A488700-023)	4.25 mL	50 mM (Potassium phosphate buffer)
1M KH_2PO_4 (Merck, Lot# A0104673-938)	45.75 mL	
760 mM $(NH_4)_2SO_4$ (Merck, Lot# A897317-824)	10 mL	7.6 mM
1.7 M $MgCl_2$ (Sigma, Lot# 49H04351)	1 mL	1.7 mM
1.7 M NaCl	1 mL	1.7 mM
Sucrose (Merck, Lot# K41912651-128)	3.42 g	10 mM
Sterile H_2O	938 mL	-
Total	1 L	

In the infiltration part, the wheat differential lines were labeled clearly. The cells were infiltrated with the cells (1-2 cm from the infiltration spot on the leaves) using sterile needless syringes as it is shown in the Figure 2.3. The infiltrated plants were placed in a glass cabin with a steam engine to provide shaded mist. The glass cabins were transferred into the growth chamber at 24 ^oC with 16 hours light. The HR symptoms were monitored over the next 2-7 days.

Figure 2.5 Infiltration of wheat leaves with *P. fluorescens* suspensions.

2.5.4 DAB staining of the infiltrated leaves

DAB staining was performed following the protocol described by Yin and Hulbert (Yin & Hulbert, 2010). 1 mL of DAB 50X solution (Millipore, Cat# E5005, Lot# 2086520) was added to 49 mL dH_2O. pH of the solution was adjusted to 3.8 by adding NaOH. Infiltrated wheat leaves were cut about 1 cm long on each site away from infiltration spot. The leaves were immediately immersed in DAB solution in sterile eppendorf tubes and incubated at 25 ^oC for 8 hours under light in a growth chamber. DAB stain was reacted with Hydrogen peroxide upon invasion. The reaction was terminated by transferring leaf samples to 96% EtOH containing eppendorf tubes and the tubes were kept at 80 ^oC for boiling purpose about 10 min. After termination of the staining reaction, samples were washed with 70% EtOH. Wheat leaves were preserved in 70% EtOH and visualize under microscopy (Leica Microsystems CMS GmbH, Germany) and naked eye. The data was recorded by a photograph.

2.6 Subcellular localization of PstHa15N21

To the localization site of the effector candidate *in planta*, we planned to take advantages of a destination vector, namely pK7FWG2. It had a GFP site on its C-terminus. Therefore; we didn't need to have a stop codon on the gene of interest for continuous expression of the gene and GFP fusion. For this purpose, we used Pst15N21-CACCFwd and Pst15N21-NoSTP-Rev primer set (Table 2.2) for PCR amplification of the gene. The following steps and general flow chart of the subcellular localization assay were illustrated in Figure 2.6.

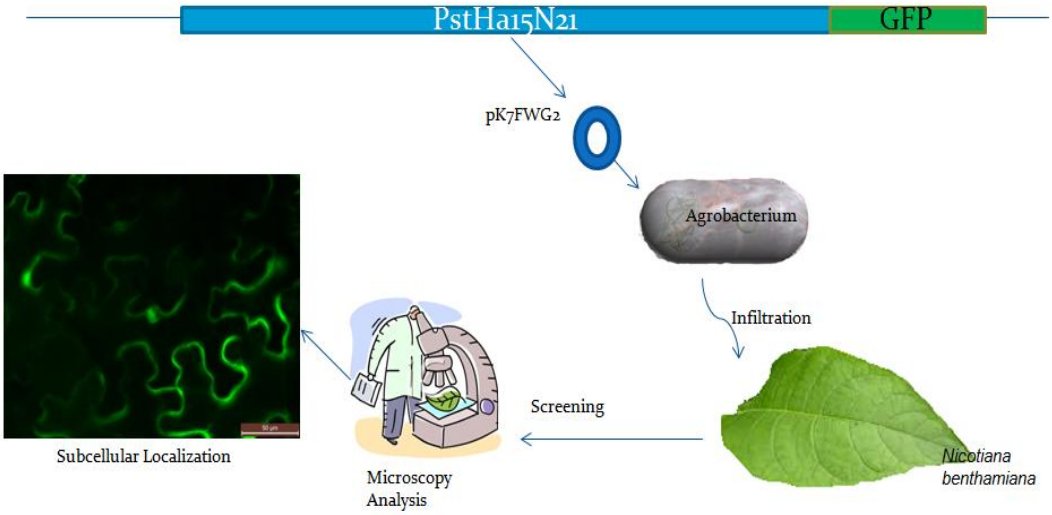


Figure 2.6 Illustration of the general outlook of the subcellular localization experiments.

2.6.1 Gateway cloning

PstH15N21 gene was amplified with Pst15N21-CACCFwd and Pst15N21-NoSTP-Rev primer set *via* PCR as it was described in section 2.3. The PCR products were merged with pENTR™/D-TOPO vector by the aid of topo-isomerase in a reaction as in Table 2.5. The products of the reaction were transformed into *E.coli* TOP10 cells with heat shock method which was mentioned in section 2.5.1.3. Verification of the transformants was done by the colony PCR reaction. Confirmed cells were named as pENTRY-PstHa15N21 and stored in %20 glycerol stock at -80 °C.

The pENTRY-PstHa15N21 plasmids were isolated from stored cells using QIAprep® Spin Miniprep Kit as described in Section 2.4. The results were measured on Nanodrop. pK7FWG2 destination vector was also isolated from its survival strain stocks. We performed a LR clonase reaction to transfer PstHa15N21 gene from the entry vector to the destination vector. The reaction components were prepared as in Table 2.7. The resulting recombinants were transformed into *E.coli* TOP10 *via* heat shock. The cells were spread into LB agar media with 100 µg/mL Spectinomycin for selection. The plates were incubated at 37 °C for overnight. The grown colonies were tested by colony PCR method. Also, double digestion was performed to verify the subcloning into pK7FWG2 destination vector. The pK7FWG2-15N21 plasmids were cut with *Xba*I and *Spe*I while empty destination vector with *Xba*I and *EcoR*I-HF restriction enzymes. The reaction products were visualized under UV after 1% agarose gel electroporation. The results were positive.

2.6.2 Preparation of electro-competent *Agrobacterium* GV3101 (pMP90)

Upon the verification of subcloning into pK7FWG2 vector, subcloned plasmids were planned to transform into *Agrobacterium* cells for further analysis. *Agrobacterium* transforms *via* electroporation method. Hence, we needed to prepare electrocompetent cells.

Streak plate was prepared from *Agrobacterium* GV3101 (pMP90) cells which were obtained from Dr. Csaba Koncz, Max-Planck Institute Plant Breeding Research Department, Cologne, Germany. Grown single colonies were inoculated into 4 mL LB medium with 2.5 µg/mL Tetracycline. The tubes were incubated for overnight at 28 ^{o}C at 150 rpm in a shaker. 1 mL of the fresh, overnight culture was transferred into 100 mL LB medium containing 5 µg/mL Tetracycline. The suspension was incubated at 28 ^{o}C at 150 rpm through the night. Next day, as A_{600nm} of the cell was between 0.5-0.7, the cells were poured into two sterile 50 mL falcon tubes. The culture was nested on ice for 30 min. The cells were collected *via* centrifugation at 3500 rpm, 4 ^{o}C for 15 min. Medium was discarded and the pellet was resuspended in 50 mL of filter-sterilized, chilled 10% glycerol. Centrifugation was repeated for another 15 minutes under same conditions. The supernatant was removed from the pellet. The washing step and centrifugation were repeated. The supernatant was poured off. Resuspension was done by adding 200 µL GYT medium (0.125% yeast extract merges with 10% glycerol and 0.25 tryptone) onto the cells. The suspension was stored by taking 50 µL aliquots in sterile 1.5 mL eppendorf tubes. The aliquots were snap-freezed using liquid nitrogen and stored at -80 ^{o}C.

2.6.3 Electroporation of pK7FWG2 into *Agrobacterium tumefaciens*

50 µL electrocompetent cells of *Agrobacterium* GV3101 (pMP90) was placed in ice to thaw. 2 µL of pK7FWG2-PstHa15N21 plasmids DNA contains about 200 ng DNA was merged with the electrocompotent cells and the mixture was rested on ice for 10 min. The cells were delivered to an ice-cold pulser cuvette having 1 mm gap. The electroporation was applied on the cells under 25 µF, 2.2 kV using an electroporator (Celljject duo, Therma Corporation). The zapped cells were treated with pre-warmed 1 mL SOC medium in a sterile 1.5 mL eppendorf tube for recovery. The sample was incubated at 28 ^{o}C for 1.5 hours at 150 rpm in shaker. The grown cells were spread to LB agar medium with 100 µg/mL Spectinomycin and incubated at 28 ^{o}C. Colonies were observed after two days and they were controlled by colony PCR method. Confirmed colonies were inoculated in sterile tubes containing 4 mL LB medium with 100 µg/mL Spectinomycin and incubated at 28 ^{o}C for two days. The grown bacteria was collected in sterile 2 mL tubes with glycerol and immobilized by snap-freezing *via* liquid nitrogen. The prepared stock was preserved at -80 ^{o}C.

2.6.4 Agro-infiltration

Agro-infiltration into the host cells (*Nicotiana benthamiana*) requires several preparation step and materials. Before preparation of *Agrobacterium* for leaf infiltration assay, we arranged the following materials which are listed in Table 2.10. To prevent the loss in the activity of acetosyringone, it was added last to the solutions which it involves.

Table 2.11 Materials and their contents that needed for agro-infiltration

Materials and reagents	Components
LB media	10 g tryptone, 5 g yeast extract, 5 g NaCl, 3.2 mL NaOH in 1L ddH_2O
Acetosyringone	0.1 M in DMSO
MES (pH 5.7)	0.5 M
$MgCl_2$	1.0 M
L-MESA	10 mM MES, 20 µM Acetosyringone in LB media
Agroinduction media	10 mM MES, 10 mM $MgCl_2$ 150 µM Acetosyringone in ddH_2O

Agrobacterium cells, which were transformed with pK7FWG2-PstHa15N21, were grown in a streak plate containing Spectinomycin (100 µL/mL) to obtain single colonies from the stocks. After two days, colonies were observed and they were inoculated into 3 mL L-MESA with selective antibiotics; Kanamycin (50 µL/mL), Rifampicin (10 µL/mL) and Gentamicin (25 µL/mL) in sterile falcon tubes. The cells were grown at 28 ^oC, 250 rpm for 24-30 hours in shaker. The required A_{600nm} value for optimum infiltration was between 0.8-1.0. Hence, the grown cells were transferred into another medium containing 5 mL L-MESA with Kan, Rif, and Gen to reach the targeted A_{600nm} values while the bacteria were still in log or lag phase. Upon reaching the desired absorbance value, the bacteria were collected by centrifugation at 3500 X g for 10 minutes in bench-top centrifuge. The supernatant was removed and the pellet was resuspended gently in agro-induction media without disturbing the cell pellets. The suspension's absorbance value (A_{600nm}) was measured *via* spectrophotometer (Shimadzu, UV-1601) and we adjusted it to 1.0 by adding agro-induction media. The cells were kept at room temperature for overnight. The host *N. benthamiana* plants were collected when they were 5 weeks old. The adult (not too old), wide, healthy leaves were selected and labeled clearly. The overnight *Agrobacterium* cell cultures were infiltrated by the aid of needless syringe into the plant leaves. The infiltration was performed through the bottom of the leaf and without tearing the tissue as it is shown in Figure 2.7. We used 0.5 mL bacteria for infiltration and tried to penetrate tissue in one go. *Agrobacterium* can invade leaf tissue from open wounds. Hence, we used gloves and worked in sterile manner to avoid cross-contamination of the leaves.

Figure 2.7 Infiltration into *N. benthamiana* using Agrobacterium.

2.6.5 Screening of the infiltrated leaf samples under microscope

The infiltrated leaves were collected at two days post inoculation. The samples were cut around 1 cm radius of the infiltration spot and placed in water. The samples were attached top of the slides and treated with water instead of glycerol. The cover slip was put gently at 45^{o} angle and closed slowly without leaving any bubbles on the sample. The slides were first visualized under light microscope (Leica, DFC 280) with GFP filter. 40X and 100X magnifications were used for imaging of the sample. Clear images were recorded and these samples were selected to visualize under confocal microscope (Zeiss, LSM 500).

2.7 Cloning into pJL48-TRBO vector for tagged protein co-immuno precipitation

Determination of the interacting factors *in planta* was planned to achieve by using pJL48-TRBO. pJL48-TRBO was obtained from Dr. Tolga O. Bozkurt, Sainsbury Laboratory, Norwich, UK. pJL48-TRBO is a high expression vector *in planta* so suitable for protein expression and protein-protein interaction studies. However, it produces low copy number plasmids. Hence, we used larger amount of cells to isolate plasmids from pJL48-TRBO empty vector. Then, we proceeded with next step. The experimental flow chart of the analysis was illustrated in Figure 2.8.

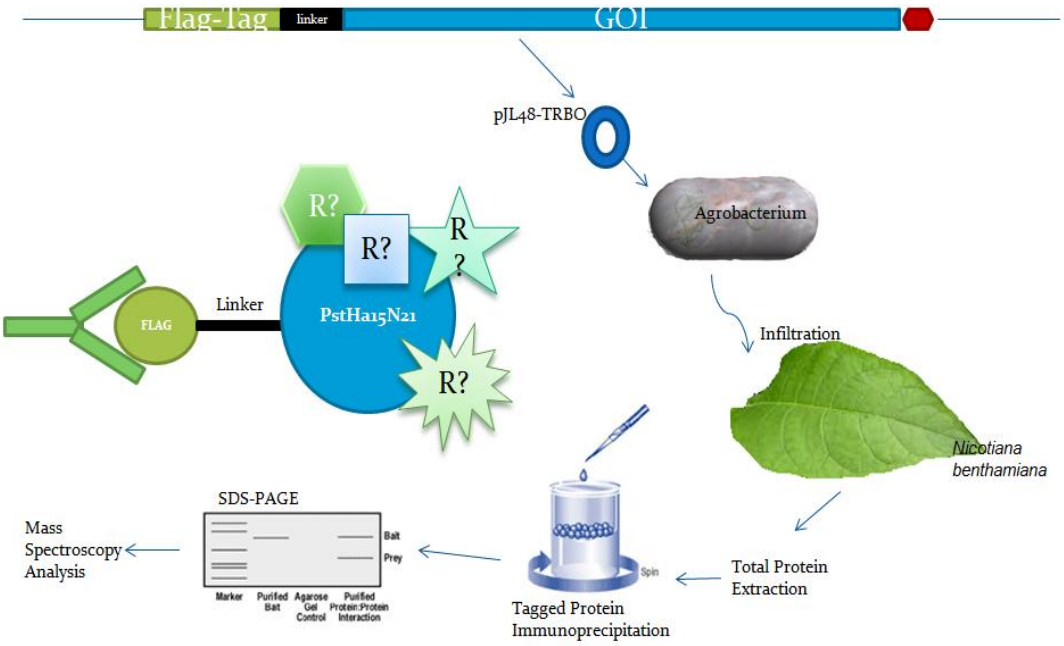


Figure 2.8 Experimental design of interacting host factors analysis.

2.7.1 Amplification and restriction enzyme double digestion

To clone our gene of interest in to the vector, we amplified our sequence with Pst15N21-Flag-Fwd and Pst15N21-Rev primer set using Q5 High Fidelity *Taq* DNA Polymerase (NEB) in a reaction as described in section 2.3. The primers had restriction cut sites which allow us to perform directional cloning into the target vector. Both isolated empty vector and PCR products were cut with *Pac*I (NEB, Cat# R05475, Lot# 0641206) and *Not*I-HF (NEB, Cat# R31895, Lot# 0031112) restriction enzymes. The contents of double digestion reaction were shown in Table 2.11.

Table 2.12 Reagents of double digestion reaction of the vector.

Reaction Components	Amounts (µL)	Final concentrations	Total amount
Vector (~ 100 ng)	16 µL	-	~ 1500 ng
*Pac*I (10,000 unit/mL)	0.5 µL	-	5 units
*Not*I HF (20,000 unit/mL)	0.25 µL	-	5 units
NEB buffer 4 (10X)	2 µL	1X	
BSA (100X, 10 mg/mL)	0.2 µL	1X	
ddH_2O	1.05 µL	-	
Total reaction volume	20		

The reactions were prepared as shown in sterile 200 µL PCR tubes and incubated at 37 ^{o}C for 3 hours. After 3 hours, the temperature was raised to 65 ^{o}C to terminate reactions. The products of the reaction were separated on 1% agarose gel in 1X TAE buffer at 60 V for 1 hour.

2.7.2 Gel extraction

Gel extraction was performed using QIAquick Gel Extraction Kit (Qiagen, Cat# 28706, Lot# 139312248). The bands were sliced and cut from the gel carefully to avoid involving any unnecessary gel part to the sample. Cutting was performed with sterile lancet under UV light in very swift manner to reduce DNA damage due to UV exposure. From this part, we followed the manufacturer's protocol. The gel slices were placed in sterile, 1.5 mL eppendorf tubes and weighted. Buffer QG was added to sample depending on the formula (for 1 mg of sample, 3 µL buffer was added). The samples were placed in a water bath and incubated for 10 min. at 50 ^{o}C to liquefy agarose gel. In 2-3 min intervals, the samples were mixed by spinning or tapping until solution turns into clear, homogeny appearance. Next, 1 volume of isopropanol was added to samples and mixed by inversions. Then, the samples were applied to QIAquick spin column provided by the kit to catch DNA. The column was centrifuged at 13000 rpm for 1 min. in bench-top centrifuge. The flow through was discarded and 0.5 mL Buffer QG was added into each column. Centrifugation step was repeated to elute any agarose leftover from the column. The flow through was disposed. The column was cleansed with 0.75 mL PE buffer addition and we let the column stand for 3-5 min. to increase washing efficiency. The centrifugation step was repeated and the flow through was disposed. To remove all traces of alcohol which may threaten downstream processes, we applied additional centrifugation at 13000 rpm for 2 min. Finally, 30 µL of nuclease free water was added directly onto the membrane of the spin column. The column stood for 3 min. The centrifugation was done at 13000 rpm for 2 min. The flow through was measured under Nanodrop and visualize under 1% agarose gel in 1X TAE buffet to verify the collected DNAs were from the right bands.

2.7.3 Ligation reaction

The ligation reaction was prepared from the reagents presented in Table 2.12. We determined ratio of the vector to the insert by looking the brightness of the bands on the agarose gel. The direction of the cloning was achieved *via* sticky ends of *Not*I and *Pac*I sites. T4 DNA ligase (NEB) enzyme was used to ligate these stick end into the vector.

Table 2.13 The reagents used in ligation reaction.

Reaction Components	Amounts (µL)	Final concentrations
10X T4 ligase buffer	1	1X
pJL48-TRBO vector (55 ng/µL)	1	
PstHa12h2 insert (21 ng/µL)	2	
T4 DNA Ligase (5 unit/µL)	0.2	1 U
ddH_2O	6.4	
Total Volume	10	

The reagents were mixed in a sterile 200 µL PCR tube and incubated at 22 ^{o}C for one hour. The sample was kept at 4 ^{o}C for overnight. Next day, we took 5 µL of the ligation product to transform them into *E.coli* TOP10 competent cells. Transformation was performed by merging 5 µL of the ligate product to 100 µL of the competent cells and applied heat shock as described in section 2.5.1.3. The cells were allowed to grow in 500 µL LB medium at 37 ^{o}C for 1-2 hours at 200 rpm in shaker. After recovery phase, the cells were spread on to their selective media which contained LB agar medium with 50 µg/mL Kanamycin. The plates were placed in an incubator and allowed to grow at 37 ^{o}C for overnight. The grown colonies on the plates were picked and tested for presence of the insert *via* colony PCR as mentioned in section 2.5.1.4. After confirmation by colony PCR, we cut plasmids from transformant cells by using *Pac*I and *Not*I restriction enzyme to see the insert as second confirmation method. However, the insert was not observed on agarose gel electrophoresis (1% agarose in TAE buffer). We sent the plasmids to MCLAB Company, USA for sequencing purposes. The output of the sequencing process was negative.

2.7.4 TA Cloning of PstHa15N21 into pGEM-T-Easy vector

In trial 2, we first attempted to clone our gene of interest into pGEM-T-Easy vector by TA cloning method. The gene of interest was amplified with Pst15N21-Flag-Fwd and Pst15N21-Rev primer set by using *Taq* DNA polymerase with Standard *Taq* Buffer (NEB, Cat# M0320S, Lot#0141203). The reaction components were prepared as in Table 2.6. However, different thermocycling conditions (Table 2.13) were applied to increase TA ends in the extension process of the PCR.

Table 2.14 Thermocycling conditions of PCR**.**

PCR steps	Temparature	Time	
Initial denaturation	95 °C	30 sec	
Denaturation	95 °C	30 sec	33 cycles
Annealing	55 °C	1 min	
Extension	68 °C	1.15 min	
Final extension	68 °C	10 min	

PCR products were visualized using agarose gel electrophoresis on 1% agarose gel in TAE buffer. Next, PCR products were cloned into vector in a ligation reaction. T4 DNA ligase was used as ligation enzyme. The other components of the reaction were presented in Table 2.14.

Table 2.15 Amounts of the reagents used in TA cloning.

Reagents	Volume (µL)
PCR product	5
pGEM®-T or pGEM®-T Easy Vector (50ng)	1
10X Ligation Buffer	1
T4 DNA ligase (5 unit/µL)	0.1
ddH_2O	2.9
Total Volume	10

The components of the reaction were mixed in a sterile 200 µL PCR tube and incubated at 22 °C for one hour and at 4 °C for overnight. The ligation products were transformed into *E.coli* TOP10 cells *via* heat shock as described in section 2.5.1.3. After recovery phase, the cells were spread on 100 µg/mL Ampicillin containing LB agar plates. The samples were allowed to grow at 37 °C for overnight in incubator. The grown colonies were tested by colony PCR as mentioned in section 2.5.1.4. The positive clones were selected and grown in 4 mL LB medium with selective antibiotic. The overnight cultures were stored by snap-freezing *via* liquid nitrogen and preserved at -80 °C.

2.7.5 Double digestion of pGEM-T-Easy-PstHa15N21 construct

pGEM-T-Easy-PstHa15N21 vector was double digested by using *Pac*I and *Not*I restriction enzymes to produce insert with sticky ends. Also, pJL48-pTRBO was cut with same enzymes. However, we used pJL48-TRBO vector with another insert (PstHa2a5) for digestion. Both vector constructs were cut using reaction components in Table 2.11 under the reaction conditions mentioned in section 2.7.1. The resulting products were run on 1% agarose gel in TAE buffer under 60 V for 1 hour for band separation.

2.7.6 Gel extraction and ligation of PstHa15N21 and pJL48-TRBO vector

The cut insert and vector were extracted from agarose gel after separated by gel electrophoresis. Observed bands were cut with the aid of a lancet under UV light. Gel extraction protocol was applied to sliced bands as explained in section 2.7.2. The resulting products were measured in Nanodrop. They were also loaded on 1% agarose in TAE buffer for agarose gel electrophoresis. The results were recorded.

The ligation reaction was performed depending on these results. Ratio of the insert to the vector was determined as 2:1 after analyzing the brightness of the bands on agarose gel. The other reagents in the ligation were prepared as presented in Table 2.15.

Table 2.16 Amounts of the components used in ligation reactions.

Reagents	**Volume (µL)**
10X T4 ligase buffer	1
pJL48-TRBO vector	1
PstHa12h2 insert	2
T4 DNA Ligase (5 unit/µL)	0.2
ddH_2O	6.4
Total Volume	10

The reaction mixture was prepared in sterile 200 µL PCR tube and incubated at 22 ^oC for one hour and 4 ^oC for overnight. The ligation products were transformed into *E.coli* TOP10 competent cells using heat shock method as mentioned in section 2.5.1.3. The cells were incubated at 37 ^oC for 1-2 hours at 200 rpm in shaker. Then, they were spread onto LB agar containing 50µL/mL Kanamycin for selection and incubated at 37 ^oC for overnight in incubator. The surviving colonies were selected and tested for presence of the insert by using colony PCR method as in section 2.5.1.4. Positive colonies were grown in 4 mL LB medium containing Kanamycin at 37 ^oC for overnight. Plasmid isolate ion was applied to the grown cells using QIAprep® Spin Miniprep Kit (Cat# 27106, Lot# 142349895) as it is described in section 2.4. The plasmids were cut using *Pac*I and *Not*I restriction enzymes to check for the insert. After confirmation of the cloning into pJL48-TRBO vector, the positive clones were stored in glycerol at -80 ^oC for further analysis.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Prediction of PstHa15N21 as an effector candidate

PstHa15N21 gene was predicted as an effector gene from haustorial cDNA library data published by Yin and his colleagues (Table 1.2) (Yin *et al*., 2009a). PstHa15N21 cDNA sequence, which was retrieved from NCBI database with Accession number; GH737567 (http://www.ncbi.nlm.nih.gov/), was converted into possible ORF using ORF finder and the full length protein sequence was obtained. Amino acid sequence of the protein was scanned for the presence of a signal peptide using 'SignalP' prediction tool. The result was shown in Figure 3.1. Possible cleavage site of signal peptide is predicted in between 17th and 18th amino acids; A and T, as indicated with the arrow on the graph.

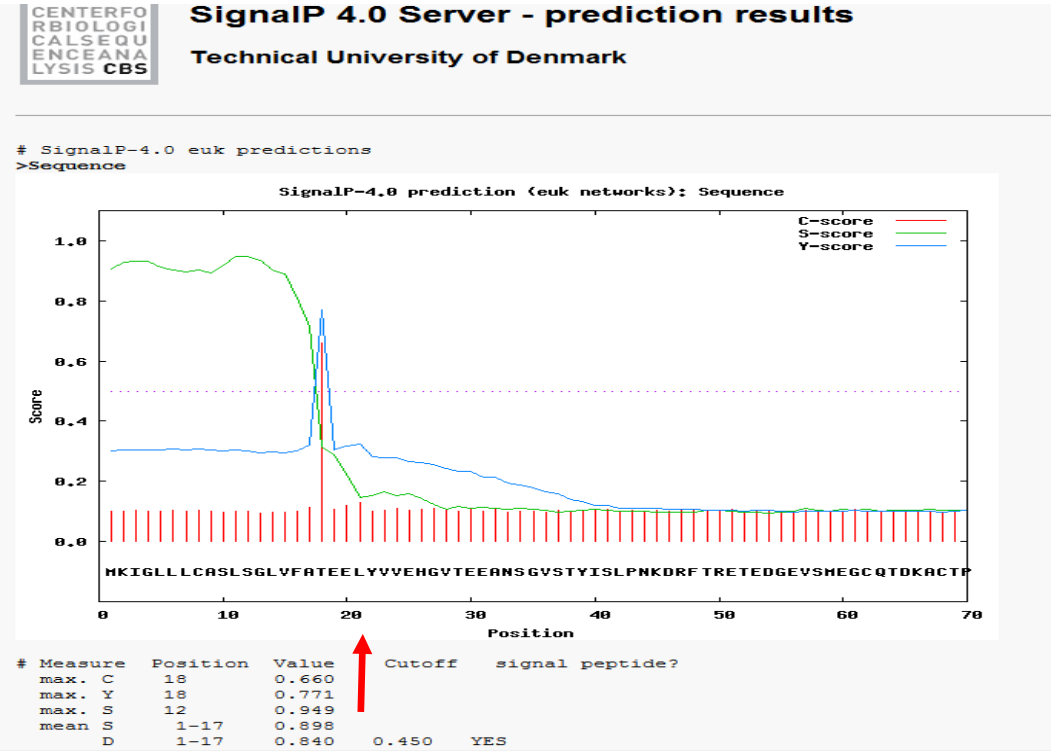
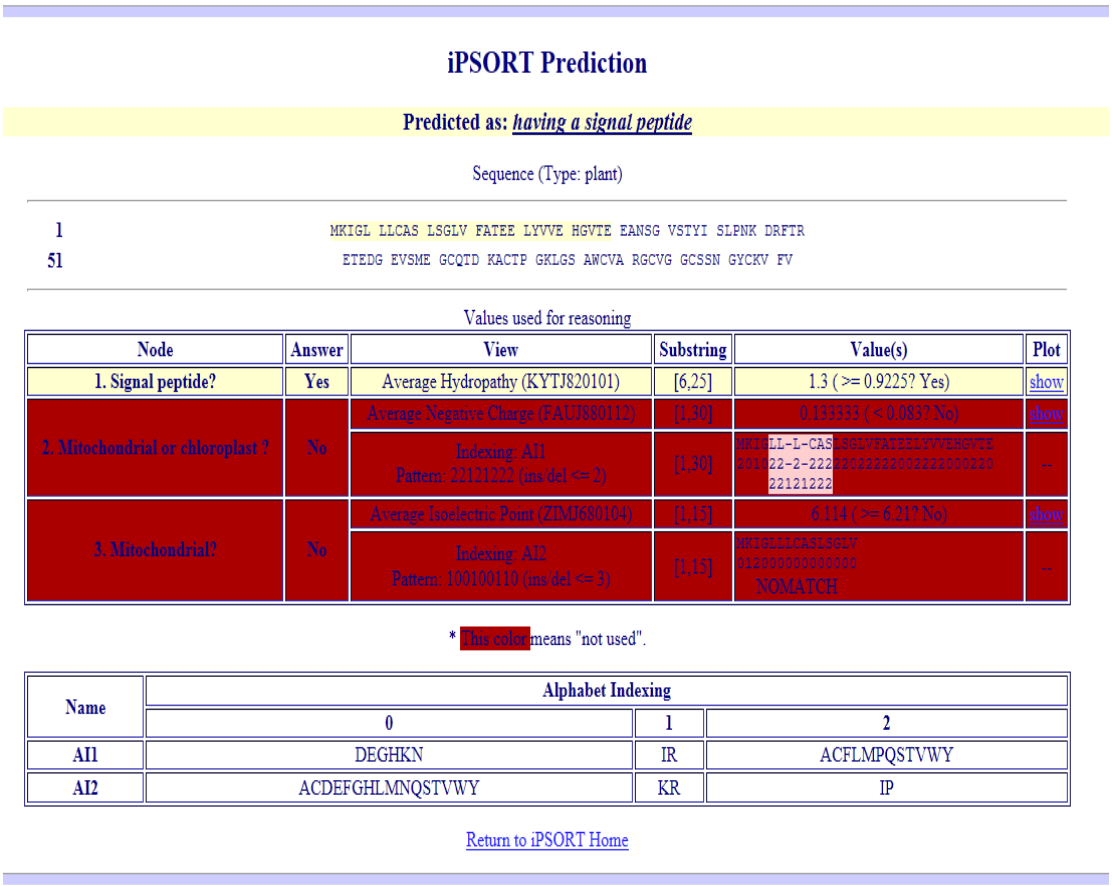


Figure 3.1 The signal peptide sequence is present in N-terminus of PstHa15N21 protein.

To confirm the presence of signal peptide, we used another prediction program, 'iPSORT' (http://ipsort.hgc.jp/) which was specifically designed for plants and fungi. The result is shown in Figure 3.2. PstHa15N21 has a signal peptide sequence in its N-terminus. 'iPSORT' also verified signal peptide determined are not targeting the protein to other organelles such as mitochondria or chloroplast.



iPSORT Prediction

Predicted as: *having a signal peptide*

Sequence (Type: plant)

```
1    MKIGL LLCAS LSGLV FATEE LYVVE HGVTE EANSG VSTYI SLPNK DRFTR
51   ETEDG EVSME GCQTD KACTP GKLGS AWCVA RGCVG GCSSN GYCKV FV
```

Values used for reasoning

Node	Answer	View	Substring	Value(s)	Plot
1. Signal peptide?	Yes	Average Hydropathy (KYTJ820101)	[6,25]	1.3 (>= 0.9225? Yes)	show
2. Mitochondrial or chloroplast ?	No	Average Negative Charge (FAUJ880112)	[1,30]	0.133333 (< 0.083? No)	show
		Indexing: AI1 Pattern: 22121222 (ins/del <= 2)	[1,30]	MKIGLL-L-CASLSGLVFATEELYVVEHGVTE 201022-2-222220222220022220002202212121222	--
3. Mitochondrial?	No	Average Isoelectric Point (ZIMJ680104)	[1,15]	6.114 (>= 6.21? No)	show
		Indexing: AI2 Pattern: 100100110 (ins/del <= 3)	[1,15]	MKIGLLLCASLSGLV 012000000000000 NOMATCH	--

* This color means "not used".

Name	Alphabet Indexing		
	0	1	2
AI1	DEGHKN	IR	ACFLMPQSTVWY
AI2	ACDEFGHLMNQSTVWY	KR	IP

Return to iPSORT Home

Figure 3.2 The Result of the iPSORT prediction tool.

PstHa15N21 is a small, secreted protein having 7 cysteine residues in its sequence. Cysteine rich residues tend to make the protein stable because of the disulfide bonds, thus it is considered that those effector candidates having many Cysteine residues are more likely to be apoplastic effectors (Jonge *et al.*, 2011) Moreover, PstHa15N21 sequence is unique one with no homology to other known nucleotide sequences according to BlastN and BlastP programs of NCBI (http://blast.ncbi.nlm.nih.gov/). No homology to any known gene sequence is an expected feature of effector candidates, since there are only very few number of known, experimentally confirmed effector genes of pathogens which are very different than that of rust pathogens. Even those show a limited homology among themselves except with some signature conserved motifs (Godfrey et al., 2010)(Jiang *et al.*, 2008)(Haas *et al.*, 2009)(Rafiqi *et al.*, 2012). Thus, PstHa15N21 is a true effector candidate of *Puccinia striiformis* f. sp. *tritici*.

3.2 Cloning of PstHa15N21 gene into pEDV6 destination vector

We used Gateway cloning strategy to clone the gene of interest into a pEDV6 expression vector, so that when different destination vectors are used, we can easily carry the gene of interest into the destination vectors from an entry vector already constructed with our gene.

The pEDV6 vector is a Gateway destination vector so we first needed to clone the PstHa15N21 gene into an Entry vector (pENTR^{TM}/D-TOPO). To achieve this, we incorporated 'CACC' sequence to its 5' end of the gene by PCR. Then, the gene was recombined into the pEDV6 vector producing the expression vector with the insert, and byproduct which cannot survive due to exchange of the lethal gene, *ccdB*, from the destination vector.

3.2.1 Amplification of the gene of interest

Amplification of PstHa15N21 from synthesized gene construct in pUC57 was performed using specific primers as indicated in Table 2.2. The forward primer bore CACC sequence and followed by ATG start codon at its 5' end to achieve directional cloning into the entry vector. The reverse primer had stop codon to terminate expression after PstHa15N21 synthesis. The reaction product was clearly identified with its expected size (Figure 3.3).

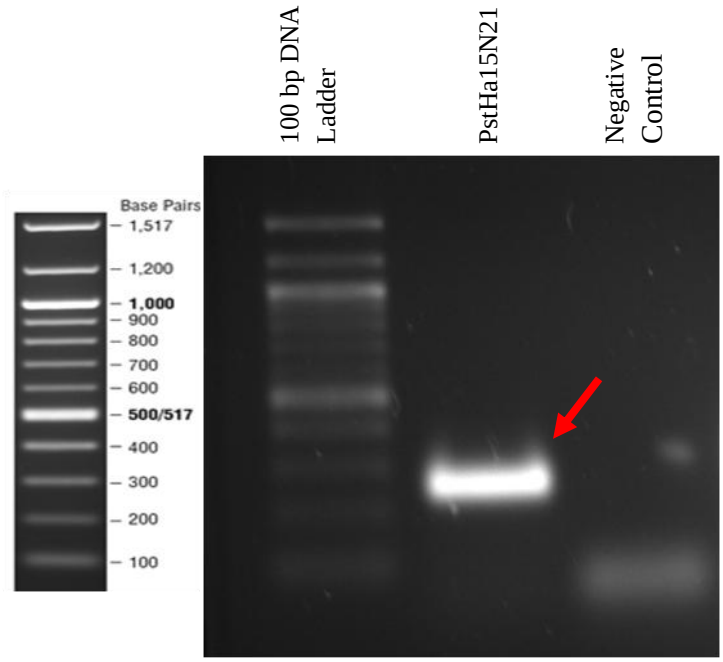


Figure 3.3 Agarose gel electrophoresis image of PCR reactions. The samples were separated on 1% agarose gel in 1X TAE buffer. First lane: 100 bp DNA Ladder (0.5 µg) (Cat # N3231S, Lot # 0831006, NEB). Second lane: 2 µL of amplicon (294 bp). Third lane: negative control (no template) for the PCR.

3.2.2 Cloning into pENTR™/D-TOPO

The cloning of the gene of interest was achieved with the aid of Topo-isomerase which is attached to the pENTR™/D-TOPO vector. The critical point for the cloning reaction is not to disturb Topo-isomerase on the entry vector by means of any sheer force. After cloning reaction, recombinant plasmid with the gene of interest was transformed into *E.coli* TOP10 cells and the grown colonies were selected in the presence of 50 μg/mL Kanamycin containing LB agar plates.

3.2.3 Cloning into pEDV6 vector

LR clonase reaction was used to transfer the gene from the entry vector into the pEDV6 destination vector. Both vectors had specific recombination sites called attachment sites. These sites recognize each other and achieve recombination by LR clonase enzyme. pEDV6 vector originally had *ccdB* gene which is a lethal gene. Therefore, *ccdB* gene bearing pEDV6 vectors or in general destination vectors can only be maintained in specific survival cells. After recombination, the newly formed byproduct cannot survive due to recombined *ccdB* lethal gene. On the other hand, the unreacted entry vector with the gene of interest was eliminated using selection media (Gen). The recombinant pEDV6 vector which now became an expression vector with the insert, selected in media containing Gentamycin.

Grown colonies were verified with three methods to eliminate all chances of false positive results. The first method was colony PCR. Using same primers as in PCR amplification, colony PCR was applied on selected colonies. The resulting PCR products were separated on a gel *via* agarose gel electrophoresis method. The results are displayed in Figure 3.4. Positive clones have 294 bp product size.

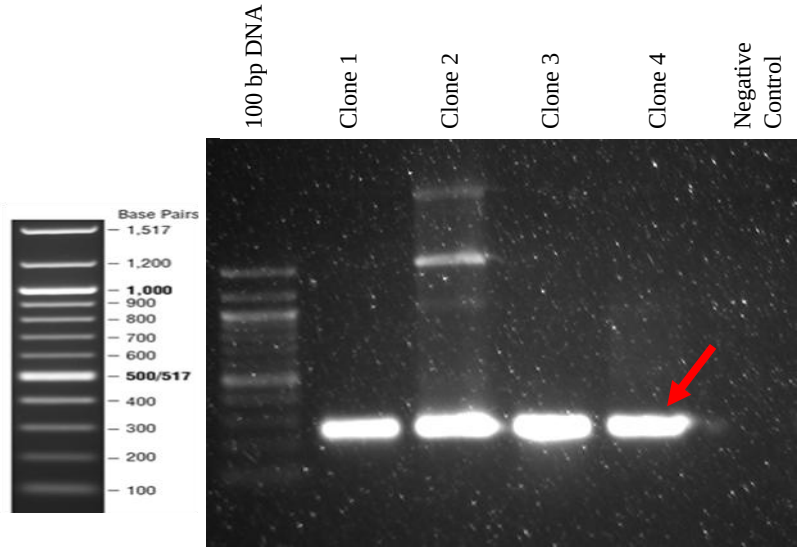


Figure 3.4 Agarose gel (1 % in TAE) image of colony PCR reaction. First lane is loaded 100 bp DNA ladder (0.5 µg). Lanes 2-5 contain 2 µL of colony PCR products. 6th lane is negative control (no PCR template).

The 2nd colony has several bands along with the bright ~300 bp band which shows the insert. Therefore, we suspected that 2nd colony may be yielding PCR artifacts. The other colonies of choice gave clear bands of insert. We needed to confirm the cloning by cutting the pEDV6 vector using *Pst*I and *EcoR*I restriction enzymes. The vector with the insert should produce 2 bands after double digestion whereas pEDV6 vector should give three bands because there is another *EcoR*I cut site in *ccdB* gene. The result is presented in Figure 3.5.

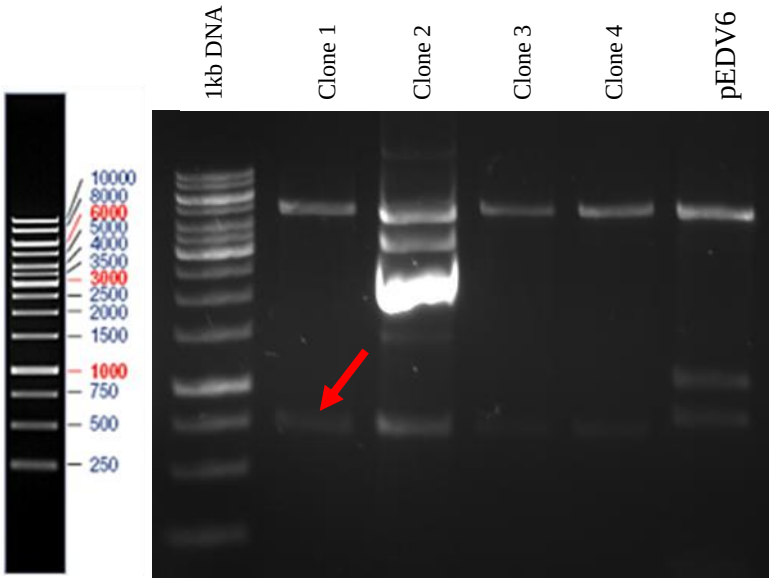


Figure 3.5 Agarose gel (1% agarose in TAE) electrophoresis image of double digestion reaction. Lane 1: 0.5 µg, 1 kb DNA ladder (Cat # SM0311, Lot #00093172, Fermentas). Lanes: 2-5 contain 2 µL of digestion reaction products of colonies. Positive colonies have two bands as expected; one being the insert and the other is the linearized vector. Lane 6: Empty pEDV6 produces three bands if double digested with *EcoR*I and *Pst*I.

1st, 3rd and 4th colonies gave positive results after using two confirmation methods. However, 2nd clone had contaminants, so it was disposed. Positive colonies were also sent to MCLAB Company, USA for sequencing. The results were also confirmed after sequencing (the data not shown) of the plasmids. The gene of interest was successfully cloned into pEDV6 destination vector.

3.2.4 Electroporation of pEDV6-PstHa15N21 expression vector into *P. fluorescens* (EtHAn)

pEDV6 vector was designed for *P.fluorescens* EtHAn for the delivery of effectors. Hence, the recombinant vector needed to transform into the bacteria. We first used heat shock transformation method on competent *P. fluorescens* EtHAn cells. However, we did not observe any colonies upon transformation. The protocol to prepare competent cells was obtained from CSIRO-Plant industry (N.Upadhyaya). The protocol states that the efficiency of transformation was low. Hence, we decided to test electroporation method.

The procedure to prepare electro-competent cells was adopted from the literature (Choi *et al.*, 2006). The electroporation was applied using pulser cuvettes and electroporator (Celljject duo, Therma Corporation) as the details presented in Section 2.5.2.2. Electroporated cells were grown on their selective media (LB agar with 100 µg/mL Gentamycin). The colonies were observed after two days instead of one day. Possible explanation for this situation is

the long recovery phase after electroporation. However, we confirmed that the observed colonies contained the gene by colony PCR. The results of the colony PCR are shown in Figure 3.6. Colony 1 and 2 were selected and stored for further wheat infiltration analysis.

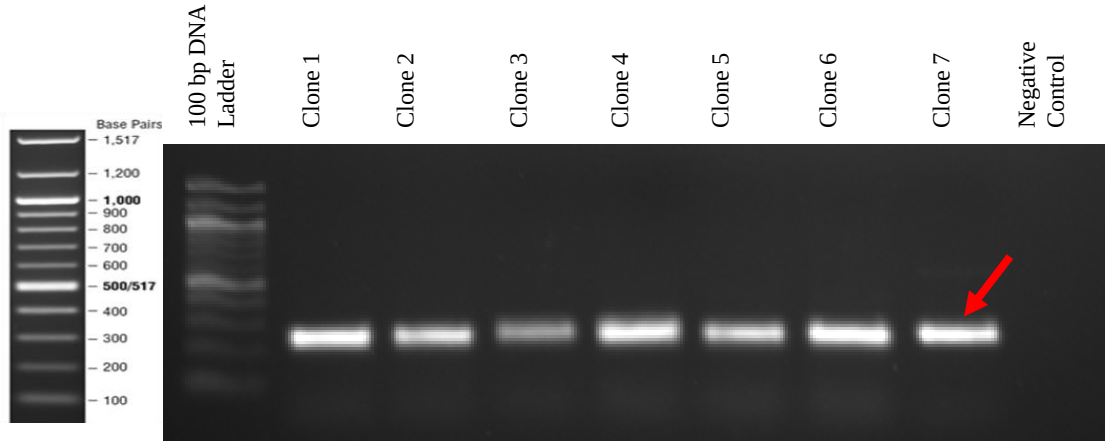


Figure 3.6 Colony PCR results of pEDV6-PstHa15N21 inserted *P. fluorescens* EtHAn cells. The samples were run on 1% agarose gel in 1X TAE buffer. Lane 1: 100 bp DNA ladder (05 µg). Lanes: 2-8 contain 2 µL of colony PCR products. Positive colonies have ~300 bp single bands. Lane 8: Negative control (no template PCR).

3.3 Wheat infiltration assay

P. fluorescens (EtHAn) mediated wheat infiltration assay was performed following the protocol which was obtained from CSIRO-Plant industry (N.Upadhyaya). The primary leaf was targeted and approximately 2.5 mL culture infiltration into the host plants was aimed. Different plant leaves behave differently during infiltration. Infiltration to some plant genotypes was easy, while to others was not due to their thick epidermis. The total of 27 yellow rust differential lines was tested for the effect of PstHa15N21 expression. pEDV6-PstHa15N21 was infiltrated into two seedling in same pot while empty *P. fluorescens* (*EtHAn*), pNR527 having *P. fluorescens* EtHAn and $MgCl_2$ was used as controls for comparison. Besides wheat differential lines (total 27 plants), we also infiltrated *N.tobaccum* plant for comparison.

After infiltration step, the plants were kept in shaded mist overnight to ease infection. The next 2 to 7 days, the plants were observed for HR symptoms while they grew under 16 hours light and 8 hours dark conditions. However, even after 7 days, we failed to see any HR symptoms. Then DAB staining was performed on wheat differential lines to detect HR symptoms if any. The leaf samples were cut approximately 1 cm long, away from infiltration site to omit HR response caused by sheer force of invasion and wounding. In Figure 3.7, 3.8, 3.9, 3.10 and 3.11 DAB stained leaves are presented except *N. tobaccum* (it is examined

without DAB staining). *N. tobaccum* showed HR like symptoms (chlorosis etc.) in all infiltrated regions on leaf blade except $MgCl_2$ infiltration (control). Hence, we thought that *N. tobaccum* had basal resistance or non-host resistance to *P. fluorescens* (EtHAn) because all bacteria infiltrated regions; including the invaded by empty *P. fluorescens* (EtHAn) showed the same response.

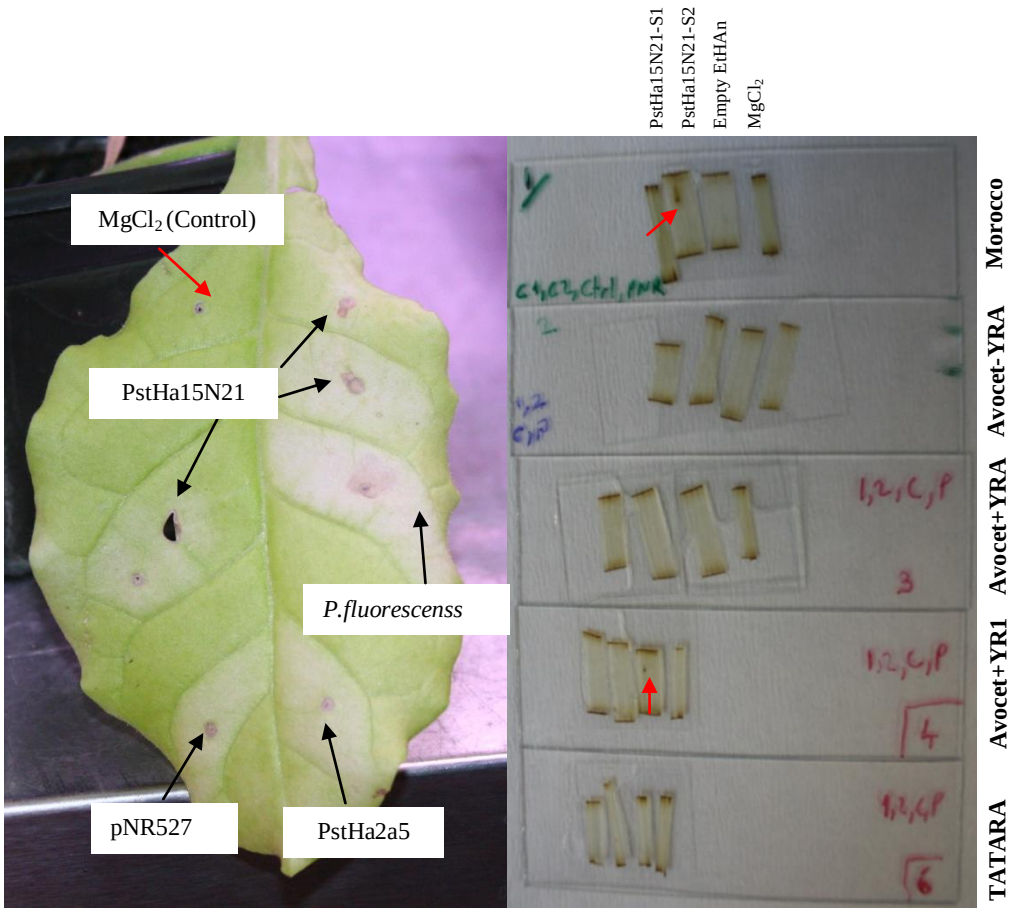


Figure 3.7 The photos of the infiltrated leaves. Photo on the left belongs to *N. tobaccum*. On the right, wheat differential lines from 1-6 (5 excluded due to early death of leaves) are presented. First two leaves on each slide belong to pEDV6-PstHa15N21/EtHAn, third and fourth leaves are empty *P. fluorescens* (EtHAn) and $MgCl_2$ infiltrated leaf samples.

HR-like symptoms in DAB staining appear as brown or dark brown spots on the leaf blade due to hydrogen peroxide accumulation. In Figure 3.7, we show results from *N. tobaccum* and first 5 of the 27 differential lines tested (names indicated on the right of each image). Wheat cultivar Morocco had a brown spot which is shown with a red arrow. A tiny spot was also observed on empty *P. fluorescens* infiltrated leaf sample of cultivar Avocet+YR1, whereas the effector infiltrated leaves of same cultivar showed no sign of similar spots. There were no traces of HR symptoms in other cultivars of Figure 3.6. We decided to examine the leaves more closely using a light microscope (Leica DM4000B microscope /

DFC 280 camera) under 10X and 40X magnifications. The photos of HR like symptoms and remote microburst appearances in the leaf samples were recorded by microscope. Interestingly, wheat line, Morocco produced a brown spot which was unlikely since this cultivar is considered universal susceptible. However, no similar response was found in other effector infiltrated samples of Morocco (Sample 1 (S1) of Morocco). Moreover, we also observed brown spot in cultivar, Avocet+YR1 (Slide #4 in Figure 3.7) which is unexpected because it was infiltrated with empty *P. fluorescens* (EtHAn) cells, it was concluded that brown color appearance was due to the shear force during the infiltration. Or any other artifact, which should be concluded more number of trials with reproducible results. Figure 3.8 represents the photos of wheat cultivar, Morocco.

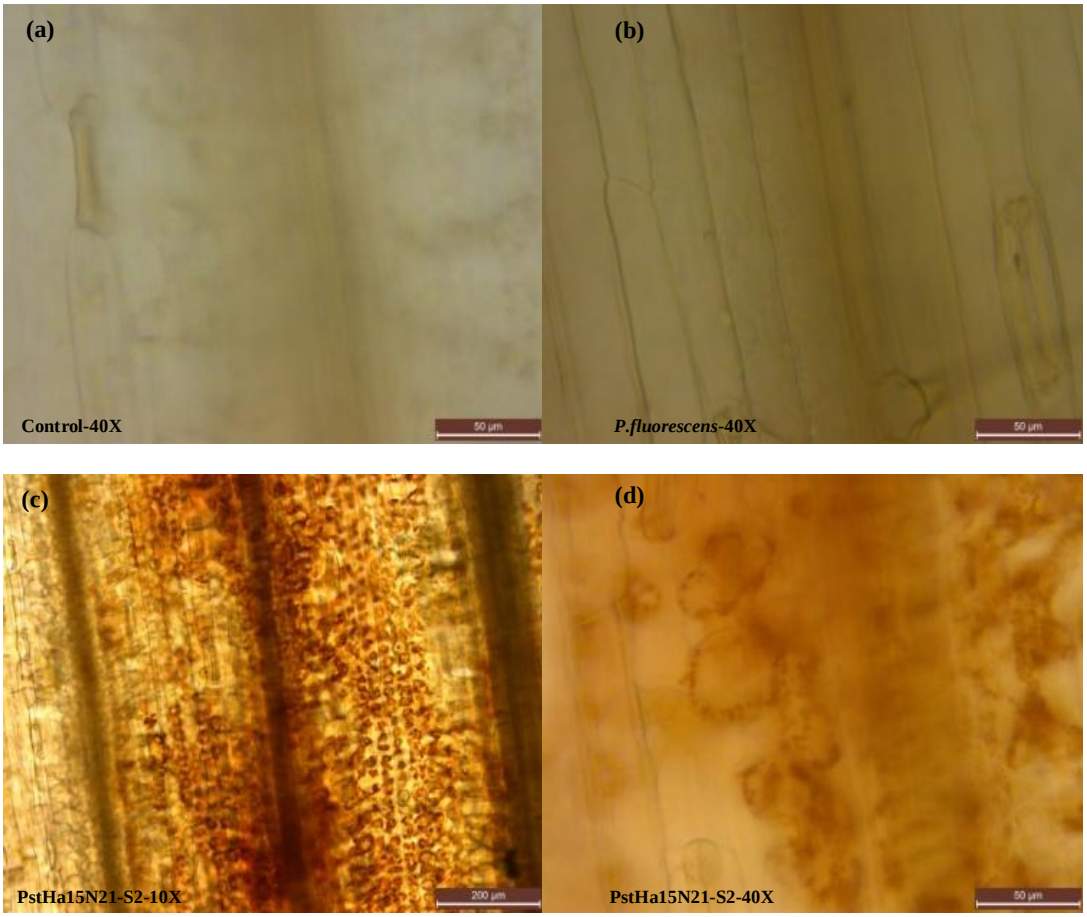


Figure 3.8 DAB stained samples of Morocco (YR differential line-No 1) infiltrated with the candidate effector. **(a)** Control, **(b)** Empty *P. fluorescens* (EtHAn) infiltrated wheat leaf, **(c)** Effector candidate, Sample 2 of Morocco (S2) under 10X magnification and **(d)** Effector candidate, S2 (40X).

As can be seen in Figure 3.8, there are clear images of hydrogen peroxide accumulation after cellular burst in the Morocco sample infiltrated with *P. fluorescens* with the effector. However, brown spots were local and around transport channels of the leaf blade. Therefore, sheer force during infiltration to invade more space in leaf blade may have led to clusters of cell death due to physical wounding.

In Figure 3.8, the samples from 7 to 16 are displayed; YR differential lines 8, 13, and 14 showed brown spots marked with red arrows. The spots are not present in the second replicates of effector infiltrated ones. Hence, we scanned them at cellular level under light microscope.

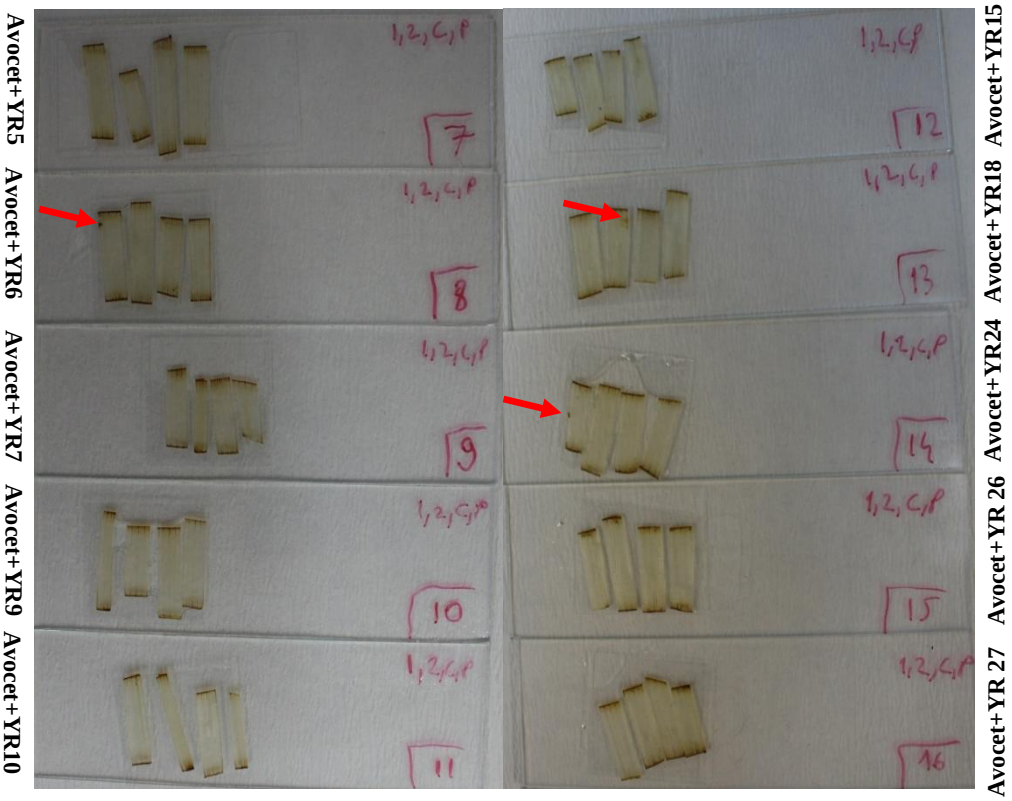


Figure 3.9 DAB staining photos of infiltrated YR differential lines. On the left, wheat lines from 7 to 11 are displayed. Right panel shows differential lines from 12 to 16 (Table 2.1). Possible HR-like symptoms were shown *via* red arrows. In each slide; the samples are ordered as PstHa15N21-S1 and S2, *P. fluorescens* (EtHAn) and $MgCl_2$ infiltrated control leaves from left to right, respectively.

The results of the microscope imaging were informative (Figure 3.10). The spot on the line 8 (Avocet+YR6) which was infiltrated with the effector containing bacteria was local; possibly induced by wounding. It was also very close to the cut site. The replica of the same plant line did not show any brown color, thus no hydrogen peroxide accumulation, no cell death. The line 13 (Avocet+YR18) and line 14 (Avocet+YR24) have large brown regions in one of the replicates but similar spot was not apparent in the other replicate. However, when investigated under the microscope, PstHa15N21 introduced line 13 showed very clear hydrogen peroxide accumulation in both replicates, whereas as expected leaf samples of the control groups produce no symptoms. Hence, the result of DAB staining reaction indicates that PstHa15N21 may be an avirulence (*Avr*) gene, AvrYr18, It was recently reported that YR18 resistance gene encodes an ABC transporter (Krattinger *et al*., 2009). Thus, it makes sense for an effector with possible apoplastic functions to interact with a membrane bound resistance gene. It is also known for a long time that Yr18 indeed not an R-gene rather it is referred as durable resistance genes, this fact too, is in accordance with faint brown color due to lower accumulation of hydrogen peroxide. Durable resistance is different from major-gene resistance which follows gene-for-gene manner. It has broad and extended resistance efficiency to the disease (Johnson, 1984) (McDonald & Linde, 2002). It is expected to see more mild responses in this type of resistance. In the Yr differential line 14 (Avocet+Yr24), we spotted a hydrogen peroxide accumulated region which was absent in control groups. An HR symptom is present in first leaf sample of the cultivar but absent in the second leaf (replica) of the same cultivar which suggests that the cell death may be formed by sheer force during infiltration. We further investigated the accumulation site under light microscope which is shown in Figure 3.11. The brown region seemed to be local and incoherent. The second effector infiltrated replica leaf sample did not have any accumulation site which means either infiltration failed or HR in first leaf caused by physical damage. The brown spot was local and dense. Therefore, we concluded that the brown spot may be occurred wounding or other sheer stresses.

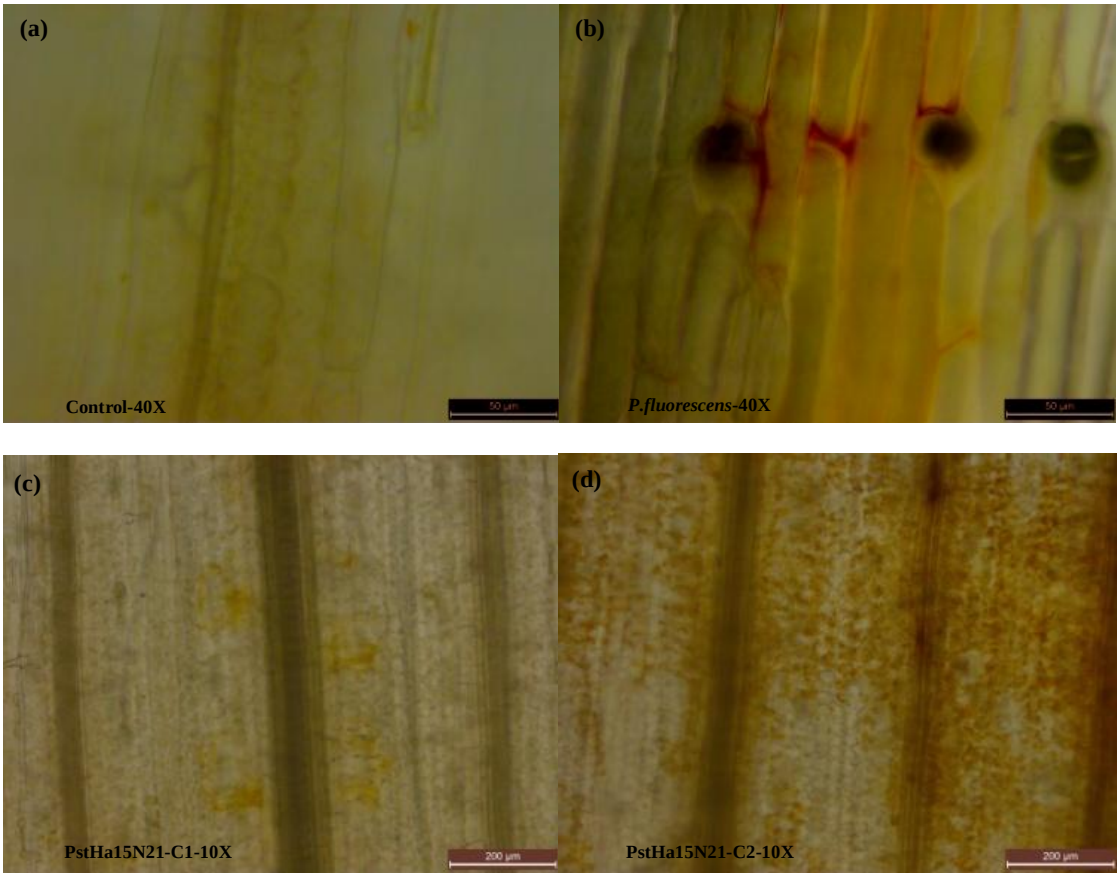


Figure 3.10 DAB staining results of YR differential line 13; Avocet+YR18. (a) Control ($MgCl_2$), (b) Empty *P. fluorescens* (EtHAn) infiltrated wheat leaf, (images of control groups shown in 40X magnification to emphasize that there are no visible cell burst) (c) Effector candidate infiltrated sample 1(S1) under 10X magnification and (d) Effector Sample 2, (S2) (10X) (Effector infiltrated samples shown in 10X magnification to prevent dense image of brown colored accumulation sites).

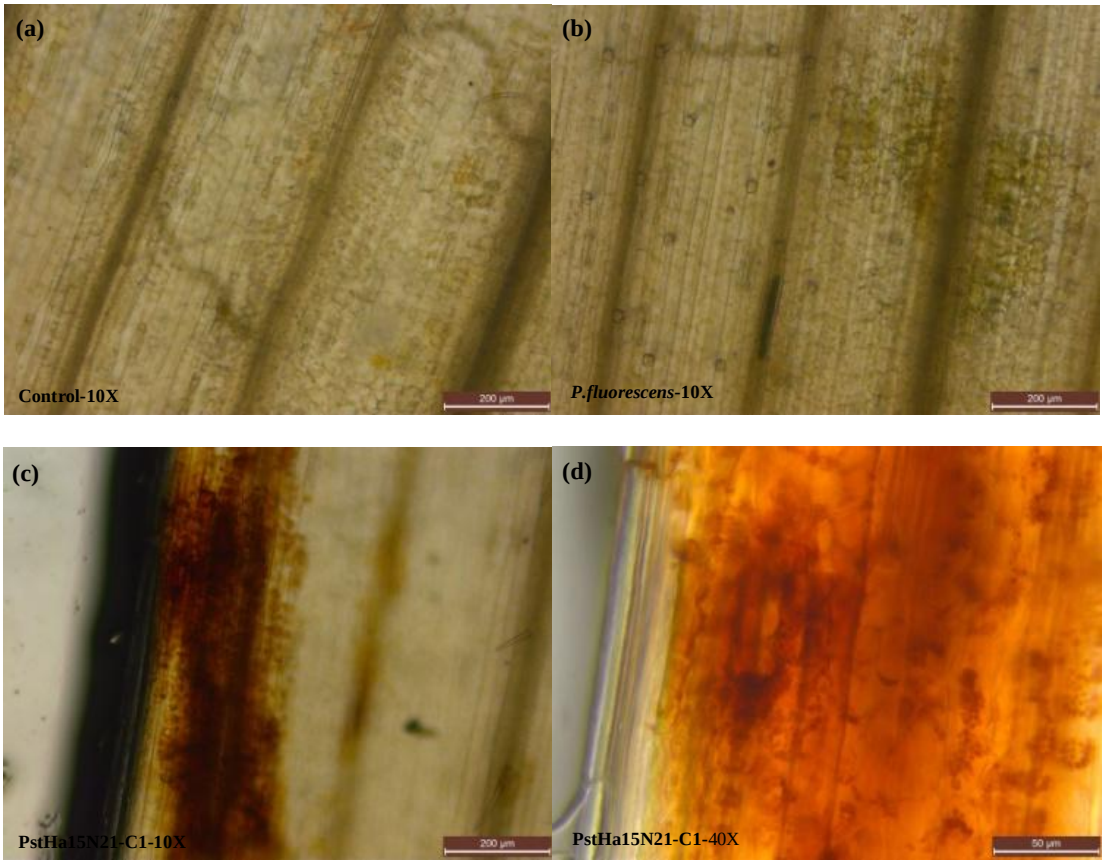


Figure 3.11 DAB staining results of YR differential line 14; Avocet+YR24. (a) Control, (b) Empty *P. fluorescens (*EtHAn) infiltrated wheat leaf, (c) Effector candidate, S1 under 10X magnification and (d) Effector candidate, S1 (40X).

The samples between 17 and 26 are displayed in Figure 3.12. There were 2 brown spots on leaf samples covered with slides. We further investigated these brown spots and scanned all other samples under light microscope for hydrogen peroxide accumulation. Except for the differential lines no 17 and 20, no significant symptom was observed. 17th sample had brown spots (Figure 3.13) nearly all over the leaf blade in all leaves including empty *P. fluorescens* and *P. fluorescens* with pNR527 vector (another compatible vector used as control group). This result suggests that the differential line 17 (Cultivar Avocet+YRSP) may show basal resistance or non-host resistance against *P. fluorescens* (EtHAn) just as in *N. tobaccum* in Figure 3.7.

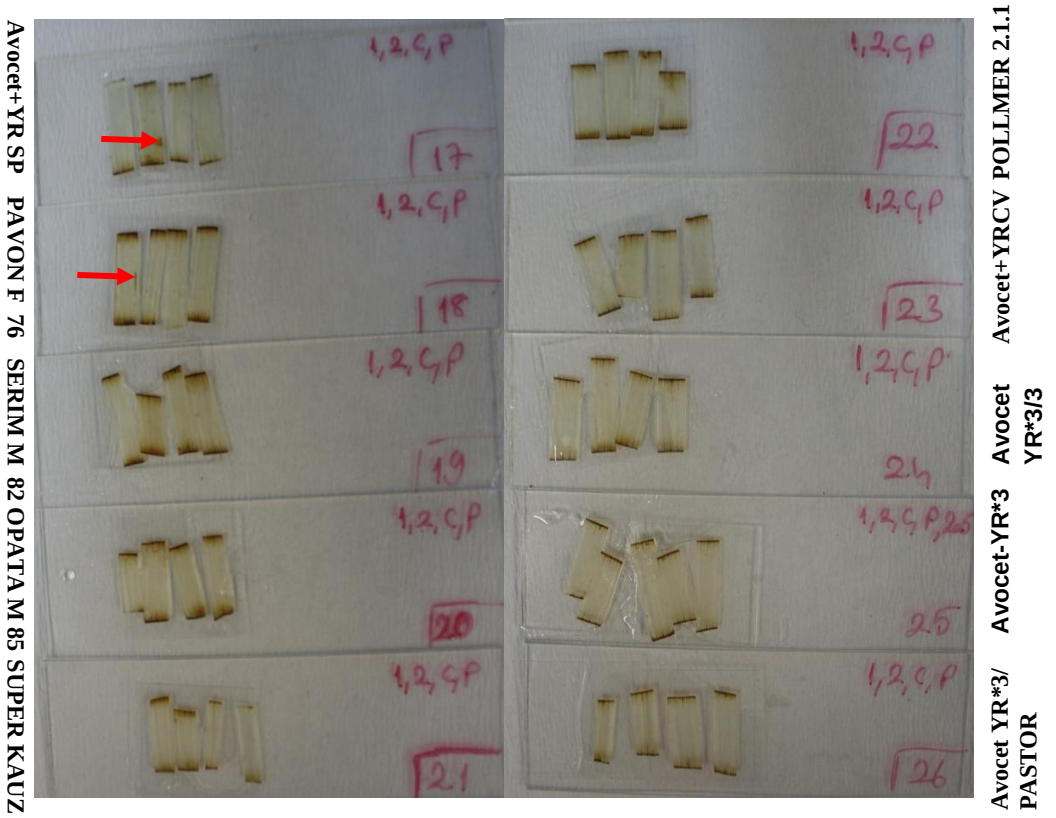


Figure 3.12 DAB staining photos of infiltrated YR differential lines. On the left, differential lines from 17 to 21 were displayed. Right photo belongs to differential lines from 22 to 26. Possible HR-like symptoms were shown *via* red arrows. The samples are ordered as PstHa15N21-S1 and S2, *P.fluorescens* (EtHAn) and control leaves from left to right, respectively.

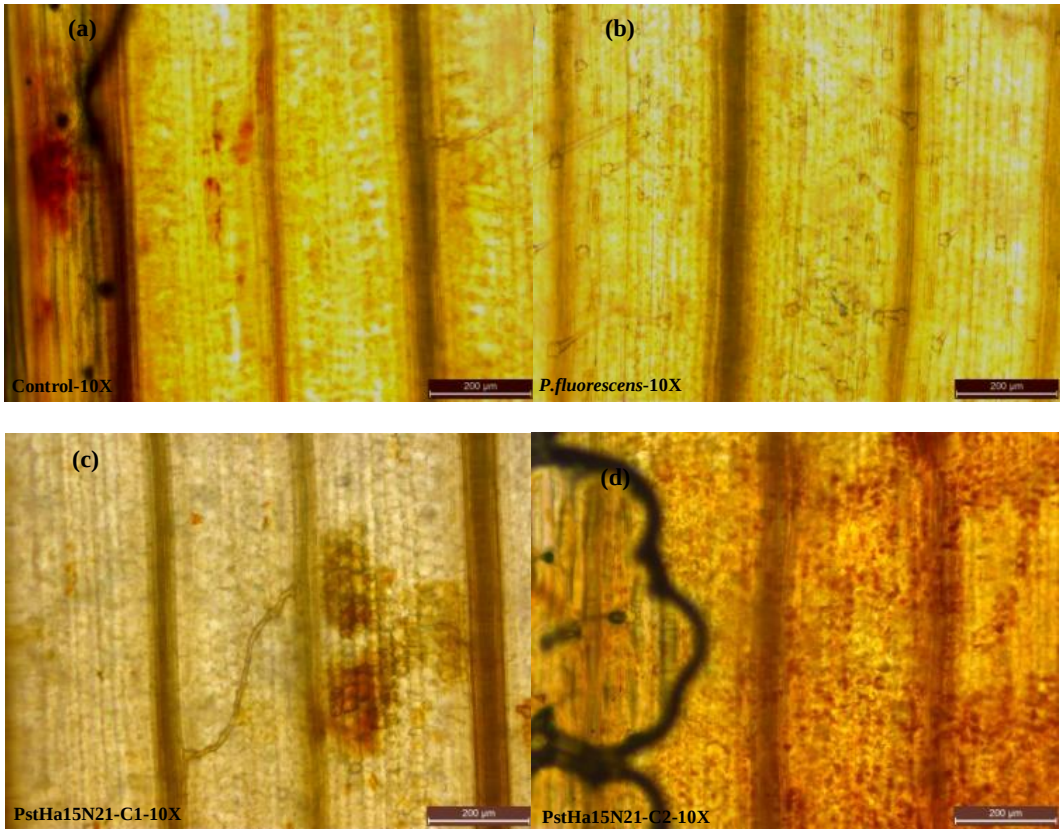


Figure 3.13 DAB staining results of YR differential line 17; Avocet+YRSP. (a) Control (*P. fluorescens* (EtHAn) with PNR527), (b) empty *P. fluorescens* (EtHAn) infiltrated wheat leaf, (c) Effector candidate, S1 under 10X magnification and (d) Effector candidate, S2 (10X).

In Figure 3.14, light microscope images of the differential line 20 (Opata M85) are presented. There are signs of HR symptoms in all samples. However, severity of the cell death increased in PstHa15N21-infiltrated leaf samples. $MgCl_2$-infiltrated control group had also faded brown accumulation sites remote from infiltration site, but they were rather insignificant compared to effector infiltrated ones. Although we spotted HR symptoms in differential line 20, they are not uniform and continuous through the leaf blade. Moreover, they are local and microbursts are rather low than we expected.

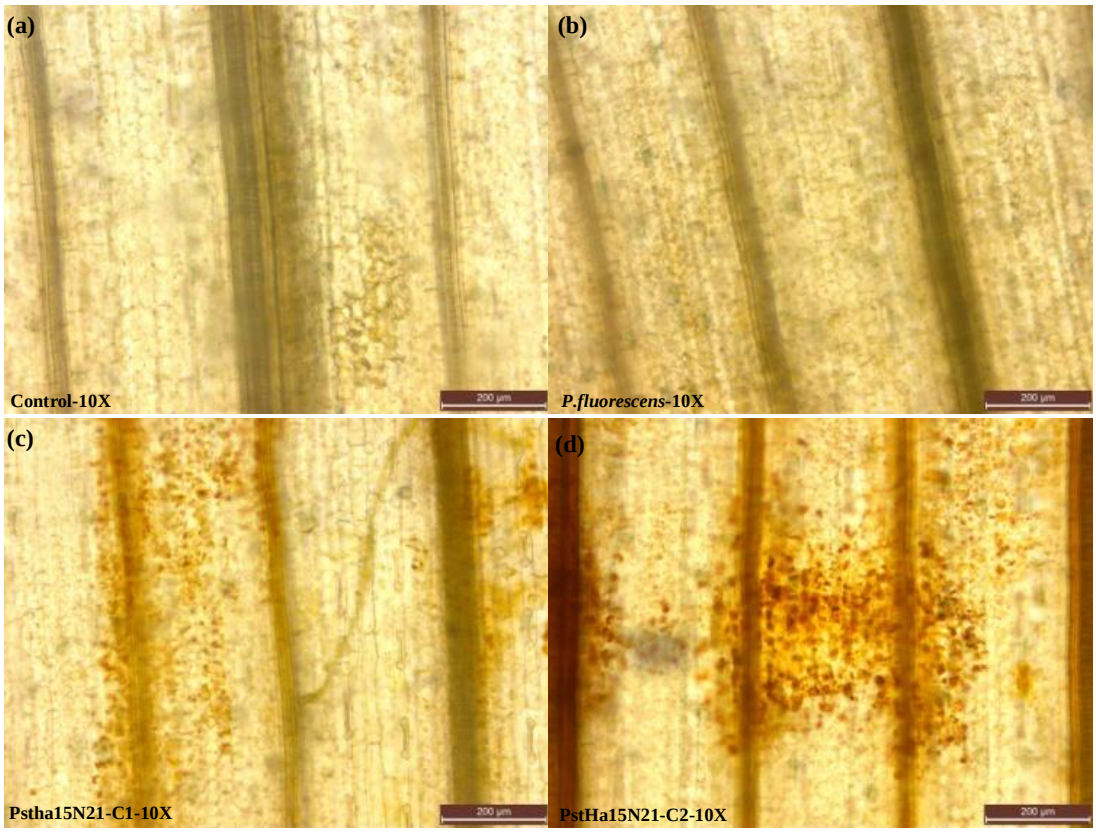


Figure 3.14 DAB staining results of YR differential line 20; Opata M 85. (a) Control, (b) Empty *P.fluorescens* (EtHAn) infiltrated wheat leaf, (c) Effector candidate, S1 under 10X magnification and (d) Effector candidate, S2 (10X).

We observed clear hypersensivity responses (HR) on differential line 5, Siete Cerros T66 as shown in Figure 3.15. The chlorosis of the leaf sample was clear and large as in the case of real infection stage. Intriguingly, the responses were absent in both control groups and second leaf sample infiltrated with PstHa15N21 to serve as replica. Moreover, the symptoms were present in secondary leaf of the infiltrated sample. We speculated that the HR symptoms might be formed by contamination *via* yellow rust spore in the environment. Hence, we kept the pot for further incubation in growth chamber. However, neither any spore formation occurred nor any HR symptoms were observed in other plant leaves. Moreover, plant leaves died swiftly and we lost the infiltrated cultivar. Hence, both DAB staining results and microscope images of the Cultivar Siete Cerros T66 is absent in result part. To track down the actual cause of HR symptoms in this cultivar (Figure 3.15), we will repeat the infiltration assay. Additional trials are also needed for confirmation of avirulence of the PstHa15N21 effector candidate in yellow rust differential line 5 called Siete Cerros T66.

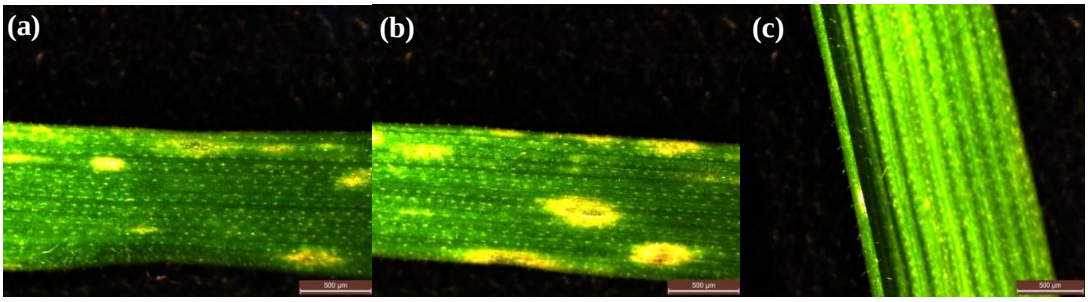


Figure 3.15 Photos of HR symptoms on infiltrated wheat line, Siete Cerros T66. (a) Infiltrated primary leaf sample (b) Secondary leaf of the same leaf sample (c) Control ($MgCl_2$ infiltration)

3.4. Cloning into pK7FWG2 expression vector

Subcellular localization of PstHa15N21 was studied using pK7FWG2 expression vector. pK7FWG2 is a Gateway destination vector which has a GFP sequence on its C-terminus for detection of the protein of interest *in planta*. We designed specific reverse primer without stop codon and forward primer bearing CACC and ATG site on its 5' end as we used in cloning to pEDV6 vector. The amplification (from synthesized gene construct which is shown in Figure 2.3) was done using Q5 High Fidelity *Taq* DNA Polymerase (NEB) in PCR. PCR products were loaded on 1% agarose gel for gel electrophoresis and the result was shown in Figure 3.16.

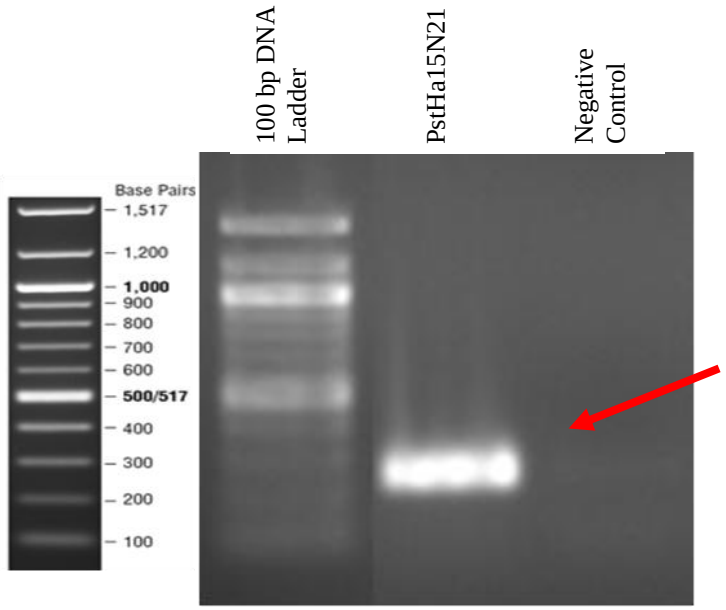


Figure 3.16 Agarose gel electrophoresis image of PCR reactions. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 100 bp DNA ladder (0.5 µg) (Cat # N3231s, Lot # 0831006, NEB) as marker. Second lane has 2µL of PCR products which has the gene of interest (~300 bp) as shown with red arrow. The third lane is negative control (No template) for the PCR.

Amplified gene of interest was needed to clone into pENTR™/D-TOPO vector before pK7FWG2 destination vector. Hence, we recombined the PCR product with pENTR™/D-TOPO vector using interaction between CACC site of the insert and topo-isomerase. The recombinant plasmids were transformed in *E.coli* TOP10 competent cell *via* heat shock method as mentioned in section 2.5.1.3. Transformants were selected on LB agar medium with Kanamycin (50 µg/mL). Colony PCR was applied on grown colonies to eliminate possibility to have false positive results. The output of the colony PCR reaction was displayed in Figure 3.17.

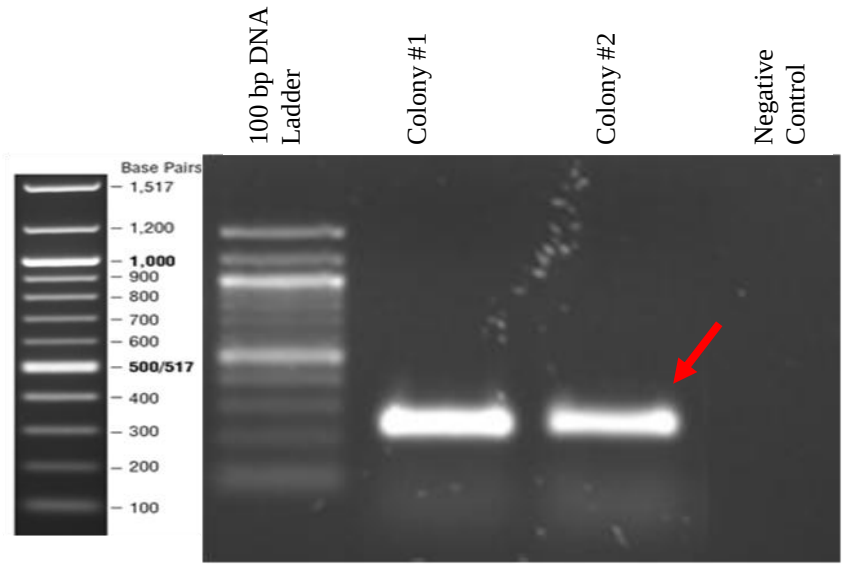


Figure 3.17 Agarose gel image of colony PCR reaction. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 100 bp DNA ladder (0.5 µg) as marker. Lanes 2 and 3 contain 2µL of colony PCR products. Positive colonies have ~300 bp single bands. Lane 4 is negative control (No template).

Subcloning into pK7FWG2 destination vector was performed using LR clonase enzyme after cloning into the entry vector. The reaction products were transformed into *E.coli* TOP10 cell *via* heat shock method. Transformants were selected on LB agar containing Gentamycin (100 µg/mL). Colonies were tested for presence of the insert in the destination vector using both colony PCR and double digestion method. Colony PCR results of the insert were shown in Figure 3.18.

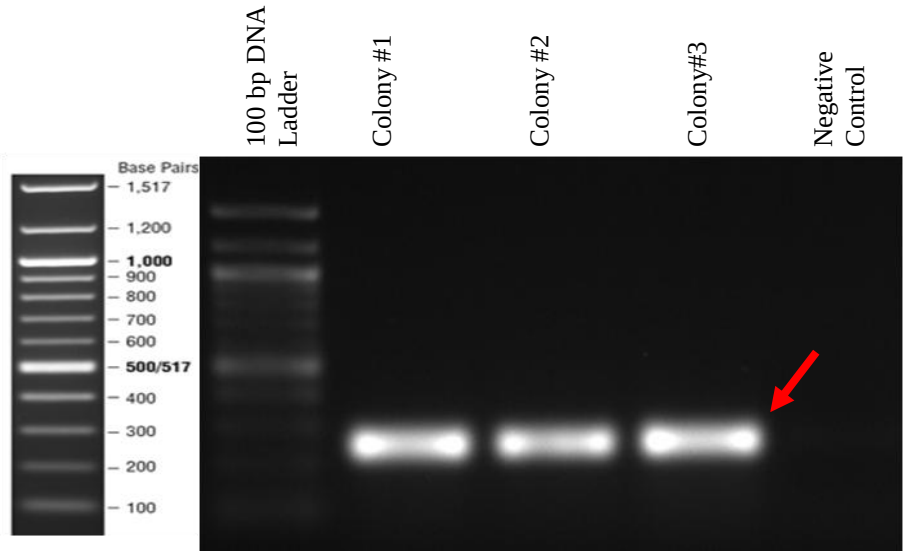


Figure 3.18 Agarose gel image of colony PCR reaction. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane was loaded with 100 bp DNA ladder (0.5 µg) as

marker. Lanes 2, 3 and 4 contain 2 µL of colony PCR products. Positive colonies have ~300 bp single bands as shown with red arrow. Lane 5 is negative control (No template).

Double digestion of the destination vector was the second confirmation method for success of cloning into destination vector. Colony PCR may sometimes give false positive results because we use the cells themselves as PCR template. We needed to ensure that was not the case. Hence, we cut the destination vector of both recombinant plasmids and empty vector with *Xba*I and *Spe*I restriction enzymes. Products of the digestion reaction were analyzed *via* agarose gel electrophoresis and the result was presented in Figure 3.19. Verified recombinant plasmid was transformed into *Agrobacterium* GV3101 (pMP90) *via* electroporation method. Transformed *Agrobacterium* cells were also confirmed by colony PCR method.

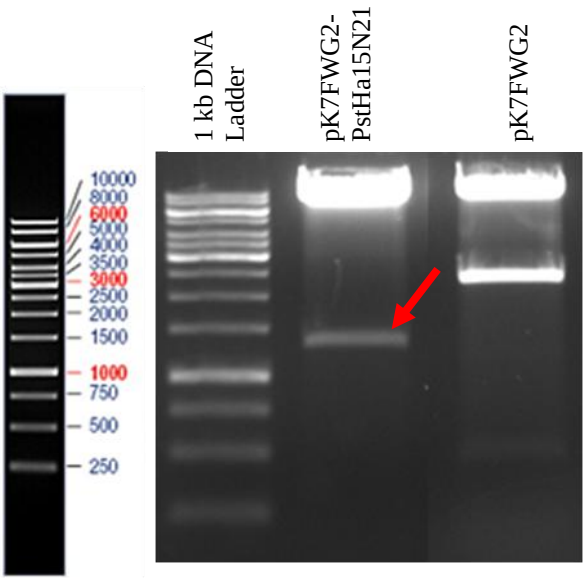


Figure 3.19 Agarose gel electrophoresis image of double digestion reaction. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 1 kb DNA ladder (0.5 µg) (Cat # SM0311, Lot # 00093172, Fermentas) as marker. Lane 2 contains 2 µL of digestion reaction products of pK7FWG2 vector isolated from colonies. Positive colony produce smaller band around 1400 bp while empty pK7FWG2 vector produce larger band due to presence of *ccd*B gene which is longer than the insert. Lane is empty pK7FWG2 vector used as control.

3.5 Subcellular localization of PstHa15N21

Agrobacterium GV3101 (pMP90) with pK7FWG2 expression vector was infiltrated into 5 weeks old *N. benthamiana* leaves. Same amount of *Agrobacterium* cells were penetrated into plant leaves. However, injection was not performed in one turn in some leaves. We opened several wounds to infiltrate same amount of culture in these leaves. After infiltration, the plants rested for 2 days. Then, we collected *Agrobacterium*- infiltrated leaf samples and kept them in water to prevent dying.

Visualization of GFP expression was made under light microscope (Leica DM4000B microscope / DFC 280 camera). Mock infiltrated (water) *N. benthamiana* leaf was used as control group. The subcellular localization of the gene of interest was recorded as it is shown in Figure 3.20.

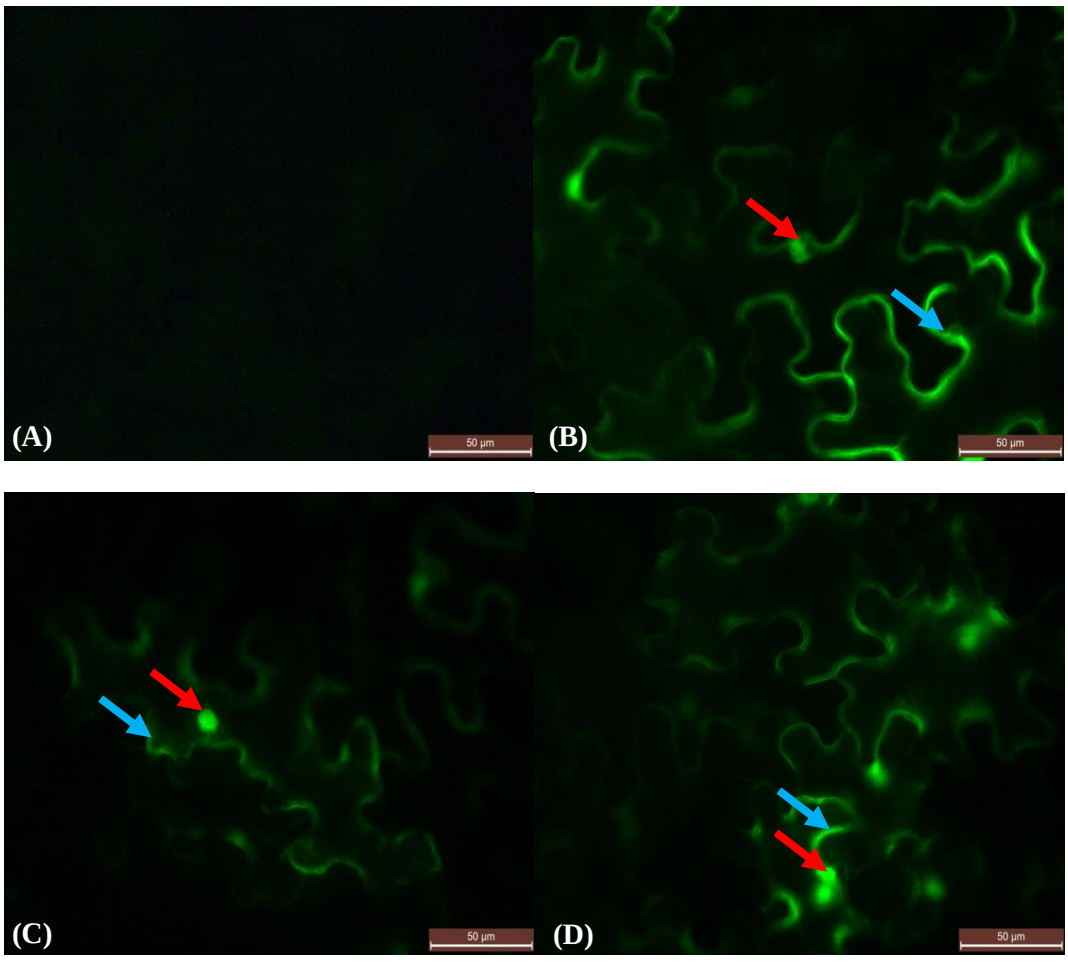


Figure 3.20 The subcellular localization PstHa15N21 effector candidate in *N. benthamiana*. Red arrows show the nucleus whereas blue ones indicate cytosol of the host cell. (a) Control, (b), (c) and (d) are PstHa15N21 infiltrated wheat leaves under 40X magnification**.**

The subcellular localization of PstHa15N21 was studied in *N. benthamiana* to comprehend the target site of the effector candidate *in planta*. The accumulation sites of the protein were both nucleus and cytosol as shown in Figure 3.20. The localization site of the effector was predicted in chloroplast or nucleus using 'WolfPSORT' program which uses homology ratio to guess accumulation site in cell (data not shown). However, we observed clearly that the effector candidate localized in cytosol and nucleus region *in planta*. Still, it was typical and average translocalization in host because GFP also follows non-specific translocation in host. We observed that the GFP expression of PstHa15N21was rather low. We can safely say that the effector could be an apoplectic one due to the loss of activity. To get clearer image of subcellular localization, we visualized our sample under confocal microscope, also (Zeiss, LSM 500). The images of confocal microscope analysis were displayed in Figure 3.21. Cytosolic accumulation of the effector shows that PstHa15N21 may have cytosolic functions. However, accumulation rate in nucleus was substantially higher than cytosolic aggregation. Moreover, membrane targeted protein localization was not observed, suggesting that effector does not interact with recognition patterns on membrane structure. Our effector protein bears cysteine rich residues in its sequence. This feature generally belongs to apoplastic effectors along with smallness and having secretory signals in N-terminus (Jonge *et al*., 2011). Therefore, the non-specific localization in host is possible for an apoplastic effector.

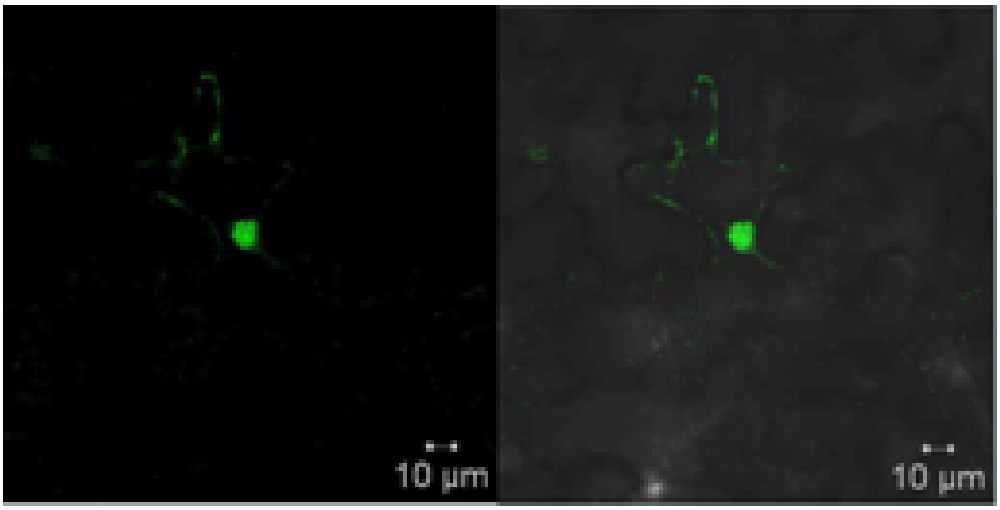


Figure 3.21 Images of PstHa15N21 fused to GFP.

In summary, the translocation pattern of PstHa15N21 has features of non-specific dissemination of GFP. Transient expression *in planta* has some limitations because the real infection process of the disease could not be achieved. In agroinfiltration, the concentration of the effector couldn't be adjusted at the same level as in actual disease development. Moreover, although *N. benthamiana* is an excellent model organism to study subcellular localization, the effector of interest may not attain proper translocation as it does in its actual host. To overcome this limitation, there is a need for expression system in wheat to study subcellular localization without inducing HR symptoms for *Pst*-host interaction analysis. We used a C-terminus GFP fusion system to avoid masking N-terminus signals of the effector which may manage translocation in host. We also removed N-terminal signal peptide from *in planta* cleavage site to prevent transfer of the effector to outside of the plant cell because this signal is predicted to be removed upon entry into host cell. Hence we did not want this signal to interfere with translocation in the host.

3.6 Cloning of PstHa15N21 into pJL48-TRBO vector

PstHa15N21 was cloned into pJL48-TRBO vector to conduct agroinfiltration assay in search for host interacting factors. pJL48-TRBO is an over-expression vector and uses *Agrobacterium* mediated gene transfer. Hence, we can express the gene of interest in *N.benthamiana* in large amounts and we can retrieve the effector and factors interacting with it. We designed the construct of our gene with a FLAG-tag on its N-terminus and had synthesized it in that way. Also, there was a linker sequence to ensure the tag part does not disrupt the folding. Thus, the activity of the effector would not be lost.

The vector of choice has a small multiple cloning site, in which only 3 restriction enzyme cuts site are present. Therefore, we designed our primers so that they had *Pac*I and *Not*I cut sites on their 5' and 3' ends, respectively to achieve directional cloning. They also had additional 3 nucleotides on their ends to provide the enzyme a better landing position on the insert. The amplification was performed as in section 2.7.1 and products of the reaction were run on agarose gel (1%) electrophoresis as shown in figure 3.22. The red arrow shows PstHa15N21 gene. The length of the amplicon was greater than the gene due to presence of FLAG-tag and linker sequence.

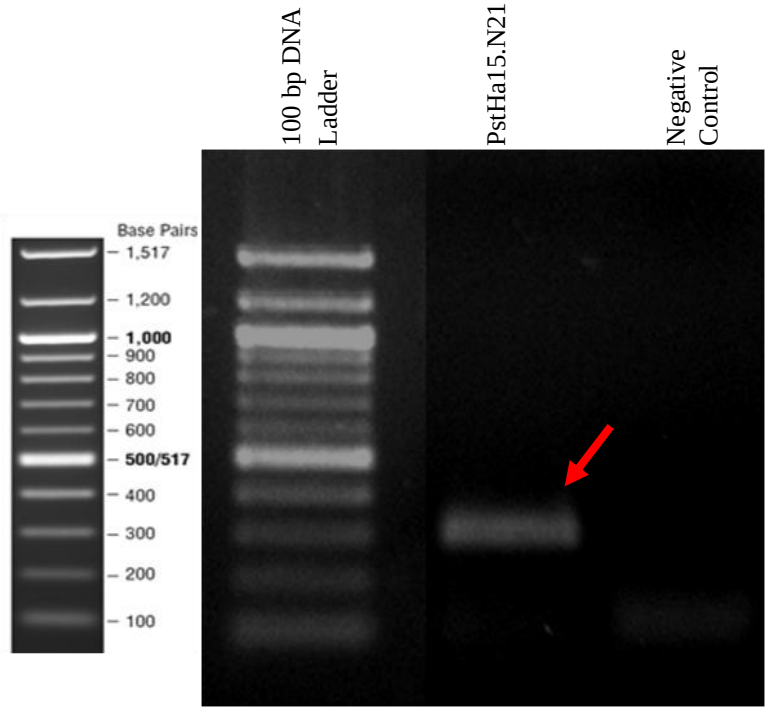


Figure 3.22 Image of PCR product on agarose gel. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 100 bp DNA ladder (0.5 µg). Second lane has 2µL of PCR products which has the gene of interest (~300 bp). Red arrow shows PstHa15N21.

Amplified PstHa15N21 gene with FLAG-tag was cut with *Not*I and *Pac*I restriction enzymes. PstHa15N21 band was observed on agarose gel which was shown in Figure 3.23. The band was extracted from the gel and measured using Nanodrop. The data is presented in Figure 3.23. The yield of extraction was efficient to perform ligation reaction. We lost little DNA during extraction process.

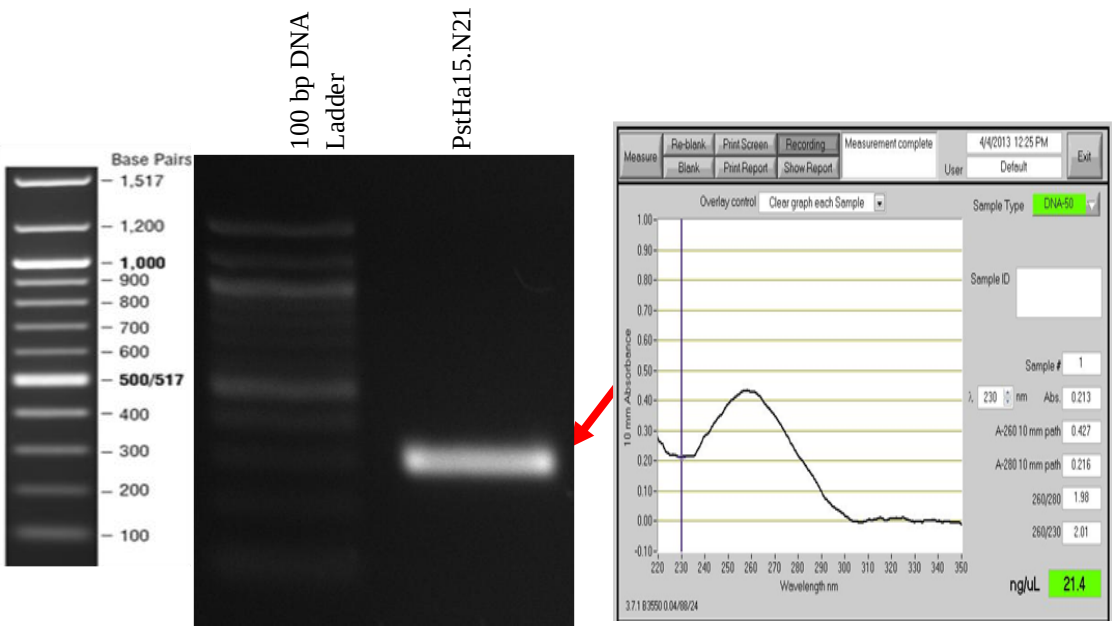


Figure 3.23 Image of PCR product on agarose gel and NanoDrop result of the band. The samples were separated on 1% agarose gel (in TAE buffer). First lane is loaded with 100 bp DNA ladder (0.5 µg) as marker. Second lane has 2µL of PCR products which has the gene of interest (~300 bp) marked with red arrow.

We isolated the vector of interest (pJL48-TRBO) from 8 mL of cell culture. We used a large amount of cell because pJL48-TRBO is a low copy number plasmid. Therefore, it makes ligation reaction harder. Isolated plasmids were measured using Nanodrop and the result was recorded (Figure 3.24/a).

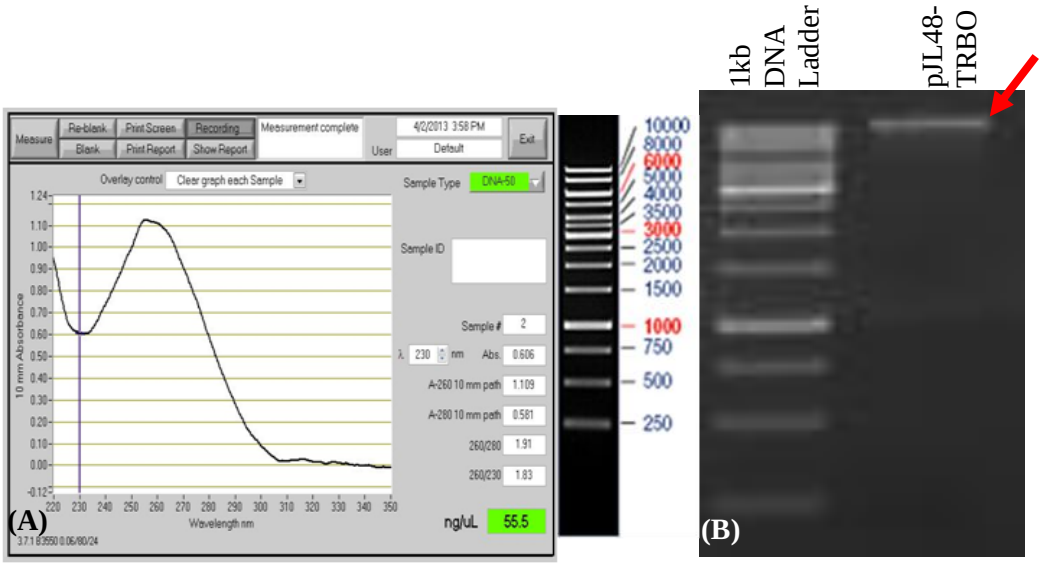


Figure 3.24 Preparation of pJL48-TRBO. **(a)** Nanodrop result of the pJL48-TRBO plasmid isolation. **(b)** Agarose gel electrophoresis image of double digestion reaction. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 1 kb DNA ladder (0.5 µg), whereas the second lane has pJL48-TRBO marked with red arrow.

Isolated plasmid was cut with *Pac*I and *Not*I restriction enzymes. Digested pJL48-TRBO plasmid was separated on 1% agarose gel and then gel extraction was performed. 1 µL of the digested plasmid was loaded on agarose gel (Figure 3.24). Ligation reaction was performed between pJL48-TRBO and the gene in different 'vector: insert' ratios depending on the data provided in Figure 3.23 and 3.24. The ligation products were transformed into *E.coli* TOP10 competent cells. Growing transformant cells were tested using both colony PCR and double digestion method (Figure 3.25). Interestingly, colony PCR gave positive results while the insert was not observed after double digestion reaction. We hypothesized that the insert was not observed because pJL48-TRBO is a low copy number plasmid. Hence, we sent the plasmids with insert to MCLAB, USA for sequencing. The result of the sequencing was negative (data not shown).

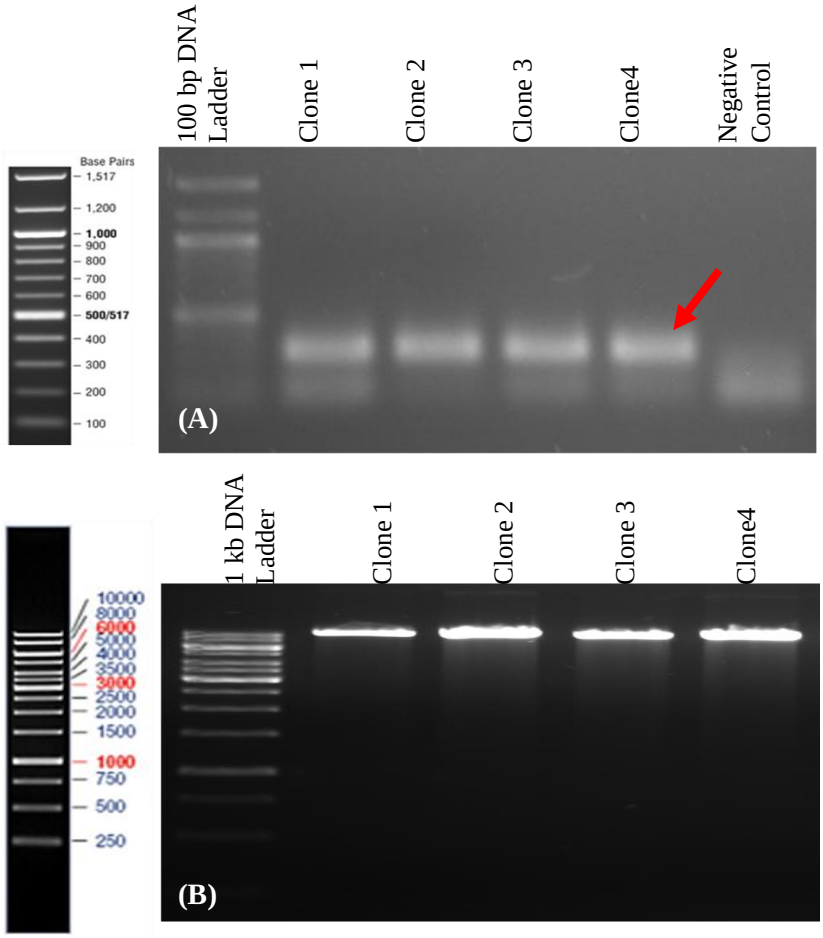


Figure 3.25 Confirmation of transformation of PstHa15N21 into *E.coli* TOP10 cells. **(a)** Agarose gel image of colony PCR reaction. The samples were separated on 1% agarose gel (in 1X TAE buffer). First lane is loaded with 100 bp DNA Ladder (0.5 µg) as marker. Lane no: 2-5 contains 2 µL of colony PCR products. Positive colonies have ~300 bp single bands marked with red arrow. Lane 6 is negative control (No template). **(b)** Agarose gel image of double digestion reaction using *Pac*I and *Not*I restriction enzymes. First lane is loaded with 1 kb DNA Ladder (0.5 µg). Lanes from 2 to 5 contain 20 µL reaction products. The insert was not observed.

We tried the ligation reaction in various experimental conditions but we failed in all cases. To investigate why colony PCR gave positive result, we also repeated the experiment and used empty pJL48-TRBO vector as template to see whether non-specific amplification occurs. One of the possible explanation for cloning failure, proximity of the cuts sites in multiple cloning site of the vector. There are only 22 nucleotide long DNA sequences present between *Pac*I and *Not*I sites. Therefore, it is possible that the enzymes may not cut the vector of choice at the same time. To avoid this problem, we used pJL48-TRBO-PstHa2a5 as the plasmid template in cloning, which was a previously cloned effector.

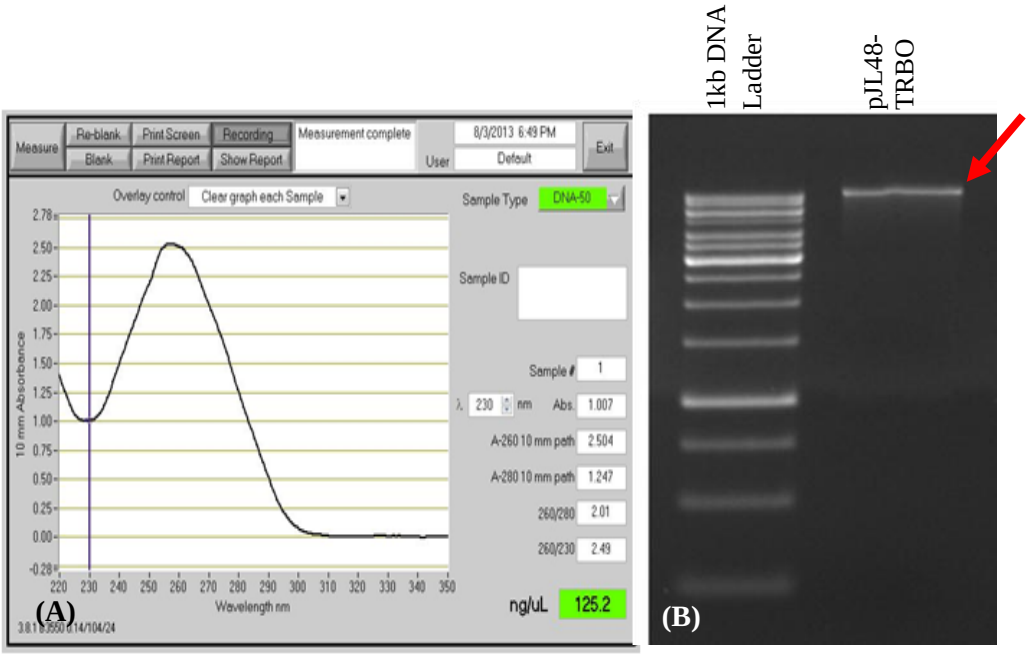


Figure 3.26 pJL48-TRBO vector preparations. **(a)** NanoDrop result of isolated pJL48-TRBO vector with PstHA2A5 insert. **(b)** Gel photo of double digested product after gel purification. First lane was loaded with 1 kb ladder (marker). Second lane, cut pJL48-TRBO vector was loaded after gel extraction. Red arrow shows the cut pJL48-TRBO vector with an expected size of ~10,000 bp. (electrophoresis was performed by 1% agarose gel in 1X TAE buffer).

We also directly cut PCR products. We added several nucleotides after cut sites to avoid any dissociation in the end of the PCR product in synthesized construct. However, restriction enzymes possibly did not cut the PCR products properly and did not produce any stick end. To eliminate this possibility, we decided to clone our PCR product into another intermediate vector called pGEM-T-Easy. Using TA cloning, we were able to clone PstHa15N21 PCR products into pGEM-T-Easy. Cloning reaction was verified *via* colony PCR method. The insert was cut from the pGEM-T-Easy vector *via* double digestion. The digested inserts with sticky ends were recovered by gel extraction. Prepared vector and insert were ligated and then transformed into *E.coli* TOP10 cells. Transformation was verified with both colony PCR and double digestion method. The insert was observed after double digestion as a faded band due to low plasmid number of the vector (Figure 3.27).

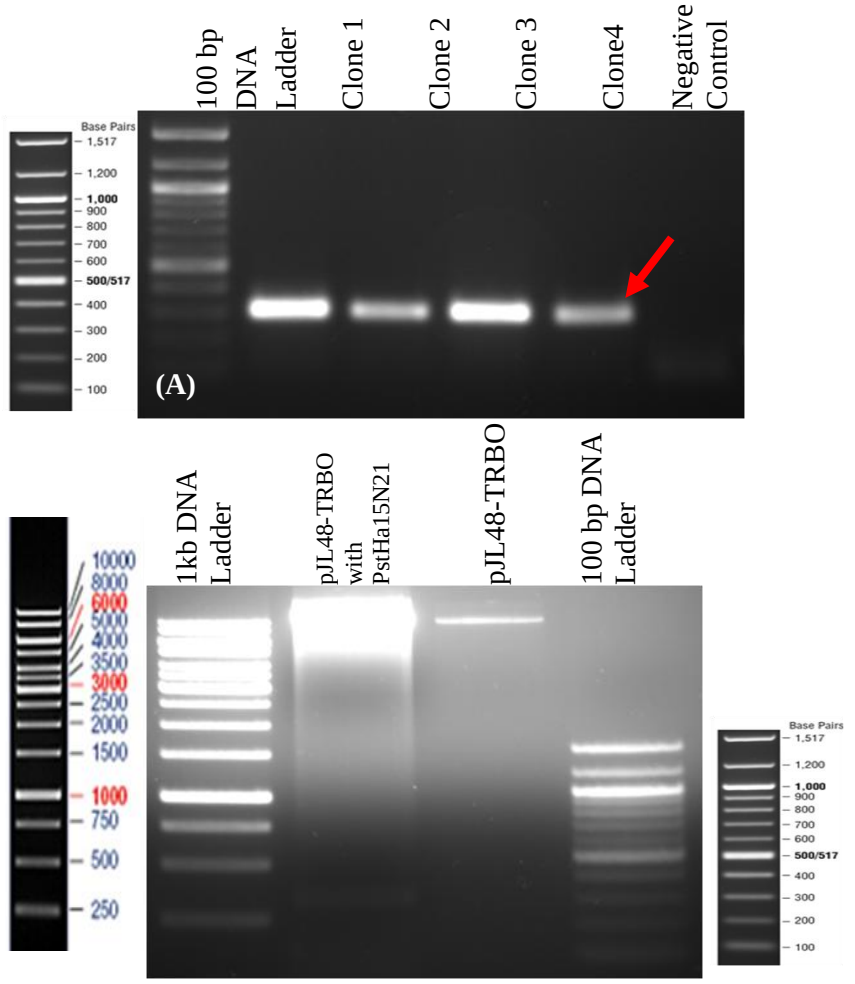


Figure 3.27 Confirmation of transformation of pJL48-TRBO with PstHa15N21. **(a)** Agarose gel image of colony PCR reaction. First lane is loaded with 100 bp DNA ladder (0.5 µg). Lane no: 2-5 contains 2 µL of colony PCR products of pJL48-TRBO with the gene. Positive colonies have ~300 bp single bands marked with red arrow. Lane is negative control (No template). **(b)** Agarose gel image of double digestion reaction using *Pac*I and *Not*I restriction enzymes. First lane is loaded with 1 kb DNA ladder (0.5 µg). Second lane contains 20 µL reaction digested plasmid. Third lane was empty pJL48-TRBO. (The samples were separated on 1% agarose gel in 1X TAE buffer).

Confirmed clones were stored properly at -80 ^{o}C after cloning into pJL48-TRBO vector was achieved. We had a hard time while cloning into the vector due to its narrow multiple cloning sites. We also used an intermediate vector to produce sticky ends of the insert *via* double digestion. Possibly, directly cutting PCR products with restriction enzymes was not an efficient way. Upon achieving cloning, we will proceed with agroinfiltration of the cloned vectors into the host, *N. benthamiana.* Then, we will perform co-immunoprecipitation to track down interacting host factors.

CHAPTER 4

CONCLUSION

Wheat yellow (stripe) rust is one of the most devastating and disastrous cereal diseases in the world. Its causative agent, *Puccinia striiformis* f. sp. *tritici* is an obligate biotrophic fungus. Interaction of pathogen with its host is poorly understood. Pathogen effectors are the proteins secreted by haustoria for feeding and interacting with the host cell. Identity and delivery mechanism of effectors and their functions are yet to be discovered. Thus, any information obtained on the effector in this thesis study, as preliminary as it might be, it is still a gain. Moreover, these findings will initiate further investigation to gather more insight on molecular complexity of plant-pathogen interactions and plant innate immunity.

With the advances in genome sequencing projects, effectors of filamentous fungi can now easily be mined using bioinformatics tools. However, there is a need for robust expression systems to test the predicted effector candidates in high throughput manner. It was reported that *P. fluorescens* (EtHAn) can deliver effectors into wheat with its TT3S (Thomas, Thireault, Kimbrel, & Chang, 2009; Yin *et al*., 2009b; Yin & Hulbert, 2010).

In this thesis study, we selected an effector candidate, PstHa15N21, from a predicted effector list which were obtained from haustorial cDNA library (Yin *et al*., 2009b). PstHa15N21 effector was cloned into pEDV6 vector (which stands for 'effector detector vector'). Then, PstHa15N21 was tested for avirulence by observing the presence of HR symptoms in yellow rust differential lines.

HR symptoms were observed in cultivar 13 (YR18/3*AOC) by light microscope. Presence of HR indicates that PstHa15N21 is likely to be an avirulence (Avr) gene which may interact with the R-gene. The R-gene, YR18 is present in the YR18/3*AOC cultivar. YR18 is a membrane-bound ABC transporter and it was recently reported that it shows durable resistance to multiple fungal pathogens in wheat (Krattinger *et al*., 2009). YR18 doesn't possess NB-LRR sequence and it is different than other cloned R-genes (Keller *et al*., 2009).

PstHa15N21 is a cyteine-rich, small and secreted protein which is likely to be an apoplastic effector. Hence, the interaction between YR18 and PStHa15N21 is possible due to observed mild HR. To test if it is apoplastic protein, PstHa15N21 was cloned into pK7FWG2 as a GFP fusion for finding the subcellular localization. If the effector was translocated only in the cell periphery, we could safely say that PStHa15N21 is encoding a apoplistic protein and interacting with Yr18 encoded receptor, thus concluding that PstHa15N21 is indeed AvrYr18 leading to the HR. On the other hand, our observation suggested that PstHa15N21

was localized both in the cytosol and the nucleus showing a non-specific translocation pattern in *N. benthamiana*. However, this observation might be an artifact, since the huge plant vacuole of tobacco cells occupy the almost entirety of the cell pressing the cytosol toward cell periphery and preventing the observation of the cytosol separate from the cell periphery. Therefore, it becomes very difficult to detect if the effector is associated on the periphery of the cell or in the cytoplasm. In order to confirm whether the effector is an apoplastic or cytoplasmic, experiments are needed to be repeated with co-expression of both effector and Yr18 in *N. benthamiana*, so that the Yr18 R-gene receptor is present in the system. If the membrane association can be shown by infiltrating the purified effector protein with the SP domain, we could support that the PstHa15N21 is an apoplastic avirulence effector gene, when compared with protein the SP domain-GFP fusion as a control. Our observation currently does not exclude the effector being an apoplastic protein. We will also repeat the experiment on dehydrated plant seedling where the vacuole shrinks leaving a room for the cytoplasm.

Also, we detected HR like symptoms in YR differential line 1, 5, 14, 17 and 20. However, these cases were inconsistent; not reproducible in the replicates. This occurred most likely because of physical wounding of the cells, not because of incompatible interaction among the plant and pathogen resulting HR. Cultivar no 17 (YRSP/6*AOC) may show some levels of basal or non-host resistance to *P. flourescens* EtHAn because all the infiltrated leaves. Cultivar no 5 (Siete Cerros T66) produced HR in both primary (infiltration site) and secondary leaves of the cultivar. This was an unexpected result, since the secondary leaves were not infiltrated, they cannot produce HR. It is very likely that an intact pathogen contaminated that sample unintentionally.

Pseudomonas fluorescens EtHAn mediated wheat infiltration assay is a practical, rapid and effective method to test avirulence of *Pst* effectors. However, HR symptoms were mild and very few across the infiltrated regions of leaf surfaces in our experiments. Therefore; in the future work, we will repeat the infiltration experiments to obtain reproducible and robust HR symptoms. Moreover, we will use other YR18 bearing wheat cultivars (Appendix E) for avirulence tests to support the data we obtained in this thesis.

PstHa15N21 was also cloned into a pJL48-TRBO overexpression vector to track down host interacting factors. In future analysis, we will infiltrate *N. benthamiana* leaves with PstHa15N21 bearing *Agrobacterium*. Then, we will perform tagged co-immunoprecipitation assays to catch interacting factors of the host plant. Mass spectrometry analysis will be applied to determine the identity (sequence) of these proteins. Although *N. benthamiana* is not the real host of PstHa15N21, infiltration to this plant is much easier and much efficient, since *Agrobacterium* can infect the *N. bentamiana* for expressing a foreign gene. Thus, we hope to obtain some information about the interactions of PstHa15N21 in *planta*.

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APPENDIX A

PstHa15N21 GENE SEQUENCE

(Obtained from NCBI databases)

```
LOCUS       GH737567                459 bp    mRNA    linear   EST 05-JAN-
2010
DEFINITION  PSTha15N21 Puccinia striiformis f. sp. tritici haustoria cDNA
library Puccinia striiformis f. sp. tritici cDNA clone 15N21 5',
            mRNA sequence.
ACCESSION   GH737567
VERSION     GH737567.1  GI:222428916
DBLINK      BioSample: LIBEST_024285
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 459)
  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
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I;
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      421 ccacgaaaaa aaaaaaaaaa aaaaaaaaaa aaaacatgt
//
```

APPENDIX B

SEQUENCE OF pJL48-TRBO VECTOR

(Obtained from Lindbo, 2007)

>pJL48 vector sequence (10606bp):(nucleotide1=first nucleotide of TMV)

GTATTTTTACAACAATTACCAACAACAACAAACAACAGACAACATTACAATTACTATTTACAATTACAATGGCATA
CACACAGACAGCTACCACATCAGCTTTGCTGGACACTGTCCGAGGAAACAACTCCTTGGTCAATGATCTAGCAAAG
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CGTGCATTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATATCTGATGATGCAAATTCCCTACGGATCATTG
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APPENDIX C

SEQUENCE OF pEDV6 VECTOR

(Obtained from Peter Doods)

>pEDV6; 6985bp

ACCTTCGGGAGCGCCTGAAGCCCGTTCTGGACGCCCTGGGGCCGTTGAATCGGGATATGCAGGCCAAGGCCGCCGC
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APPENDIX D

SEQUENCE OF pK7FWG2 VECTOR

(Karimi *et al*., 2002)

>pK7FWG2 ;11880 bp

CGACGTCGCATGCCTGCAGGTCACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTACAAA
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APPENDIX E

TABLE OF SOME YR18 CONTAINING WHEAT LINES

Table 1: The size of bands in six allele specific markers of the *Lr34* gene for each cultivar tested in this study. The field test results of leaf rust are also indicated. Rust resistance was recorded on a scale from 0 (no pustules = resistant) to 100 (leaf area totally covered with pustules = highly susceptible) on a field plot basis.

Cultivar	*cssfr1* (bp)	*cssfr2* (bp)	*cssfr3* (bp)	*cssfr4* (bp)	*cssfr5* (bp)	*cssfr6* (bp)	*Lr34* status	Field test of leaf rust
Albatros^{seq}	517	–	150+517	150	751	451+135	+	40 MS
Aldane	–	523	229	229+523	523	589	–	MR
Altay2000	–	523	229	229+523	523	589	–	80 S
Aytın 98	–	523	229	229+523	523	589	–	80 S
Atilla 12	–	523	229	229+523	523	589	–	100S
Atlı2002	–	523	229	229+523	523	589	–	100 S
Basribey 95	–	523	229	229+523	523	589	–	40 MS
Bezostaya1^{seq}	517	–	150+517	150	751	451+135	+	60–80S
Bolal 2973	–	523	229	229+523	523	589	–	60–100 S
Dağdaş 94	–	523	150	150+523	523	589	–	60 S
Demir	–	523	229	229+523	523	589	–	80–100S
Dropia^{seq}	517	–	150+517	150	751	451+135	+	100 S
Esperia^{seq}	517	–	150+517	150	751	451+135	+	40 MS
Flamura 85	–	523	229	229+523	523	589	–	100 S
Gelibolu^{seq}	517	–	150+517	150	751	451+135	+	80–100 S
Gerek 79^{seq}	–	523	229	229+523	523	589	–	MR-MS
Göksu 99^{seq}	517	–	150+517	150	751	451+135	+	80 S
Golia	–	523	229	229+523	523	589	–	100 S
Guadalupe	–	523	229	229+523	523	589	–	100 S
Gün 91^{seq}	–	523	229	229+523	523	589	–	80–100 S
İkizce 96^{seq}	–	523	229	229+523	523	589	–	80 S
Kaklıç 88^{seq}	–	523	229	229+523	523	589	–	40 MS
Kate A1	–	523	229	229+523	523	589	–	100 S
Kınacı 97	–	523	229	229+523	523	589	–	MR
Kırgız 95	–	523	229	229+523	523	589	–	60 S
Kırkpınar 79	–	523	229	229+523	523	589	–	100 S
Krasunia^{seq}	517	–	150+517	150	751	451+135	+	60 S
Kutluk 94	–	523	229	229+523	523	589	–	60–100 S
Nina	–	523	229	229+523	523	589	–	100 S
Pehlivan	–	523	229	229+523	523	589	–	100 S
Prostor^{seq}	517	–	150+517	150	751	451+135	+	80–100 S
Sagittario	–	523	229	229+523	523	589	–	100 S
Saraybosna^{seq}	517	523	150+517+320+229 320+229	150+523+320+229 320+229	751	451+135	+	100 S
Saroz 95	–	523	229	229+523	523	589	–	100 S
Selimiye	–	523	229	229+523	523	589	–	80 S
Seval	–	523	229	229+523	523	589	–	100 S
Sultan 95^{seq}	517	–	150+517	150	751	451+135	+	60–80 S
Syrena^{seq}	517	–	150+517	150	751	451+135	+	80 S
Tekirdağ^{seq}	517	–	150+517	150	751	451+135	+	100 S
Tina	–	523	229	229+523	523	589	–	100 S
Tosunbey	–	523	229	229+523	523	589	–	60 S
Uzunyayla	–	523	229	229+523	523	589	–	100 S
Yakar 99	–	523	229	229+523	523	589	–	60–80 S
Yıldız 98^{seq}	517	523	150+517+320+229	150+523+320+229	751	451+135	+	80 S
Yunak	–	523	229	229+523	523	589	–	100 S
Zencirci 02^{seq}	–	523	229	229+523	523	589	–	100 S

seq indicates sequenced cultivars.

–/+ indicate the absence and the presence of the resistance allele of the *Lr34* gene, respectively.

MR = Moderate Resistant, MS = Moderate Susceptible and S = Susceptible

APPENDIX F

P. FLUORESCENS HEAT SHOCK COMPETENT CELL PREPARATION

The protocol to prepare heat shock competent *P. fluorescens* EtHAn cells was adapted from LBM protocols. The bacteria were incubated for overnight in 4 mL LB medium. 300 µL of the overnight culture was poured into 30 mL LB and incubated at 33 ^{o}C at 200 rpm in a shaker to reach A_{600nm} value between 1.8 and 2.0. The grown cells were centrifuged at 5000 rpm for 5 min. at 4 ^{o}C. The supernatant was removed and the pelleted cells were resuspended in 7.5 mL of chilled 0.1 M $CaCl_2$ solution by pipetting. The samples were nested on ice for 30 min. The cells were harvested by centrifugation at 5000 rpm, 4 ^{o}C for 5 min. The supernatant was discarded and the pellet was resuspended in 1.5 mL chilled 0.1 M $CaCl_2$. 50 µL of DMSO was added into the cells and gently swirled. The suspension was placed in ice for 15 min. After resting, 50 µL of DMSO was added into the suspension. The cells were again rested in ice bath for 15 min. 100 µL aliquots of the cell suspension was poured into sterile 1.5 mL eppendorf tubes. The cells were freezed by liquid nitrogen, immediately and store at -80 ^{o}C.

Competent *P. fluorescens* EtHAn cells were thawed in an ice bath for transformation. 5 µL of pEDV-PstHa15N21 plasmid was added into the cells and the mixture was left on ice for 30 min. Heat shock was applied to the cell at 42 ^{o}C for 2 min. in a water bath. 1mL of prewarmed (33 ^{o}C) SOC medium was added to the cell. Then, the mixture was incubated for 4 hours. The cells were grown on selective media consisting LB agar with 100 µg/mL Gentamycin for overnight. However, the transformation failed even the experiment was repeated 4 times.