DETERMINATION OF GENES INVOLVED IN THE YELLOW RUST DISEASE OF WHEAT

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ABSTRACT

DETERMINATION OF GENES INVOLVED IN THE YELLOW RUST DISEASE OF WHEAT

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It is important to understand the underlying plant defense mechanisms in order to establish best strategies to reduce losses due to diseases in cereals. The current available information is mostly on model organisms and their plant-pathogen interactions. However, this study is focused on the identification of genes involved in the resistance mechanism of one of the most devastating diseases of wheat, yellow rust. The strategy undertaken was to use differential display method (DD) together with microarray technology, on yellow rust differential lines of wheat (Avocet-Yr1 and Avocet-Yr10) infected with the virulent and avirulent *Puccinia striiformis* f. sp. tritici races (Pst: PST17, PST45, 169E136 and 232E137) together with appropriate control infections. DD primer combinations of ninety allowed the detection of fourteen differentially expressed genes which were also confirmed by real-time QRT-PCR analysis. All of but one were found to be novel sequences in wheat genome. Among those, two very important genes were identified as full ORF including 5' and 3' end untranslated regions (UTR); namely cyclophilin like protein

(putative antifungal activity) and ubiquitin conjugating enzyme (E2). The sequence homology analysis of the cloned gene fragments reveled that the genes detected have roles in ubiquitinylation, programmed cell death (apoptosis), putative antifungal activities, disease resistance, pathogen related responses, including a few with no known function.

In addition to DD analysis, using wheat Affymetrix "GeneChip", we identified 93 differentially expressed ESTs of wheat in response to avirulent pathogen attack. We also investigated the differential expression profiles of wheat leaves during the virulent infections and determined 75 differentially regulated ESTs. 1Selected ESTs were further analyzed using QRT-PCR analysis and 15 were confirmed to be differentially regulated.

For the further characterization of the identified genes, such as determination of their putative roles in disease response, functional studies have to be performed. For this purpose, BSMV (Barley Stripe Mosaic Virus) mediated virus induced gene silencing (VIGS) method is optimized in this thesis for wheat. We have successfully managed to silence the endogenous *PDS* gene (Phytoene desaturase) of wheat which can be used as a positive control for the monitoring of silencing of the genes we have identified. Our results show that BSMV mediated VIGS can be used efficiently and effectively to silence wheat genes that we identified through differential display and microarray analysis and can be used to study the functions of those genes

Key words Avocet differential lines, differential display, microarray, *Puccinia striiformis* f.sp. tritici, yellow rust, wheat, VIGS, *PDS,* BSMV

ÖZ

BUĞDAY SARI PAS HASTALIĞINDA ROL ALAN GENLERIN SAPTANMASI

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Tahıllarda hastalıklara karşı ürün kaybını azaltabilecek en iyi stratejileri geliştirmek için, bitkilerdeki savunma sistemi mekanizmasının ortaya çıkarılması çok önemlidir. Bu konuda şu anki bilgimiz model organizmalar ve onların patojenleri ile olan etkileşimleri ile sınırlıdır. Bu çalışmada, buğdayın en tahrip edici patojenlerden biri olan sarı pasa karşı savunmada kullandığı genleri tanımlama amacına yoğunlaşılmştır. Farklılık gösterim ve Mikroarray yöntemleri kullanılarak, buğdayın iki farklı hattında (Avocet Yr10 ve Avocet Yr1) hastalığa sebep olan ve bitkide dirençlilik oluşturan patojen suşları kullanmı (Pst: PST17, PST45, 169E136 and 232E137) ile ortaya çıkan gen ifadesindeki değişmeler saptanması amaçlanmıştır. Farklılık gösterim tekniği ile 90 primer kombinasyonu sonucunda bulunan genlerden 14 tanesi gerçek zamanlı polimeraz zincir reaksiyonu ile de doğrulanmıştır. Bu genlerden biri hariç hepsi buğdayda daha önce tanımlanmamış yeni genlerdir. Bu genlerden iki tanesi tam olarak proteine dönüşebilecek şekilde üç üstü ve beş üstü

uçları da dahil olmak üzere tanımlanmıştır . Bunlardan bir tanesi cylophilin (olası antifungal) benzeri bir gen diğeri ise ubikütin conjugating E2 tipi enzim olarak tanımlanmıştır. Tanımlanan genlerin homoloji çalışmaları sonucunda ubikütine bağlı protein parçalanması, programlanmış hücre ölümü, hastalık dirençlilik gibi mekanizmalarda rol alan genlere karşılık geldikleri saptanmıştır.

Farklılık gösterim tekniğine ek olarak, mikroarray yöntemi kullanılarak bitkide hastalığa karşı direnç oluşumu sırasında ifade düzeyleri değişen 93 tane gen saptanmıştır. Bunun yanı sıra hastalığın bitkide oluşumu esnasında, yani dirençliliğin olmadığı durum da ifade düzeyleri değişen 75 tane gene saptanmıştır. Bunlarda 15 tanesi gerçek zamanlı polimeraz zincir reaksiyonu ile çalışılıp gen ifadelerindeki değişimler doğrulanmıştır.

Tanımlanan genlerin ileri düzeyde karakterize edilebilmeleri için, yani hastalık esnasında ne gibi roller üstlendiklerini anlamak için fonksiyonel testlere ihtiyaç vardır. Biz bu sebeple bu tezde BSMV'ye (Barley Stripe Mosaic Virus) bağlı virus ile gen susturma yöntemini buğday için optimize ettik. Bitkide bulunan *PDS* genini başarıyla susturduk. Bu gen susturalarak tanımlamış olduğumuz genlere pozitif kontrol geni olarak kullanılıp , susturma deneylerinin izlenmesi sağlanabilecektir. Sonuçlarımız, BSMV kullanılarak buğdayda mickroarray ve farklılık gösterim tekniği ile saptadığımız genlerinin etkin bir biçimde susturulabileceğini göstermektedir ve bu yöntemle bu genlerin fonksiyonları saptanabilecektir.

Anahtar kelimeler: Avocet farklı hatları, farklılık gösterimi, Mikroarray, sarı pas, buğday, Virus ile gen susturma, *PDS,* BSMV

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LIST OF ABBREVIATIONS

µg : Microgram

µL : Microliter

µM : Micromolar

[α^{32}P]-dATP : [α^{32}P]-deoxyadenosinetriphosphate

A : Absorbance

APS : Ammoniumpersulfate

BSA : Bovine Serum Albumin

cRNA : Complementary RNA

DNA : Deoxyribonucleic acid

dNTP : Deoxy-nucleotidetriphosphate

ds : Double stranded

DTT : Dithiothretiol solution

EDTA : Ethylenediaminetetraacetic acid

DD : Differential Display

hrs. : Hours

Dpi : Days post infection

HR : Hypersensitive response

SAR : Systemic acquired resistance

LRR : Leucine rich repeat

M : Molar

mg : Miligram

min. : Minute

mL : Mililiter

mM : Milimolar

NBS : Nucleotide binding site

NEB : New England Biolabs

QRT-PCR: Quantitavie realtime polymerase chain reaction

ng : Nanogram

nm : Nanometer

PCR : Polymerase Chain Reaction

pmol : Picomole

PR : Pathogenesis related

RNA : Ribonucleic acid

ROS : Reactive oxygen species

rpm : Rotation per minute

RT-PCR Reverse transcriptase polymerase chain reaction

TEME : N, N, N',N'-Tetramethyl ethylene diamine

U : Unit

UBC : Ubiquitin conjugating enzyme**,**

UV : Ultraviolet

v/v : Volume per volume

CHAPTER I

INTRODUCTION

1.1 Wheat

Wheat is one of the most important crops for human nutrition. In addition to its basic caloric value, wheat, with its high protein content, is an important source of plant protein in the human diet. Wheat has a critical role as a food crop which originates from its wide adaptability and flexibility in its end products. An enormous increase in food production is necessary to supply sufficient food for a growing world population. It has been estimated that the world will need to produce as much food during the next 50 years as was produced since the beginning of agriculture 10,000 years ago (James, 1997). It is thought that wheat will more likely become an even more important crop in the world with maize close behind (Hoisington, 1998).

When compared to world consumption of wheat, Turkey is the highest. Turkey is among the most important wheat producers worldwide. However, when our yield (2.08 t ha^{-1}) is compared to top-level producers like China, India, USA, Russia, and major European producers (7 t ha^{-1}) it remains to be insignificant. One of the important factors that decrease the yield is disease state. Several pathogens such as fungi, virus, bacteria, and nematodes attack wheat and cause yield losses. On average, these cause 20–37% yield loss and 70 billion USD throughout world representing the economical impact of this damage. Epidemics of yellow rust occasionally occur and decrease the yield significantly.

1.2 Wheat-fungi interactions

Dramatic increase in agricultural productivity after the mid 19th century was a result of discovery of genetics by Mendel, and pursued studies of other researchers. By the work of researchers, vast improvements in agriculture have been achieved. However, those improvements have also led to a decline in the genetic diversity of the crops because only chosen varieties which have certain traits were selected to be cultivated. For short term, this does not cause serious problems, but long-term viability of species requires genetic variation (Frankel, 1970).

Some of the most devastating and universal crop diseases are caused by fungal pathogens. However, one of the greatest danger is the rust epidemics that can be formed as a result of the airborne rust pathogens; such as *Puccinia graminis tritici*, *Puccinia recondita*, and *Puccinia striiformis*, that cause stem (black) rust, leaf (brown) rust, and stripe (yellow) rust disease of wheat. Among the fungal diseases of the wheat, rusts are the major pathogens. Epidemics of wheat rust can spread between continents because of the extensive spreading abilities of rust spores (Roelfs, 1989). Rusts in general, reduce plant vigor and limit seed maturing in adult plants and cause most damage before or during flowering.

Rusts are also mentioned in the earliest records of wheat cultivation. They played important roles in the course of early civilizations by destroying the major food source. Aristotle (384-322 B.C.) writes of rust being produced by "warm vapors" and mentions the devastation of rust and the years, when rust epidemics took place. Theophrastus reported that rust was more severe on cereals than legumes. Excavations in Israel have revealed urediniospores of stem rust that have been dated at about 1300 B.C.

1.3 Yellow rust

Puccinia striiformis Westend. f. sp. *tritici*, causal agent of yellow rust disease of wheat is a biotrophic fungus that needs living host tissue for its growth, development and reproduction. It is a major disease of the leaves of wheat in higher elevations and cooler climates and it is among the most important wheat diseases worldwide. The disease takes its name from the yellow colored spores of the fungus. It is also called stripe rust. It has been the most devastating disease in Turkey during the last 25 years. In an infected field, especially, when the environmental conditions are suitable, yield losses can reach up to 100% (Roelfs, 1989).

Puccinia striiformis does not have a sexual stage and does not require an alternate host to complete its life cycle. Minimum, optimum and maximum temperatures for infection are 0, 11, and 23 °C, respectively (Hogg *et al*. 1969). Urediniospores of yellow rust are produced in large numbers and can be wind-transported considerable distances.

Stripe rust is the only rust of wheat that consistently spreads beyond the initial infection period. Urediniospores of yellow rust are produced in large numbers and can be blown considerable distances by wind. In general, spores move from West to East due to the winds resulting from the rotation of the earth. Urediniospores of yellow rust are sensitive to ultraviolet light. Therefore, they are possibly not transported as far as those of leaf and stem rust in a viable state. However, Hogg reports that spores of yellow rust were wind-transported more than 800 km in a viable form (Hogg 1969). Recent introduction of yellow rust to Australia is considered to be aided by man through jet travel. However, the spread of disease from Australia to New Zealand (2000 km) is probably through airborne spores. One of the examples of migration of stripe rust is the 8156 virulence. The disease was first recorded in Turkey and later in India and Pakistan (Roelfes *et al*, 1989).

The disease requires high humidity and low temperatures, with the optimum reported at 9-13°C for infection; thus, stripe rust is a disease of more Northern or Southern latitudes and high elevations

1.4 Disease control

One way to control disease is usage of chemicals (fungicide). However, this application has some drawbacks such as cost of the chemicals, limited shelf life, negative effects on the environment, and acquired resistance to applied chemical in the pathogen. Therefore, growing genetically resistant cultivars has been the main mechanism to control the disease. Advantages of growing resistant cultivars include reduced price (cost spread to all users of the cultivar), requirement of no action by the farmer after cultivar's selection, and possessing no known environmental impact. Therefore, developing and planting resistant cultivars are the most economical method of controlling the disease. Genes present in the wild relatives of the crops constitute an important source of single-locus variation and in the past years many studies have been performed successfully to transfer resistance genes to cultivated plants from wild relatives using classical breeding practices. However, most of those genes are broken by the pathogens. Therefore, finding those genes that provide durable resistance has been important. In order to achieve generation of durable resistant plants, mechanisms underlying plant pathogen defense must be revealed.

To date, more than 100 resistance genes (*R*) against many rust diseases in wheat and its relatives have been identified. However, only three of them have been characterized at molecular level, in other word "cloned". These are *Lr21* (Huang *et al*., 2003) and *Lr10* (Feuillet *et al*., 2003) genes that provide resistance to leaf rust pathogen and ad Pm3 (Yahiaoui *et al*., 2004) which is responsible for the resistance against powdery mildew disease. In addition to those genes, Yr10 gene is the only yellow rust disease resistance gene in wheat that has been submitted in NCBI. Sequence is available in the GenBank (Laroche *et al*., 2002).

1.5 Plant disease resistance mechanisms

It is in plants' nature to resist pathogens. Generally, it is an exception if a pathogen can infect a plant. Plants do have innate immunity against pathogens in which the attacked plant cells respond to pathogen individually. Plants are continually exposed to pathogen attack. The organisms that attack plants include viruses, mycoplasma, bacteria, fungi, nematodes, protozoa, and parasites. Plants have evolved a defense mechanism that is distinct from the vertebrate immune system. Since they do not have a circulatory system and antibodies, and their cells are not mobile, they have developed a system in which every individual cell can recognize and respond to microbial attack autonomously. This is called as innate immunity.

The majority of potential pathogens are stopped even before they can initiate their lifecycles on plants which is referred to basal defense. This protection includes preformed barriers, such as waxy cuticles and antimicrobial compounds. Even if the pathogen is capable overcoming basal defense, plants have at least two sets of genetically defined overlapping defense responses: The hypersensitive response (HR) and systemic acquired resistance (SAR). Each plant cell is capable of defending itself *via* combination of basal and induced defenses. Interactions of the pathogen and wheat lines that we used in our experiments are summarized in Table 1.1.

1.5.1 Hypersensitive response (HR)

Hypersensitive response (HR) is one of the most powerful weapons in plants' arsenal against pathogens. It is characterized by rapid, localized cell death at the site of pathogen attack (Hammond-Kossack, 1996). The molecular basis of HR-mediated disease resistance was first clarified by the studies of Flor in 1942. His studies with the flax-flax rust pathosystem led to the development of the gene-for-gene model, which states that, for every dominant resistance (*R*) gene in the plant, there is a matching dominant avirulence (*avr)* gene in the pathogen (Flor, 1942).

HR is initiated by plant's recognition of specific signal molecules produced by the pathogen. These elicitors are produced directly or indirectly by *avr* genes and *R* genes are thought to encode receptors for those elicitors. Once the signal for the presence of pathogen is recognized by the (*R*) gene, a chain reaction of signaling events starts and as a result, a cascade of cellular events such as; rapid ion fluxes, oxygen burst, production of reactive oxygen species (ROS), accumulation of antimicrobial compounds and cell-wall reinforcements in cells surrounding the area of cell death are initiated. These responses are named as hypersensitive response (HR) also referred as rapid programmed cell death (PCD), since they are accompanied by the localized cell death at the site of invasion (Hammond-Kosack 1996; Shirasu and Schulze-Lefert 2000). One reasonable question is "Why would the plant prefer its own cells to suicide?" PCD (programmed cell death) is thought to be responsible for the limitation of pathogen growth by blocking its access to further nutrient sources and limiting its proliferation. Those cells going in apoptosis also believed to produce signals to neighboring cells that can play important roles in the induction of a variety of defense related genes. HR has been described for all the pathogens of the plants; therefore it is a common mechanism that present in plant's arsenal against invading pathogens.

PCD activity allows plant to prevent spreading of the infection. If the plant does not have the *R* gene product to recognize the pathogen, race specific resistance does not take place, in such an event, even the basal resistance mechanism is found to be suppressed (Shirasu and Schulze-Lefert, 2000). A pathogen carrying the *avr* gene possesses an avirulent phenotype on the host plant that carries the corresponding *R gene.*

Table1.1 Examples to possible wheat-yellow rust interactions.

Pathogen	**169E136 or Pst17**	**169E136 or Pst17**	**232E136 or Pst45**	**232E136 or Pst45**
Plant	Avocet-Yr10	Avocet-Yr1	Avocet-Yr10	Avocet-Yr1
Interaction	Avirulent	Virulent	Avirulent	Avirulent
	Incompatible	Compatible	Incompatible	Incompatible
Outcome	Resistance/HR	Disease	Resistance	Resistance

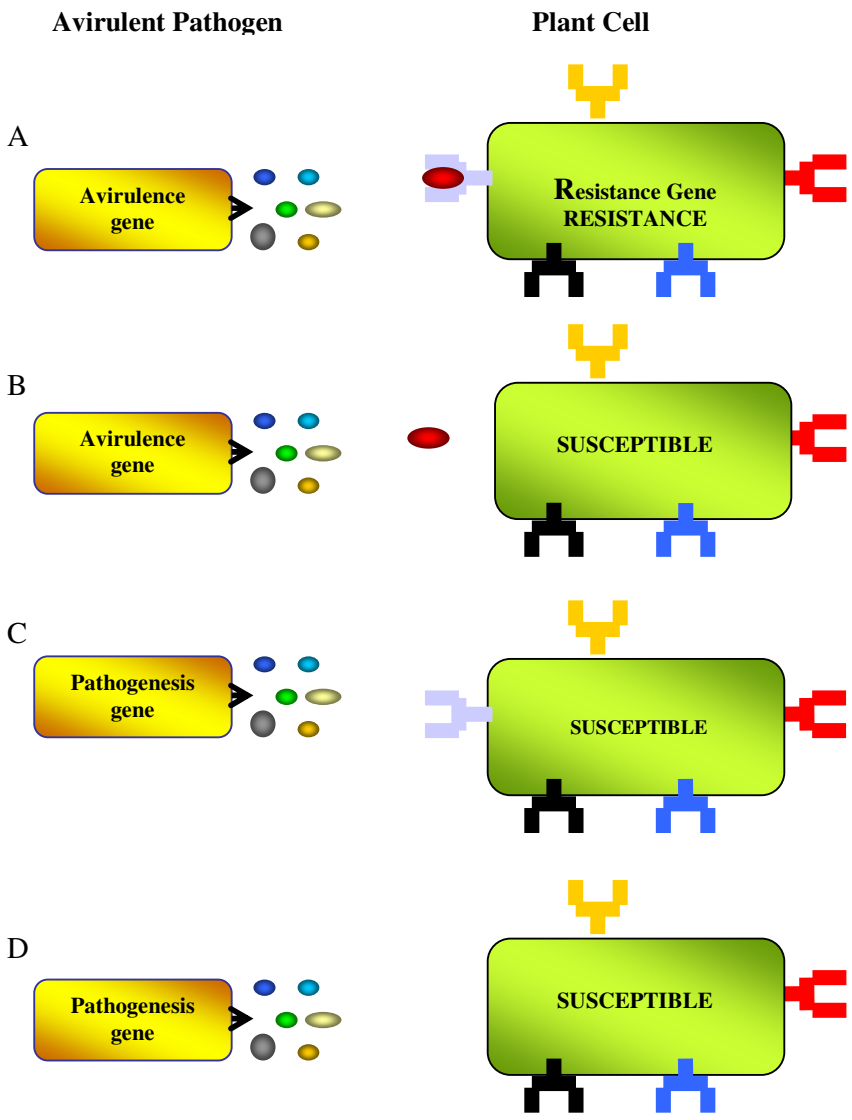

Figure1.1 A schematic diagram explaining the gene-for-gene interactions between the host and the pathogen. **A:** The pathogen sends a variety of pathogenesis proteins in order to suppress host defense mechanisms and propagate efficiently. However, the host plant possesses an *R* gene which can detect one of those pathogenesis gene products (Avr). This is called as incompatible interaction. **B:** Pathogen carries the same *avr* gene plus other pathogenesis genes but the host lacks the *R* gene that can recognize any of those virulence products. The outcome of this kind of interaction (compatible interaction) is disease. **C:** The pathogen produces the pathogenesis

signals but lacks the *avr* gene matching the R product. The interaction (compatible interaction) results in disease. **D**: both the pathogen and the host lack the matching *avr* and *R* genes respectively which lead to disease (compatible interaction).

1.5.2. Systemic Acquired Resistance (SAR)

Systemic acquired resistance (SAR) is a "whole-plant heightened, subsequent resistance response" that is triggered by HR and other necrotic reaction responses that occur following an earlier localized exposure to a pathogen. SAR is analogous to the animal innate immune system. SAR is important for plants to resist disease because once formed it acts non-specifically against a broad spectrum of pathogens (Dempsey, 1999). SAR reduces the severity of disease caused by all classes of pathogens, including normally virulent pathogens.

A large amount of evidence indicates a role of salicylic acid (SA) in SAR. In one of the experiments, it was shown that transgenic plants, carrying the bacterial *nah*G gene that encodes the salicylate hydroxylase which inactivates SA, are more susceptible to both pathogens that normally induce a resistance response and to pathogens that normally cause disease (Dempsey, 1999). For occurrence of SAR, movement of an as-yet uncharacterized molecule from infected tissues to uninfected tissues is required which is thought to be induced by HR. Nitric oxide which is a secondary signaling molecule in mammalian cells is suggested to fulfill this task. It is shown that nitric oxide is involved in triggering of PR and reactive oxygen species-dependent cell death (Cohn *et al*., 2001).

1.5.3 Avirulence genes (*avr* genes)

Avirulence (*avr*) genes were identified by Flor in the 1950s. The first *avr* gene has been cloned from bacteria in 1984 (Staskawicz, 1984) and from fungi in 1991, and viral pathogens, and since then, numerous bacterial and fungal *avr* genes have been identified and cloned. The *avr* genes disable a pathogen to induce disease on a specific variety of the host plant, by which, *avr* genes specify the host range of the pathogen at the species and at the variety level.

It might be confusing to imagine what kind of selective advantage a pathogen would have when it carries the various *avr* genes which are detected by the host surveillance system. Recent studies show that *avr* genes are actually virulence genes that have evolved to suppress host defense mechanisms. An example is *Pseudomonas syringae* effector AvrPtoB, which was was found to have a role of suppressing basal defence in *Arabidopsis* (Torres, 2006). Mutational analysis showed that *avr* genes provide a selective advantage to the pathogen in the absence of the corresponding *R* gene (Bonas *et al*. 2002). Therefore, it is suggested that *avr* gene products were previously virulence factors that were later targeted by plant *R* gene products in the course of plant pathogen co-evolution.

To date, a lot of *avr* genes have been cloned from a wide range of plant pathogens including bacteria, virus, and fungi. More than 40 *avr* genes have been cloned from bacteria and most of them were from *Xanthamonas* and *Pseudomonas* species. Of those cloned *avr* genes, little or no homology was observed between each other with the exception of the *avrBs3* and avrRxv/yopJ families (Van der Biezen, 1998).

Plant viral *avr* genes encode essential virus components, such as the coat protein, replicase and the movement protein. In most of the cases it is observed that amino acid substitutions that do not alter the function of the viral Avr proteins abolish recognition specificity of the Avr protein.

A few number of avirulence genes have been cloned from fungal pathogens and the products of these genes are also diverse. The majority of them were cloned from fungi that colonize intracellular spaces in plant tissues (Lauge, 1998).

1.5.4 Disease Resistance Genes (*R* genes)

R genes encode R proteins that recognize pathogen effectors and/or avirulence proteins in plants. *R* genes comprise a large and diverse group of related sequences in plant genome. *R* genes encode receptors to recognize the specific signals directly or indirectly produced by pathogen a*vr* genes.

During 1990s, many *R* genes have been isolated. Isolation of *R* genes revealed that, in contrast to *avr* gene products the majority of the cloned *R* genes encode structurally related proteins possessing common extracellular or intracellular leucine-rich-repeat domains (LRR). Most of the *R* genes seem to be members of an ancient gene family that encode nucleotide binding proteins (Young, 2000). In Arabidopsis, the number of NBS-LRR sequences range from 200-300. In rice, estimates are even higher as much as 1500. Because of their distinctive structure, these proteins are known as nucleotide binding site leucine-rich-repeat (NBS-LRR) proteins. Majority of the cloned *R* genes are in this group. The NBS-LRR proteins are cytoplasmic, and can be further divided into those that carry amino terminal homology to the toll and Interleukin-1 receptor genes (TIR: NBS: LRR family) or a coiled-coil (also called as LZ: leusine zipper) domain (CC: NBS: LRR family) (Jones, 2001).

In addition to NBS-LRRs, there are three other classes of *R* genes:

Members of the 2nd class contain a serine/threonine kinase catalytic region. The only examples of this class are the *Pto* from tomato (Martin *et al*., 1993) and *Rpg1* from barley (Brueggeman *et al*., 2002). However the function of the *Pto*

mediated disease resistance depends on the Prf presence of an NBS-LRR type resistance protein, Prf (*Pseudomonas* resistance and fenthion sensitivity).

Members of the 3rd class lack the NBS (nucleotide binding site) region but have a transmembrane domain and an extracellular LRR region. Cf proteins from tomato are examples of this class (Dixon, 1996).

Members of the 4th class have an extracellular LRR region, a transmembrane domain and a cytoplasmic serine/threonine kinase region. An example of this class is the *Xa21* gene of rice (Song, 1995). Examples to different classes of *R* are shown in Figure 1.2.

NBS domain that is found in most of the cloned *R* genes is probably involved in signal transduction. This domain is composed of several amino acid motifs that are highly conserved among family members. Recently cloned Rx protein of potato is a cytoplasmic NBS-LRR protein. The presence of NBS region suggests that these proteins may play a role in the activation of a kinase (Cohn *et al*. 2001).

In the carboxy terminal to the NBS region, R proteins usually contain an LRR domain that is thought to be involved in protein-protein interaction and pathogen recognition. It is considered that LRR region present in many *R* genes is a major factor responsible for the specificity of the pathogen recognition. The first report providing evidence for this hypothesis is from the studies with 11 alleles of flask rust resistance which provide resistance to different strains of the rust pathogen. Amino-acid comparisons of L6 and L11 proteins have revealed that the two proteins only differ at 33 positions in the LRR region and are identical in the remainder of the protein. Constructed chimeric proteins consisting of L2 LRR region and the N-terminal regions of the L6 and L10 alleles were shown to express L2 specificity not L6 or L10 specificities. Additionally, the TIR domain can also affect specificity. For example L6 and L7 alleles are identical in the entire amino-acid sequence and differ only in the TIR region for only 11 amino acid residues, which leads to different recognition specificities (Ellis 2000).

Recently, new reports indicate that LRRs are not only involved in the perception of the specific signal produced by the Avr proteins. Studies with the Arabidopsis NBS-LRR *R* gene *RPS5* revealed a mutation in the LRR that inhibit the function of multiple *R* genes indicating that LRRs are not only involved in recognition but also in downstream signaling (Bonas *et al*. 2002). One explanation to this kind of inhibition in function might be that, another molecule, which is involved in the downstream signaling pathway and can intereact with wild type the LRR region can no longer bind to that region. I believe this could be SGT1 protein, which is known to be interacting LRR regions of the R proteins and have roles in downstream signaling.

As a conclusion, R proteins must have two functions: first to recognize a pathogen-derived signal; and second, to initiate a coordinated plant defense response.

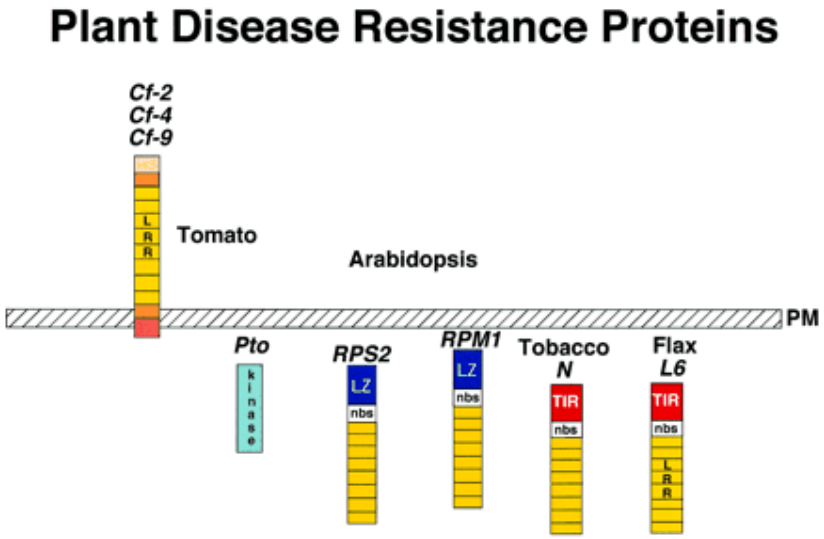

Figure 1.2 Examples to the different classes of *R* genes (Staskawicz, 2001)

1.5.5 Molecular mechanisms in R- Avr protein interactions

Since Flor identified the gene for gene mechanism in plant diseases resistance it was believed that plant R proteins and pathogen Avr proteins interact directly. A simple mechanism is explained with receptor ligand model in which avirulence gene products generate signals, and resistance genes encode cognate receptors. The receptor participates in an initial step in a signal transduction cascade leading to the activation of a variety of defense responses.

After isolation of Avr and R pairs from pathogens and plants, researchers tried to show direct interaction of R proteins and their Avr pairs. However most of those attempts have failed to find a direct interaction between Avr proteins and their cognate R proteins.

Despite intensive studies, a direct physical interaction between Avr and R proteins has only been shown for the AvrPtoB-Pto or AvrPto-Pto (Kim, 2002; Scofield, 1996 and Tang, 1996) and AvrPita-Pi-ta pairs (Jia *et al*., 2000). However, experimental evidence of direct physical interactions between R proteins and Avr proteins was limited. In the AvrPto-Pto system for resistance to take place another protein Prf (NBS-LRR) is required which interacts with Pto. In this system, Prf is considered to be the real R protein guarding Pto. Based on these observations and the lack of direct interaction led to rise of 'the guard hypothesis' (Reiner *et al*, 2002) which states that there can be a third protein (or more) that is responsible for the recognition of Avr protein by *R* gene product. However, a recent study revealed that flax R (L5, L6, L7) genes directly interact with the flax fungus Avr proteins (avrL567) (Dodds, 2006).

One evidence for guard model was that dual recognition specificities of some R proteins. For example Arabidopsis *RPM1* resistance gene: it recognizes two distinct pathogen avirulence genes; AvrRpm1 or AvrB. These results were explained by guard model: the different Avr proteins target the same host molecule and R protein recognizes this target protein (Ellis *et al*. 2000). Indeed 2 years later, it has been found that RPM1 interacts with RIN4 (RPM1-interacting protein) protein which also interacts with AvrRpm1 and AvrB. RIN4 protein level reduction also

inhibits HR and disease resistance mediated by RPM1 disease resistance gene (Dang *et.al.,* 2002).

All these studies indicate that the initially postulated models for gene-for gene interactions were too simple. Much has to be done to reveal the mode of interaction of the host and pathogen gene products and to refine the current models. Today, it is believed that both mechanisms are present in plants. Schematic models for both types of interaction mechanisms are presented in Figure 1.3

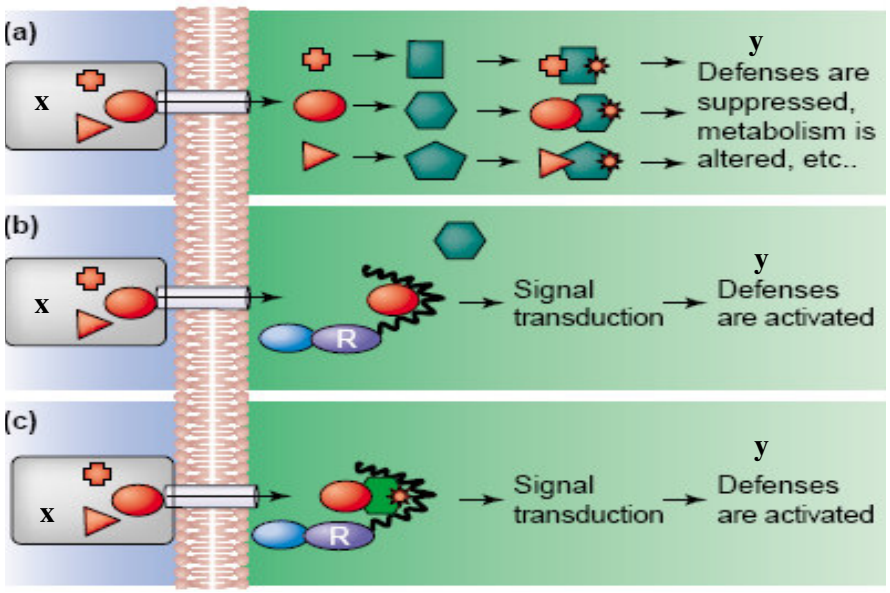

Figure.1.3 Interactions between pathogen Avr proteins and plant R proteins. The pathogen (x) has contacted to a plant cell and is expressing a variety of virulence proteins (y), which are transferred into host. When they get inside they target and supress host defense mechanisms. (a) The plant cell does not express an R protein which can recognize any virulence proteins sent by the pathogen. Thus, disease takes place (b) Depiction of simple receptor–elicitor hypothesis, in which an R protein directly binds and detects the avirulence protein. This recognition starts a cascade of

events resulting in triggering of HR and other defense responses (c) Schematic interpretation of the guard hypothesis, in which an R protein (guard) detects a modified host protein (guardee, star), whether or not in complex with the avirulance protein (McDowell, 2003).

1.5.6 The role of ubiquitinylation in plant disease resistance

One of the systems that plants use to control the level and the activity of proteins involve the use of ubiquitin/26S proteasome ubiqutinylation, a selective protein turnover system. It appears to be particularly important in the aspects of plant growth, development and defense in plants. (Callis and Vierstra 2000; Hellmann and Estelle 2002). Ubiquitin (Ub), a 76 amino acid conserved protein, is used as a tag for selective target protein degradation. (Hershko and Ciechanover 1998). E1 is a ubiquitin activating enzyme, activating Ub in an ATP dependent manner. The ubiquitin moiety from E1 is transferred to E2 (ubiquitin conjugating enzyme) in the ubiquitinylation process. E3 is either a single large gene product or a complex of different proteins including the Skp1-Cullin-F-box-Rbx1 protein (SCF) components (Sullivan *et al*. 2003). Schema for different kinds of E3 ligases are summarized in Figure1.4. Depiction of role of ubiquitinylation in plant disease resistance is presented in Figure 1.5. E3 ubiquitin ligase provides specificity for ubiquitinylation cascade complex which recognizes the ubiquitinylation signal and binds to the target protein. This SCF complex mediates the degradation of multiple proteins involved in diverse signaling pathways through an ubiquitin-proteasome protein degradation pathway. There are more than 1200 genes encoding for E3 complex and 42 genes for the E2 in Arabidopsis indicating the specificity and selectivity of targeting in Ub/26S proteasome system (Vierstra 2003).

There are reports presenting that the ubiquitinylation system also plays an important role in disease resistance mechanisms of plants;

Two major components in plant disease resistance are RAR1 and SGT1 proteins. In plants, the decreased levels of RAR1 and/or SGT1 lead to decrease in resistance against pathogens (Austin *et al*. 2002; Azevedo *et al*. 2002). The discovery of the requirement for the *Rar1* gene in resistance to powdery mildew by several unlinked CC-NBS-LRR genes was an important step in understanding the disease resistance mechansisms (Schulze-Lefert and Vogel, 2000). Studies in barley indicated that, *RAR1* functions downstream of the pathogen recognition by the R protein and upstream of H_2O_2 accumulation and host cell death (Shirasu *et al*., 1999)

The zinc finger motif called CHORD (Cys- and His-rich domain) domain in RAR1 protein shares homology with yeast SGT1 which participates in SCF type multiprotein ubiquitin ligase (E3) complex (the SCF [Skp/Cullin/F-box] complex) (Kitagawa *et al*., 1999; Matsuzawa and Reed, 2001). It is found that only a subset of R proteins are dependent on RAR1, whereas SGT1 is essential for disease resistance and HR triggered by a wide range of R proteins including non-leucine-rich repeats (LRR) type R proteins (Peart *et al*., 2002).

SGT1 is a novel subunit of SCF type E3 ligase complex and regulates the activity of SCF-type E3 ligases through its interaction with SKP1 (Shirasu *et al*., 1999). Arabidopsis *SGT1* was identified in mutational analysis for loss of *RPP7*- and *RPP5*-mediated resistance (Austin *et al.*, 2002; Tor *et al.*, 2002), and in a yeast two-hybrid screen it was shown to interact with the RAR1 protein (Azevedo *et al*., 2002). Previously, RAR1 and SGT1 were reported to be interacting with 26S proteasome subunits. RAR1 was found to be interacting with COP9 signalosome which acts as a lid for proteasome complex, whereas SGT1 was found to be interacting with SKP1 which is involved in E3 complex.

RAR1-SGT1 physical interaction and their interactions with ubiquitination-related proteins suggests that, the two proteins are signaling components that may be

involved in protein degradation processes (Austin *et al.*,2002; Azevedo *et al.*, 2002; Muskett *et al.*, 2002; Shirasu *et al.*, 1999; Tor *et al.*, 2002, Tornero *et al.*, 2002) (Figure 1.5). Evidence supporting this idea came from the recent studies of Bieri *et.al* and Holt *et.al* which reports that RAR1 and SGT1 protein levels play role to keep certain *R* gene levels at steady state. (Bieri *et al.*, 2004; Holt *et. al.*, 2005)

There are a number of recent studies that proves the important role of ubiquitinylation plant disease resistance. Arabidopsis (snc1) mutant constitutively activates defence responses without the need of interaction of host with pathogens. One mutant that suppresses snc1-mediated constitutive resistance is modifier of snc1 (mos5) is a ubiquitin-activating enzyme gene in Arabidopsis (Goritschnig, 2007). The E3 ubiquitin ligase activity of Arabidopsis PLANT U-BOX17 and its functional tobacco homolog ACRE276 were reported to be required for PCD and defense responses. (Yang , 2006). The U-box protein CMPG1 (ubiquitin ligase) was reported to be needed for efficient activation of defense mechanisms mediated by multiple resistance genes in tobacco and tomato. (Gonzalez-Lamothe, 2006). A duplicated pair of Arabidopsis RING-finger E3 ligases were found to be involved in the RPM1- and RPS2-mediated defense responses (Kawasaki, 2005).

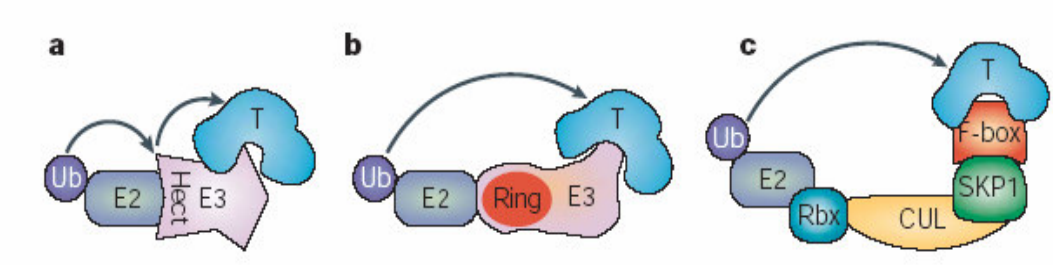

Figure 1.4 Different types of ubiquitin ligases. a. E3 type ubiquitin ligase b. ring finger type ubiquitin ligase c. SCF type ubiquitin ligase

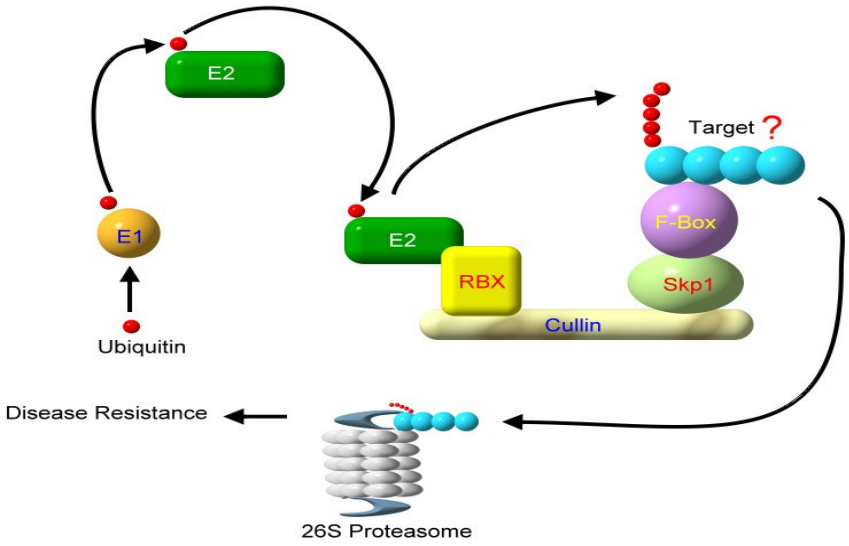

Figure 1.5 Depiction of role of ubiquitin mediated protein degradation in plant disease resistance.

1.6. Expression analysis in plants

The completion of sequencing of human genome and the flowering plant *Arabidopsis thaliana* has opened a new era on genetic research. To identify and analyze those genes involved in various biological processes have become important. Despite enormous efforts, of the 26,000 genes encoded in the Arabidopsis genome, less than 10% has been studied until now and the challenge for he following decade will focus on the remaining 23,000 genes. Global analysis of gene expression which is referred to as genome-wide expression profiling, is considered to be one of the most promising tools in functional genomics. In the past few years, several technologies for expression profiling have been developed. These methods depend on three different principles: hybridization of probes to microarrays, counting of sequence tags from cDNA fragments and gel-based analysis of cDNA fragments, (Zabeau 2001).

1.6.1. DNA Microarray

DNA microarray has attracted tremendous interests among biologists. This method is hybridization based and allows the researcher to monitor the whole genome on a single chip. Thus, the researchers can have a better picture of the interactions among thousands of genes simultaneously. The two microarray based methods are cDNA and oligonucleotide microarrays. An array is an orderly arrangement of samples. It provides a medium for matching probe and target DNA samples based on base-pairing rules. In the cDNA microarray method, probe cDNA (500~5,000 bases long) is immobilized to a solid surface such as glass and exposed to a set of targets either separately or in a mixture. In the oligonucleotide microarray, an array of oligonucleotide (20~80-mer oligos) or peptide nucleic acid (PNA) probes is synthesized on-chip or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA, hybridized, and the identity/abundance of complementary sequences is determined. The two major applications of the DNA microarray technology are: identification of sequence (gene/gene mutation), and determination of expression level (abundance) of genes.

The major strength of the technology is its potential to allow the analysis of tens of thousands of genes simultaneously. Another advantage of microarrays is that large data sets from different experiments can be combined together in single base. Despite its strengths, the method has limitations. The principal limitation is the requirement of sequence knowledge or an available cDNA clone. Another limitation is the difficulty in distinguishing among different transcripts which share a high degree of homology. Especially in plants substantial fraction of the genes are members of gene families. Sensitivity of the hybridization and the requirement of large amounts of RNA are also problems for the technology. Finally the costs of the chips are high for our purposes and it can only be used once.

1.6.2 Serial analysis of gene expression (SAGE)

This technique is principally based on counting sequence tags of 14-15 bases from cDNA libraries. By counting these tags, an accurate number of transcripts present in the mRNA pool can be determined. It has been widely used in human systems but in plants, reports are rare. The major advantage of the SAGE is that it can determine the absolute measure of gene expression instead of predicting the relative measure of gene expression levels. Another advantage is that independent data sets can be studied in a single database allowing comparison of different data sets. The main disadvantage of the technique is the requirement of large number of ESTs (expressed sequence tags) in order to monitor rarely expressed genes. Another limitation of the method is that the obtained tags are very short and therefore unambiguous.

1.6.3 Differential Display

Higher organisms contain thousands of genes encoded in their genome of which only a small fraction, perhaps 10-15% (Arthur Pardee and Peng Liang, 1992), are expressed in any individual cell. The choice of which genes are expressed determines all life processes. The course of normal cellular development as well as pathological changes that arise in diseases such as cancer are all directed by changes in gene expression. An important point is to identify and characterize those genes that are differentially expressed in order to understand the molecular nature of disease state and subsequently, to devise rational therapies.

DD is a powerful method to detect genes that are differentially expressed between two cell types or any other conditions. The general strategy of the method is to synthesize cDNAs from mRNA subsets by reverse transcription and to amplify those fragments by PCR. Then these short fragments are displayed on DNA sequencing gel.

The principal advantage of DD is that it allows genome-wide analysis in any species without prior sequence knowledge and that both known and unknown genes can be studied. When compared to SAGE it is more versatile and the obtained tags are 10-30 times longer which eliminates the need for large EST databases.

Differential display method was developed first by Drs. Arthur Pardee and Peng Liang in 1992 to allow rapid, accurate and sensitive detection of altered gene expression (Science 1992). In our study we employed a modified form of differential display method which was developed at Clontech.

As an initial step, cDNA is constructed by using oligo dT with reverse transcription taking the advantage of poly-A tail present on most eukaryotic mRNAs. In the next step PCR is performed using two primer combinations: a 5´ arbitrary P primer and a 3´ T primer which contains two additional 3´ bases to allow the $T_9N_{-1}N$ primer to bind the 5´ end of the poly A tail (Figure 1.7).

Initial 3 steps of PCR are performed at low stringent conditions to allow the arbitrary primers to bind the template. Subsequent PCR reactions are carried out at high stringent conditions. Products are labeled radioactively during the PCR when α-^{32}P dATP is used. When [γ-^{32}P] ATP is used as one of the primers is labeled before PCR. The PCR products are displayed on a sequencing gel. A schematic diagram of differential display and expected results are illustrated in Figure 1.3 and 1.4, respectively.

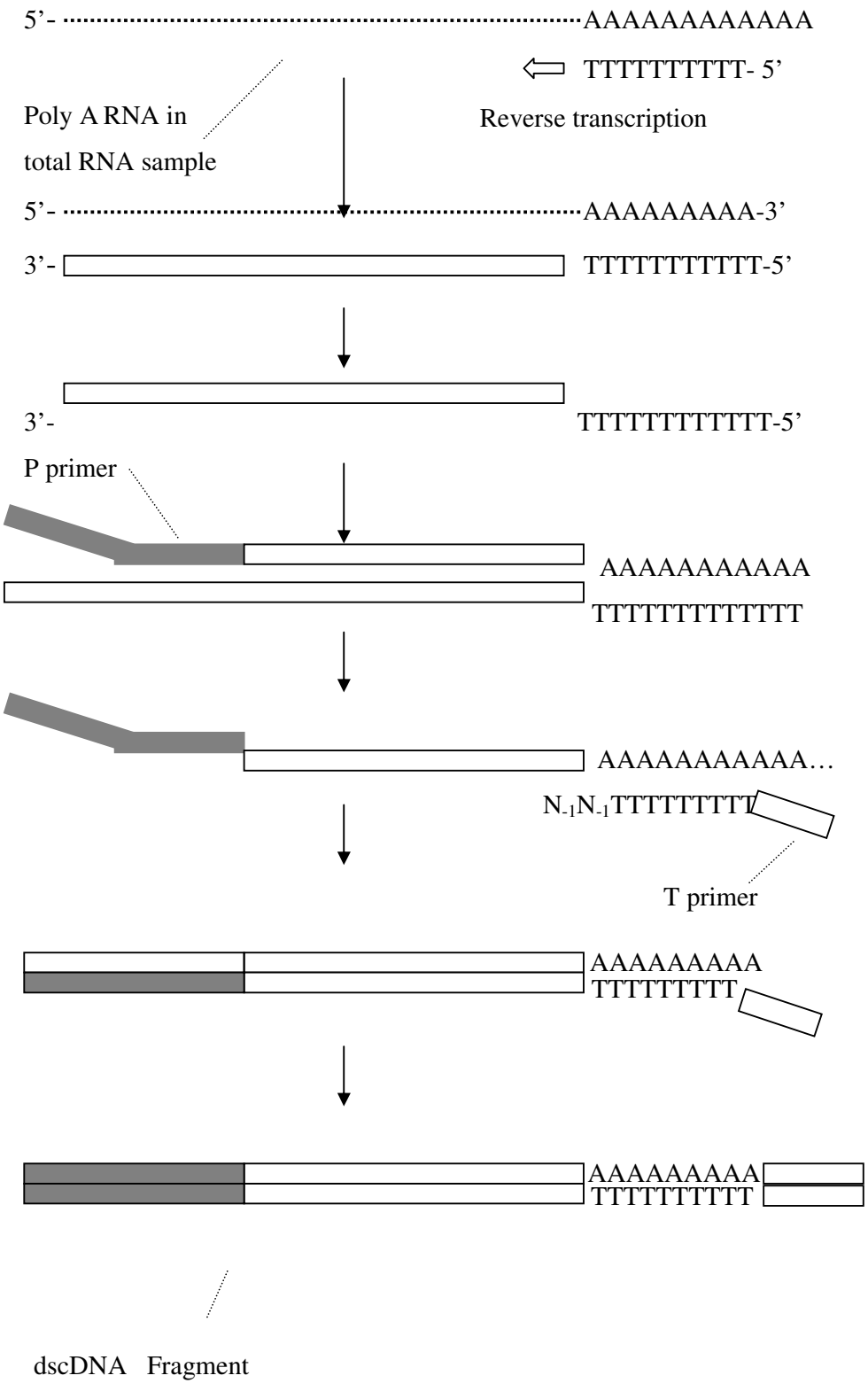
5'- AAAAAAAAAAAA
TTTTTTTTTT- 5'
Poly A RNA in
total RNA sample
Reverse transcription
5'- AAAAAAAAA-3'
3'- TTTTTTTTTT-5'
3'- TTTTTTTTTTT-5'
P primer
AAAAAAAAAAA
TTTTTTTTTTTT
AAAAAAAAAAA…
N-1N-1TTTTTTTT
T primer
AAAAAAAAA
TTTTTTTTT
AAAAAAAAA
TTTTTTTTT
dscDNA Fragment

Figure 1.6 Schematic diagram of the Differential Display

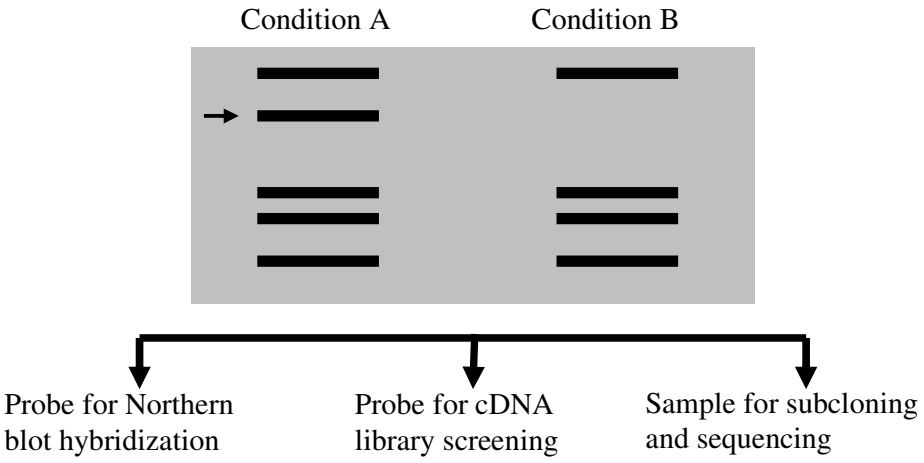

Figure 1.7 Schematic diagram of expected results in a DD analysis. The arrow indicates the differentially expressed fragment.

1.7. Virus induced gene silencing (VIGS)

Virus induced gene silencing is a newly developed sequence specific gene suppression method. Short targetted gene sequences can be silenced *via* cloning of the part of the gene into viral delivery vectors. There are number of plant species available for gene silencing using VIGS method with the development of various viral vectors. In the literature these viral vectors were constructed for efficient silencing. Some examples are TMV/N. Benthamiana (Kumagai, 1995), PVX/N. Benthamiana (Ruiz, 1998), TRV/N. Benthamiana (Ratcliff *et al*. (2001), CbLCV/Arabidopsis (Turnage *et al*., 2002). Barley stripe mosaic virus has been developed for barley silencing.(Holmberg *et al*., 2002) Scofield and his collogues used this BSMV-VIGS system for efficient silencing of hexaploid wheat species. (Scofield *et al.,* 2005). Post Transcriptional Gene Silencing is a name used for plant RNAi. Gene silencing could be succeed by different methods and VIGS is an efficient, rapid and cheap technique to suppress the function of targeted gene. The

reverse genetic is another definition used for gene silencing in which the targetted gene function is determined. This method doesn't need any transgenic plant production. Either *Agrobacterium* mediated or *in vitro* transcription generated viral vector deivery works and provide sequence specific gene silencing or specific mRNA blockage. The only limitation of VIGS is the requirement of sequence information of the gene to be targeted.

BSMV has a tripartite genome consisting of RNAs: α, β, γ. BSMV mediated gene silencing needs *in vitro* transcriptions of three different viral vectors α, β, and γ. To silence the specific gene of interest, the γ vector is modified by insertion of a short sequence of target gene. For example, to silence the *PDS* gene (Phytoene Desaturase) either sense or antisense fragments of it is cloned into γ vector. Finally, α:: βΔβa:: γ.bPDS4 *in vitro* prepared transcripts are inoculated into barley or wheat plants.

1.8 Aim of the study

Aim of our study is to determine the genes involved in yellow rust disease resistance of wheat and understand the defense response mechanisms. Currently, plant disease resistance mechanisms are still unrevealed and not very clear. Questions like "How does R proteins recognize their Avr targets?", "How do they generate signals?" "What are the other proteins involved in downstream signaling?", "How is it possible to maintain durable resistance?" still remain to be answered.

Map-based cloning of genes has not been very successful in wheat because of the large ($1.6x10^{10}$ bp) and repetitive nature of the wheat genome (Smith and Flavell, 1975). Therefore, we aimed to identify the genes that play role in disease resistance using expression based methods such as differential display and microarray. Our aim using differential display was to determine novel genes which were not previously identified in disease resistance mechanisms in wheat and other cereals. For this purpose, we have used two different wheat nearly isogenic lines (carrying different

YR disease resistance genes) and infected both of them with avirulent yellow rust strains that cause resistance symptoms on those lines.

In addition to the DD analysis, after the release of the first Affymetrix gene chip which has 60.000 ESTs and genes, we decided to use the microarray technology as well, in order to have a larger view on wheat –yellow rust interaction. In order to identify the mechanisms involved against pathogen attack and determine the changes in the gene expression during the pathogen invasion on the susceptible plant, we have designed a microarray experiment using the currently released Affymetrix wheat chip. We have used the wheat line "Avocet Yr1" which carries the *Yr1* disease resistance gene, for the experiments. We have done 3 inoculations, 1 causing disease on Avocet-Yr1 plant, second resulting in a hypersensitive response and activation of the defense responses of the Avocet-Yr1 plant. The third inoculation was mock inoculation. We have used 2 biological replicates and 1 chip replicate for each inoculations corresponding to 9 wheat microarray chips in total.

After cloning of some genes to identify their sequences and confirming their differential expressions, we considered to choose a method to study the functions of some of the genes. Thus, we have decided to investigate if their loss of function leads to break down of disease resistance. For this purpose, we applied the VIGS method. One advantage, we had, was having the knowledge of most of our target's 3 prime ends. This allows the silencing to be more specific. Our aim at first was to verify if VIGS using BSMV (Barley Stripe Mosaic Virus) vectors which were shown to be silencing barley and rice genes, can also silence wheat genes. We selected to silence the *PDS* gene (phytoene desaturase) which is involved in the protection of caretenoids from degradation by exposure to light hence formation of green color in plants. We used it as a control gene for the VIGS experiments.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant and pathogen materials

The wheat yellow rust resistant differential lines, Avocet-Yr10 and Avocet-Yr1, were developed in the Plant Breeding Institute at the University of Sydney by Dr. Colin R. Wellings in the Avocet susceptible background. The seeds were provided by Dr. Amor Yahyahoui of ICARDA, Syria. The strains PST17 / PST45 set and 169E136 / 232E137 set were provided by Dr. Xianming Chen and Dr. Lesley A. Boyd, respectively. All are avirulent on Avocet-Yr10 line and the strains PST45 and 232E137 are avirulent only to Avocet-Yr1 line.

2.2 Plant growth and infection conditions

The PST17 and PST45 infected Avocet-Yr10 and Avocet-Yr1 15 day old seedlings were prepared in the presence of RNA*later* (Ambion, Inc.), together with mock infected control samples, collected at 0, 3, 12, 24, and 96 hours after infection (hai), and mailed by Dr. Xianming Chen. The samples used for QRT-PCR analysis were prepared as in the followings in our laboratory. Two weeks old Avocet-Yr10 seedlings were infected with 169E136. Similarly grown Avocet-Yr1 seedlings were infected with 169E136 (virulent) and 232E137 (avirulent) using freshly generated spores. Both plants were mock infected without yellow rust spores. Since, currently Yr10 compatible yellow rust strain is not identified in the region, as a control, mixed races of brown rust spores, *Puccinia triticina*, were used for infection on Avocet-Yr10 seedlings. Infected seedlings were incubated at 10 ^{o}C at extreme high humidity for 24 hrs in the dark. Following the incubation period, the normal growth conditions were set to 17 ^{o}C 16 hrs day and 8 hrs dark periods. Samples were collected at 0, 12, 24 and 48 hai and stored at -80 ^{o}C.

2.3. RNA isolation from plant leaf tissue

Total RNAs from leaf aamples that belong to specific time points (infected and mock infected) of resistant and susceptible plants were isolated individually.

Homogenization:

Tissue samples were powdered using a mortar and liquid nitrogen. Powdered tissue was homogenized in 1 mL of TRIzol Reagent per 50-100 mg of tissue in a 2 *mL* sterile tube.

Phase Separation:

Homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 mL of chloroform per 1 *mL* of TRIzol Reagent was added. Sample tubes were capped securely. Tubes were shaked vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 min. The samples were centrifuged at 15,000 rpm for15 min at 4^{o}C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

RNA precipitation:

The upper phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 mL of isopropyl alcohol was used per 1 mL of TRIzol Reagent used for the initial homogenization. Samples were incubated at room temperature for 10 min and centrifuged at 15,000 rpm for 10 min at 4^{o}C. The RNA precipitate observed was a gel like pellet on the side and bottom of the tube.

RNA Wash:

Supernatant was removed and the RNA pellet was washed once with 75% ethanol, adding at least 1 *mL* of 75% ethanol per 1 *mL* of TRIzol Reagent used for

the initial homogenization. Samples were mixed by vortexing and centrifuged at 10,000 rpm for 5 min at 4°C.

Re-dissolving the RNA:

At the end of the procedure, the RNA pellet was briefly dried (10 – 15 min). RNA was dissolved in DEPC treated sterile water and incubated for 10 min at 55°C-60°C. (Invitrogen TRIzol protocol)

2.4 Concentration determination of the isolated RNA samples

RNA samples were diluted 1/1200 to 0.5 mL in double distilled water and their absorbance values were measured at 230 nm, 260 nm, and 280 nm in NanoDrop® ND-1000 UV-Vis Spectrophotometer. RNA absorbs maximum light at 260 nm, but it is also required to know the absorbance values of proteins at 280 nm and phenolic compounds at 230 nm in order to evaluate the possible contaminations.

Concentration determination of DNA samples was achieved according to the equation given below:

$1A_{260}$ = 40µg/mL RNA

Conc.of RNA (mg/µL) = A_{260} value x dilution factor x 40 mg/mL RNA/A_{260}

The intactness of RNA were checked by running RNA samples on 1 % formaldehyde-agarose.

2.5 Synthesis of first strand cDNA:

Following components were combined in a 200 µL sterile PCR tube: 50 pmol oligo dT(20), 10 pmol, total RNA (1 ng-5µg), 0.625 mM dNTP (DNA Amp) mix,

sterile distilled water up to 12 µL. Mixture was incubated at 65 ^{o}C for 5 min and quickly chilled on ice, spinned briefly and the following components were added: 1X first strand buffer (Gibco BRL), 0.005 M DTT (Gibco BRL), 30 U RNase inhibitor (Ambion). The content of the tube was spinned briefly and incubated at 50 ^{o}C for 2 min, finally 200 U of SuperScript III (Gibco BRL) reverse transcriptase enzyme was added. Reaction were carried out at 50 ^{0}C for 1 hr and stopped by incubating at 70 ^{o}C for 15 min.

2.6. Differential display (DD)

Differential Display conditions were optimized in our laboratory (Bozkurt O, 2002 Ms thesis). All incubation reactions for Differential Display were performed in MJ Research PTC 100 type thermocycler.

2.6.1 Differential display reverse transcriptase-PCR (DDRT-PCR)

DDRT-PCR was performed on single stranded-cDNAs (ss-cDNA) using different primer combinations; P1-P10/T1-T9 (primer sequences are available in Clontech (Palo Alto, CA, USA) kit (Ref. 637405) and also presented in Table2.1. cDNAs from different time points upon infection were combined to be used in DDRT-PCR experiments and amplified with 1 unit of *Taq* DNA polymerase (Fermentas) in the presence of 1X PCR Buffer (75 mM Tris-HCl with pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix, 0.02 µL of 3000 Ci/mmole; [α-^{32}P]dATP, 1.5 mM $MgCl_2$, 10 pmol P-primer, 10 pmol T-primer and sterile distilled water up to 20 µl volume. PCR cycling conditions were as follows; 94 °C for 2 min initial denaturation, 2 cycles of three steps of 2 min at 94 °C, 5 min 42 °C and 5 min 72 °C; followed by 30 cycles of three steps of 94 °C / 1 min; 60 °C / 1 min; 72 °C / 1 min and 1 cycle of 10 min extension at 72 °C. The reactions were terminated by adding 4 µL of stop solution (95% formamide, 20 mM EDTA, 0.25% bromophenol blue and 0.025% xylene cyanol).

Table2.1 Sequences of the primers used in differential display analysis

Primer	Primer sequence (5'-3')	T_A (C^o)
T1	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ AA	**65,1**
T2	CAT TAT GCT GAG TGA $CT_{(9)}$ AC	**64,9**
T3	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ AG	**65,0**
T4	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CA	**67,0**
T5	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CC	**67,8**
T6	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ CG	**68,5**
T7	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ GA	**67,0**
T8	CAT TAT GCT GAG TGA TAT $CT_{(9)}$ GC	**68,6**
P1	AAT AAC CCT CAC TAA ATG GGG A	**69,9**
P2	ATTAACCCTCACTAAATCGGTCATAG	**63.7**
P3	ATTAACCCTCACTAAATGCTGGTGG	**61.3**
P4	ATTAACCCTCACTAAATGCTGGTAG	**59.7**
P5	ATTAACCCTCACTAAAGATCTGACTG	**60.1**
P6	ATTAACCCTCACTAAATGCTGGGTG	**61.3**
P7	ATTAACCCTCACTAAATGCTGTATG	**58.1**
P8	ATTAACCCTCACTAAATGGAGCTGG	**61.3**
P9	ATTAACCCTCACTAAATGTGGCAGG	**61.3**
P10	ATTAACCCTCACTAAAGCACCGTCC	**63.0**

2.6.2 Detection and analysis of differential display bands

Differential display products were denatured at 94 °C before loading on the denaturizing gel. Samples were loaded on 6 % denaturizing polyacrylamide gel (5.7% acrylamide, 0.3% N, N'methylene-bis-acrylamide, 8M urea, 1X TBE (90 mM Tris base, 90 mM Boric acid, 2 mM EDTA)). To 60 mL of gel solution, 650µL of 10% APS (ammoniumpersulfate) and 25µL of TEMED (N, N, N', N'-Tetraethyl ethylene diamine) was added and gel was poured between two SigmaCoat treated sequencing gel plates immediately. Radioactively labeled Differential Display products were electrophoresed at a constant power of 60 Watt (Biometra High Voltage Power Supply, Pack P30, and Germany). Electrophoresis continued until the xylene cyanol dye migrates 10 cm after the bromophenol blue dye goes out of the gel. After completion of electrophoresis, gel was transferred oto Whatman paper

(3MM), and covered with stretch film. The gel was left on the gel dryer (Savant SGD 2000) and dried at 78°C for 30-40 minutes. After drying procedure X-ray film was exposed to the gel. Films were developed at METU Health Center after a period of time depending (1 day to 10 days) on the half-life of the radioactive material.

2.7 Cloning of differentially expressed bands

Differentially expressed bands detected in DD and analyses were cloned in order to be sequenced. As an initial step differentially expressed bands were cut form sequencing gel and dissolved in 30 µL distilled water. Dissolved products were reamplified by PCR

2.7.1 Reamplification of differentially expressed fragments

Reactions were carried out in 50 µL of final volume and following componenents were mixed; 5 µl redissolved product, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 0.05 mM dNTP mix (DNA Amp), 1,5 mM $MgCl_2$ (DNA Amp), 1 u of *Taq* DNA polymerase, 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 50 µL volume. PCR cycling conditions were 94°C for 2 min as an initial denaturation step, 25 cycles of three steps of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and extension at 72 °C for 2 min. Re-amplified bands were loaded on 1.5% agarose gel and evaluated. Re-amplified bands were cut and redissolved in 20 µl distilled water to be cloned.

2.7.2 Ligation of re-amplified fragments to pGEM-T-Easy vector

Redissolved DNA that was cut from agarose gel was ligated to pGEM-T-Easy vector (Promega) with a final volume of 10 µL in a PCR tube. Following components were combined; 6 µL redissolved DNA, 5 ng pGEM-T-Easy vector (Promega), 1 x Ligase Buffer (Promega) and 2 units T_4 DNA Ligase enzyme (Promega). Mixture was incubated at 4°C overnight (Approximately 18 hours).

2.7.3 Preperation of *E.coli* competent cells

A single colony of *E.coli* Dh5-α cells was inoculated into 2 mL LB medium. Cells were let grown at 37°C with moderate shaking (250rpm) overnight. 1 mL of overnight grown culture was inoculated into 100 mL of LB medium in a sterile 2-liter flask and grown at 37°C, shaking (250rpm), to an OD_{590} of 0.375. Culture was aliquated into two 50-mL prechilled tubes and leaved on ice for 10 min. Cells were centrifuged for 7 min at 3000 rpm. Supernatant was poured off and each pellet was resuspended in 5 mL ice-cold $CaCl_2$ (4mM) solution. Cells were centrifuged for 5 min at 2500 rpm and supernatant was discarded. Each pellet was resuspended in 2 mL of ice-cold $CaCl_2$ (4mM) solution. Again cells were centrifuged for 5 min at 2500 rpm and supernatant was discarded. Each pellet was resuspended in 1.6 mL of ice-cold $CaCl_2$ (4mM) solution. Cells were dispensed into prechilled 500 µL PCR tube, leaved on ice for 24 h. the cells keep their ability for efficient transformation for a week at 4°C.

2.7.4 Transformation of *E.coli* competent cells with ligation products

E.coli Dh5-α competent cells were transformed with ligation products according to the following procedure. In a sterile 2 mL tube following components were combined; 5 µL ligation product, 0.02 M β-Mercaptoethanol and 30 µL *E.coli* Dh5-α competent cells. Mixture was placed on ice for 30 min, and then heat shocked at 42 °C for 45 sec. SOC Medium was added upto 100 µL.

The cells were incubated 37 °C for 45 minutes and quickly chilled on ice for 2 minutes. They were spread on plates as 50 µL/plate (plates contain LB Agar). Plates were incubated at 37 °C overnight. After the incubation, white colonies were selected among grown colonies and these colonies transferred to 100 µL LB medium containing sterile 2 *mL* tubes. Selected colonies were PCR amplified.

2.7.5 PCR amplification of colonies with Taq Polymerase enzyme

1x PCR buffer (DNAmp), 0.2 mM dNTP mix (DNAmp), 0.8 pmol M13 Forward and Reverse primers (TIB Molecular Biology), 1.2 mM $MgCl_2$ (DNAmp), 1 unit *Taq* Polymerase enzyme, 1 µL DNA from colonies and sterile PCR water up to 25 µL final volume combined in a sterile PCR tube. PCR cycling conditions were as initial denaturation at 94°C for 2 minutes followed by 30 cycles 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

PCR products were electrophoresed on 1.5% agarose gel to detect positive and false positive colonies (colonies that do not carry the expected sized DNA fragment). Positive colonies were selected and used in plasmid isolation step.

2.7.6 Plasmid isolation from colonies

Plasmids were isolated using QIAGEN QIAprep Spin Miniprep Kit acording to kit protocol.

Bacterial culture of 2 mL was harvested by centrifugation for 5 min at 15,000 rpm. Supernatant was poured off; tube was inverted and blotted to on a paper towel to remove excess media. 250 µL of Buffer P1 was added to tube and cell pellet was completely resuspended by vortexing. 250 µL 0f Buffer P2 was added, then the tube mixed by gently inverting four times, incubated at RT for approximately 5 minutes. 350 µL of N3 solution was added and mixed by inverting 4 times. Sample tubes were centrifuged at 15,000 rpm for 10 minutes at RT. A spin column, provided by

manufacturer, inserted in to collection tube. Cleared lysate, the upperphase was transferred to the spin column, centrifuged at 15,000 rpm for 1 minute at room temperature.

After the centrifugation, filtrate in the collection tube was discarded and collection tube reinserted, and well washed 750 µL of Column Washing solution Buffer PE was added to spin column, centrifuged at 15,000 rpm for 1 minute at RT, flowthrough discarded and the collection tube reinserted again and to remove residual wash buffer completely additional 1 min centrifugation was involved. The spin column was transferred to a new sterile 1.5 mL tube, the plasmid DNA was eluted by adding 50 µL of Buffer EB (elutionbuffer) and centrifugating at 15,000 rpm for 1 min. Spin column assembly was removed and plasmid DNA was stored at –20 °C.

2.7.7 Visualization of Isolated Plasmid

In order to determine DNA integration to the plasmid and to be informed about the size of the insert, plasmid DNA was digested with EcoRI restriction enzyme. 2 µg purified plasmid, 1X NE Buffer (New England Biolabs), 1 unit EcoRI enzyme (New England Biolabs) and PCR water were combined in an eppendorf tube with a final volume of 10 µL. Mixture was incubated at 37 °C for 5 hrs. After the incubation samples were run on 1.5% agarose gel.

2.8 Sequencing reactions

Plasmids were purified as described in section 2.11. Inserts were custom sequenced, and were read using SP6 primers. Sequencing reactions of the 600 ng purified pGEM-T easy (Promega) recombinant clones were performed and the sequences were read on ABI prism-310 Genetic Analyzer. The DNA sequences are presented in Appendix A.

2.9 Sequence and homology analysis

NCBI databanks were searched for finding homolog sequences that are available. Sequence analysis was performed using Editseq 4.0 (DNA Star Inc.) expert sequence analysis software.

2.10 Real time PCR analysis for confirmation of DD results

2.10.1 Plant growth and infection

Plant growth and infections were as described in section 2.2. In order to confirm plants are successfully infected, histochemical DAB staining method is used. HR (hypersensitive response) was detected by DAB staining according to Thordal-Christensen, 1997 on the 48^{th} hr of avirulently infected seedlings. The samples were sliced into small pieces and incubated overnight in 1/9 (v/v) DAB liquid chromogen (Sigma No. D7554) and buffer (Sigma No. 7429) prior to microscopic visualization samples were washed in buffer three times.

2.10.2 Clean up of total RNA for QRT-PCR

Total RNA was cleaned up by treatment of RNA with DNAse and lithium chloride precipitation. DNAse treatment was performed using Turbo DNAse (AMBION INC. Lott 095K81). Following components were combined in a 200 µL sterile PCR tube: 15 µL ssRNA which is equal to 1µg, 1 µL DNAse enzyme. Samples were incubated at 37 °C for 30 minutes and then reaction was inactivated by heating for 15 minutes at 65 °C.

2.10.3 Lithium chloride precipitation

In order to separate pure RNA from the mixture, following components were added into a 2 mL sterile tube containing 5 µg ssRNA: one tenth volume of lithium chloride (Ambion salt, 7.5M, #9480), 2.5 volume of 100% ethanol. Then tubes were frozen at -20 °C for 30 minutes for dilute small sizes. Samples were mixed by vortex and centrifuged at 10,000 rpm for 5 min at 4^{o}C. Following centrifugation, supernatants were removed and pellets were washed with 70% ethanol made with nuclease free water. Finally, samples were centrifuged at 10,000 rpm for 5 min at 4^{o}C and pellets were re-suspended in 15 µL of nuclease free water.

2.10.4 QRT-PCR analysis

Expression level differences of the genes identified were confirmed and quantified by comparative QRT-PCR. The measurements were performed in a Stratagene MX3005p QPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). QRT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each reaction were normalized using primers specific for *Actin-1* gene AATGGTCAAGGCTGGTTTCGC and CTGCGCCTCATCACCAACATA (forward and reverse, respectively). Primer pairs for real-time PCR analyses were designed using Primer3 software (Rozen and Skaletsky, 2000) and they are listed in table 2.2. Threshold (Ct) values and gene transcript numbers were used to determine the gene expression levels from different cDNA samples. Real-time PCR data were analyzed with the REST software (Bustin et al., 2005) in comparison to constitutively expressed wheat *Actin1*, gene for normalizations. The relative expression ratio (*R*) of a target gene is calculated based on *E* and the CP deviation of an unknown sample versus a control, and expressed in comparison to a reference gene (Pfaffl, 2001).

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control-sample)}}$$

Table 2.2 QRT-PCR primers for the sequenced fragments and the internal control genes (Actin-1 and 18S rRNA) for expression normalization.

Clone name	Forward Primer s (5'-3')	Reverse Primers (5'-3')	Tm (°C)	Target size bp
DD Yr1-1	AGGAGGAATTTAGTGCGGTGT	CAGCAAGCAAGGCAGTTTGT	60-61	185
DD Yr1-2	GGAGGTCGAGTTGCTTGCTCAGTCA	ACCGGAATCTGAGTAGGCTTCCAA	72-69	163
DD Yr1-3	TGTTTACAAGATCGGTGGTATTG	ATCTTAACAAGACCAGCATCACCG	62-66	456
DD Yr1-5	ACTGCGGCCGCTCATTCTC	TTCCATCTTTCCAACCACCAA	66-62	220
DD Yr1-6	CAGTACAGCAGCCCAGCAAC	AAACCCTGCAGTACCCACAA	61-60	156
DD Yr1-8	ACCTGGAGGAGTTTGGTCTGG	CTGTGGCACTAGGCTGGAGAA	62-62	200
DD Yr1-9	AACAACCGGAACTCCATGTC	GTTGTCGCAGTCCGGTAAAT	63-63	123
DD Yr1-10	GCAAGGAGACGGCAAGAGAA	CCGCTTTCAAGCAATCCAA	62-62	172
DD Yr1-11	GCTTGGAGGACAAACTGGAGA	CCGGTGGTGAAGACAATCAA	65-65	150
DD3 Yr1-50	GCATTGGAACAAGGTGAAGAA	GAGGTAAAATTGCGCACAGG	60-60	177
DD3 Yr1-74	CACCCTTTGTGGGTTCTAGGT	AAGTGGGCCATTTTTGGTAAG	60-60	100
DD3 Yr1-85	AGACGACCAACAACAGCACA	GGATTACAGATCAAGAACACAAGCA	60-61	190
DD Yr10-1	GCGAGAGTACAACCGCAAAG	CAAACGATGCAACAAAGCAA	60-60	174
DD Yr10-4	GCGCTGATGCAAACATCAGTGAAC	CATGCTCTTCAGGAGCTGCCTCGT	71-73	300
DD Yr10-5A	GGCCAGTAGCTCCCTCCA	CAACAGAACCCTTCACTTGAAAAA	61-61	105
DD Yr10-5B	AAGGCCACCTACAAGTTCTTAA	CATGAGCAACCCCTGCTCACGACA	70-75	240
DD Yr10-6	CTACCACCTAGCTTGCTGCTGGTA	GCGTGTACGTTTGCATGCGTGGTA	68-61	120
DD Yr10-8	GTCGCTGCTAGCCAGGCAATCATG	TGTGTGCAGGAGCTATTTACGTGG	73-68	100
DD3 Yr10-21	TACCTCGCGAATGCATCTAGATTG	CTCACTGTAATCAATTTGTCCATC	62-55	473
DD3 Yr10-31	AATTGCAAGGAAAGGGAAAAA	CACTGGGGATATGGAGCTTG	60-60	158
DD3 Yr10-36	ATTGTGGTGCTTTTGCCTCGA	TCTTCTTCCCATTCTTCATTACGA	59-60	100
DD3 Yr10-37	CAAAGTGCCAAGGGGAAGT	AATACCACATCGGAGCAAACA	60-60	243
DD3 Yr10-48	AAGCCTCTGAAGAGCTGGAAC	AATTAGCCCGTTGTAGCAGAAA	60-60	150
DD3 Yr10-49	GATCGTTGAGGCTGTTATTCATCT	GCTTCAAACCGACATTCACC	60-60	157
DD3 Yr10 -55	GGCGTGTCGACCAACAACTAC	CGTCACTTGACGAGCTACTGATAC	62-60	118
DD3 Yr10-73	GCTGAACCCTAAATTTCTGTCCT	CTAAAGCACCGTCCGTAAACC	60-60	154
DD3 YR10-87	CATCAGGGTCGTTAGCGTTT	CCCAGGCATCCCTTTACCT	60-61	158
DD3 YR10-88	CTGGACGAGATCAAGGAGGTT	GGCAAATACATATAATCGCAGAGG	60-61	156
DD3 Yr10-90	GGAAGACGACGAGGACGAA	CTGCTAACCACCGAACATCAC	61-60	154
Actin-1	AATGGTCAAGGCTGGTTTCGC	CTGCGCCTCATCACCAACATA	68-63	150
18S rRNA	TTTGACTCAACACGGGGAAA	CAGACAAATCGCTCCACCAA	61-62	124

2.11 Microarray experiments

In order to identify the genes involved in resistance mechanisms and determine the changes in the gene expression during the pathogen invasion on the susceptible plant, we have designed a microarray experiment by using currently released Affymetrix wheat chips in John Innes center centre genome laboratory (Norwich/UK). We have used 2 biological replicates for each inoculations and 1 chip replicate corresponding to 9 wheat microarray chips in total. Microarray data was analyzed using genespring (agilent) program

2.11.1 Plant material and growth

Wheat cultivar Avocet Yr1 single seeds were wrapped by filter papers. They were wetted by tap water and incubated at 4°C for 3 days. After 3 day period of vernalization, seeds were let grown in dark for a week, then transferred to soil and let grow in a growth chamber for 14 days. Growth conditions were 18 °C at light period for 12 h and 12 °C at dark period for 12 h.

2.11.2 Fungal inoculations

Puccinia striiformis f.sp. tritici race 169E136 being virulent on differential lines with genes Yr1, Yr2, Yr3, Yr9, and YrA genes, and *Puccinia striiformis* f.sp. tritici strain 232E137 being virulent on Yr2, Yr3, Yr9, and YrA genes except Yr1, were used as compatible and incompatible yellow rust pathogens of Avocet-Yr1 differential line seedlings, respectively. Plants were inoculated and incubated at 10 °C for 24 hours dark period, following 24 hours incubation at 16 °C. Plant leaf samples were collected at time points of 6, 12, 24, 48, 72 hours post infection (hai). Plant leaf samples were collected after infections and stored –80 °C until time of RNA isolation.

2.11.3 RNA Isolation from plant leaf tissue

The equal amounts of each time points (16 µg) were combined to obtain get 80 µg of total RNA for each treatment; mock, virulent and avirulent infections. RNA isolations were performed as described in section 2.3. Combined RNA samples were further cleaned up using Qiagen RNeasy mini kit according to the procedure of the manufacturer (Qiagen). RNA quality was ensured on the profiles obtained using Agilent 2100 Bioanalyser (Agilent).

2.11.4 Construction of double stranded cDNA

First strand cDNA was synthesized as described in the section 2.5. For the second strand cDNA synthesis, following components were combined in a 200 µL sterile PCR tube: 1X second strand buffer (Invitrogen), 20 U of DNA polymerase I, 0.2 mM of each dNTP (Invitrogen), 10 U of RNase H (Invitrogen), 10 U of T4 DNA ligase (Invitrogen), 20 µL of first strand cDNA synthesis reaction product and water up to 150 µL final volume. Reaction was carried out at 16°C for 2 h. Double stranded (ds) cDNA was cleaned up using phase lock gel (Eppendorf) and phenol chloroform precipitation. Samples were Ethanol- precipitated and resuspended in 22 µL of H_20.

2.11.5 Probe preparation: *in vitro* transcription

Invitro transcriptions were performed using Enzo IVT kit (Enzo Life Sciences). Following components were combined in a 200 µL sterile PCR tube: 22 µL ds-cDNA, 1X IVT buffer, 4 µL biotin labeled ribonucleotides, 4 µL DTT, 4 µL (RNase inhibitor, 2 µL IVT enzyme. Samples were incubated at 37 °C by shaking at 550 rpm for 4 hours. cRNA was determined and its quality was validated on Agilent 2100 Bioanalyzer. Fragmentation of cRNA 20 µg of cRNA was carried out by incubation at 94 °C for 35 min in 1X Affymetrix fragmentation buffer in 40 µL of final volume.

2.11.6 Hybridization conditions

Arrays were prehybridized with 200 µL of hybridization buffer for 15 min at 45 °C by shaking at 60 rpm. 40 µL fragmented cRNA probes were mixed with 260 µL hybridization cocktail and incubated at 95 °C for 5 min, 45 °C for 5 min and spun for 5 min at 13,000 rpm. Hybridization buffer on the arrays were replaced by

hybridization cocktail. Arrays were incubated at hybridization owen for 16 hours at 45 °C.

2.11.7 Staining, washing & analysis of arrays

Hybridization solution in the arrays was replaced with 200 µL of wash buffer. Arrays were then placed in the machine. Antibody and SAPE (streptavidin phytoeritrine) solutions were placed in the Affymetrix microarray machine and analysis were performed.

2.11.8 Microaaray data analysis

The data collected from the microarray experiment were analyzed by Genespring program of Agilent.

2.11.9. QRT-PCR of the microarray differentially expressed ESTs

For the 20 of the differentially expressed ESTs, PCR primers (Appendix B) designed in order to confirm expression changes with realtime PCR. QRT-PCR was performed as described in section 2.10.4.

2.12 Virus induced gene silencing (VIGS)

VIGS of wheat plants were achieved using BSMV vectors according to the protocol developed by Holzberg *et al. (*2002). Then, silencing of *PDS* gene is aimed which is involved in synthesis of chlorophyll. It prevents the formation of green color on leaves so it can be used as a positive marker for silencing.

2.12.1 BSMV vectors

BSMV vectors pα, pβ, pγ and pγ.bPDS4 (Figure 2.1) were obtained from Large Scale Biology Corporation (CA, USA). Vectors were dissolved in 20 µL of

TE and then cloned into *E.coli* DH5α strains as described in section 2.7.4. Amplified plasmids were isolated and purified as described in section 2.7.6.

2.12.2 Plant growth conditions

All experiments were done using wheat Avocet-YR10 differential line. All plants were grown as described in section 2.2.

2.12.3 Linearization of plasmids

Plasmids pα, pβ, pγ and pγ.bPDS4 were digested with restriction enzymes in order to be linearized. pα plasmid DNA was digested with *Ml*uI enzyme (MBI fermentas). 7 µg purified pα plasmid DNA, 1X Buffer (MBI fermentas), 10 unit MluI enzyme (MBI fermentas) and PCR water were combined in a PCR tube with a final volume of 60 µL. Mixture was incubated at 37 °C 3 hrs. pβ plasmid DNA was digested with BcuI enzyme (MBI fermentas). Alternatively it can be linearized also using SpeI enzyme. 7 µg purified pβ plasmid DNA, 1X Buffer (MBI fermentas), 10 unit BcuI (MBI fermentas) and PCR water were combined in a PCR tube with a final volume of 60 µL. Mixture was incubated at 37 °C 3 hrs. pγ plasmid DNA was digested using BssHII enzyme (New England Biolabs). 7 µg pγ plasmid DNA, 1X Buffer (New England Biolabs), 10 unit BssHII enzyme (New England Biolabs) and PCR water were combined in a PCR tube with a final volume of 60 µL. Mixture was incubated at 50 °C 3 hrs. After the incubation samples were observed on 1.5% agarose gel. Map of the plasmids are presented at Figure 2.1.

2.12.4 *In vitro* transcription of linearized vectors

Infectious BSMV RNAs is prepared from linearized plasmid cDNA by *in vitro* transcription using T7 DNA-dependent RNA polymerase using the kit mMESSAGE mMACHINE® T7 Kit; Silencer (Ambion) according to manufacaturers' instructions. Following components were combined in a 200 µL

sterile PCR tube: 80 ng template (linearized plasmid DNA), 1X Buffer (Ambion), 1X nucleotide mix (Ambion), 1 U of T7 RNA polymerase (Ambion) and sterile distilled water up to 2.5 µL. Mixture was incubated at 37 ^{o}C for 2 hrs and stored at -80 ℃ untill use.

2.12.5 Inoculation of plants with BSMV

Avocet-Yr10 plants were labeled and watered (in order to ameliorate the mechanical damage caused by inoculation) before inoculation. Transcripts of each of the BSMV genomes were mixed in a 1:1:1 ratio (2.5 µL of each for a total of 7.5 µL for plant to be inoculated). A 7.5 µl aliquot of the transcription mix was combined with 45 µl FES (50 mL 10X GP (18.77 g Glycine, 26.13 g K_2HPO_4, dd$H_2$0 upto 500 mL, autoclave 20 min), 2.5 g Sodium pyrophosphate, 2.5 g Bentonite, 2.5 g Celite and ddH20 upto 250 mL (Pogue et al., 1998) and directly applied, with two light strokes to the second leaf from the bottom of wheat plants at 12 days post-sowing. When finished inoculating plants were mist with water and covered with a plastic dome in order to minimize water loss and then returned to growth chamber. A systemic spread was determined by the appearance of mosaic symptoms on leaves after 8-12 days pdi. Leaves from inoculated plants were collected after 12-15 days pdi in order to check *PDS* gene silencing by QRT-PCR.

2.12.6 QRT-PCR for determination of gene silencing in infected wheat plants

2.12.7 Plant samples

Leaf samples from inoculated Avocet Yr10 plants collected after 12 days pdi in order to check PDS gene silencing by QRT-PCR. Samples were frozen in liquid nitrogen and transferred to – 80 ℃ until use.

2.12.8 RNA isolation and cDNA construction from inoculated plants

Total RNA samples were isolated from frozen leaf samples as described in section 2.3. Total RNA was then treated with DNAseI and purified with lithium chloride precipitation as described in section 2.10.1.

2.12.9 Determination of *PDS* gene silencing by QRT-PCR

For the detection of the level of gene silencing at molecular level QRT-PCR method was applied. Primers for the endogenous PDS gene were designed to use in QRT-PCR experiments (PDS primers 5'-3' direction: For: CCC TGA CGAGTTATCCATGCA G Rev: GGACCTCACCACCCAAAGACT) Reactions were carried out as described in section 2.10.

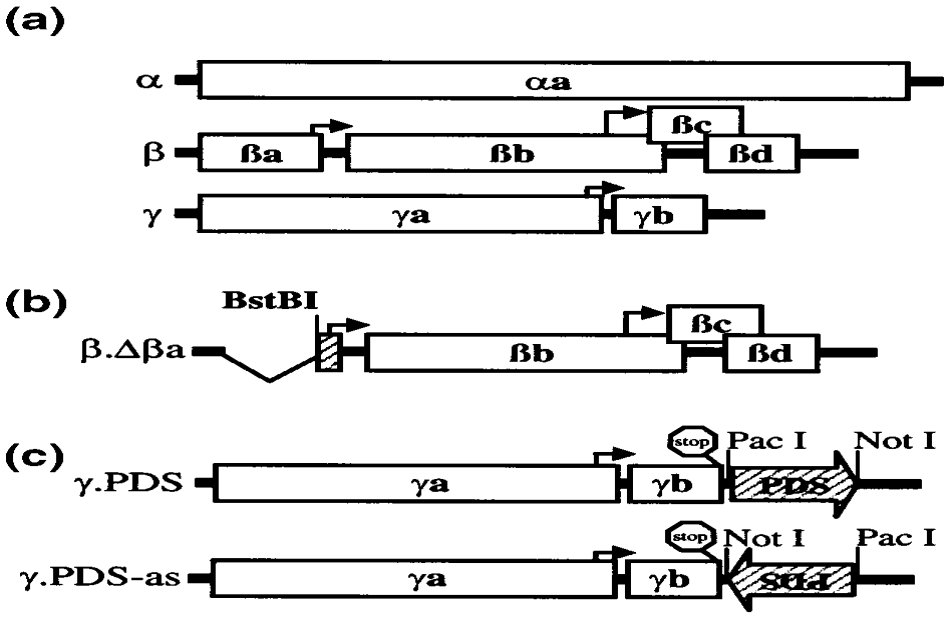

Figure 2.1 BSMV based silencing vector maps. a) Wild type BSMV vectors b) BSMV vector pα, pβ, pγ deleted (coat protein). c) BSMV vector pγ with *PDS* gene fragment inserts in sense and antisense orientation (Holzberg, 2002).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Infection controls of wheat plants

Prior to the DDRT-PCR sample preparation, infections were verified by DAB staining and auto-fluorescence HR (Kawalleck *et.al*., 1995). Accumulation of H_2O_2 and cell death is characteristics of plant defense as part of hypersensitive response. As it is demonstrated in Figure 3.1, following the 48 hrs of infection with avirulent Pst17, the autoflorescence (b) and H_2O_2 accumulation (c) were detected. 24 th hr sample did not show HR (hypersensitive response) symptoms so we used 48th hr sample for confirmations

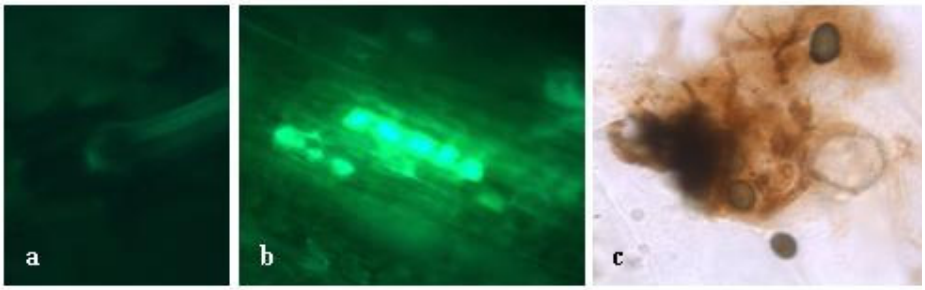

Figure 3.1 a: Avocet-Yr10 control plant (mock infection); **b**: Detection of Auto-florescence and **c**: DAB (3,3'-diaminobenzidine) staining of Avocet-Yr10 upon incompatible infection showing H_2O_2 accumulation (Leica Model DCM4000 B (Leica DFC 280 camera) at 40 X magnification).

3.2 Total RNA isolation for differential display analysis

Integrity of RNA was verified by running the samples on 1% formaldehyde-agarose RNA gel by the appearances of the intact ribosomal RNA subunits. It is a common practice that, if the RNA subunits appear non-degraded on the agarose gels, then it is considered that the rest of the mRNAs is intact as well. All RNA samples were visualized on the gel for the intactness (Figure 3.2).

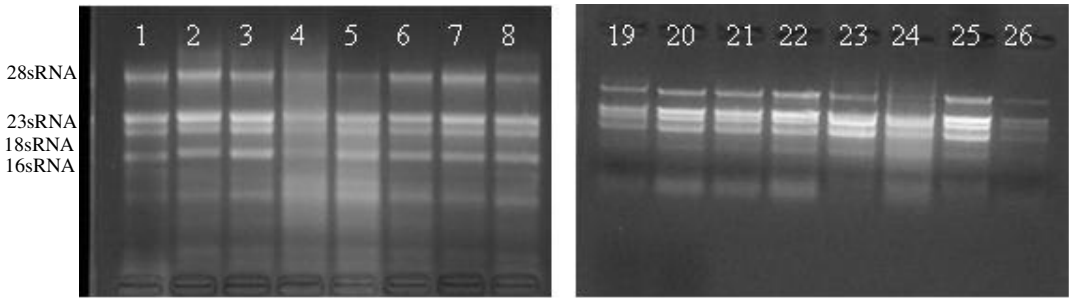

Figure 3.2 Appearance of total RNA samples isolated from leaf tissues on 1% formaldehyde-agarose RNA gel. Lanes labeled from 1 to 8 are from Avocet Yr10 and lane 19-26 are from Avocet YR1 lines. Lanes 1-4 belong to infected samples corresponding to 3,12,24,96 hrs pi (post infection), respectively while lanes 5-8 are uninfected control samples corresponding to 3,12,24,96 hrs pi. Lanes 19-22 belong to infected samples corresponding to 3,12,24,96 hrs pi respectively, while lanes 5-8 are uninfected control samples corresponding to 3,12,24,96 hrs pi.

3.3 DD analysis

We have applied the DD RT-PCR method according to Clontech's Delta DD method in which the ss-cDNA is used as template in DD analysis. PCR cycles of this approach were optimized by performing different number of PCR cycles and running the samples on agarose gel (data not shown). The experiments were carried out using custom made P and T primers. Sequences of the primers used are listed in Table 2.1. P primers were designed to anchor the 5′ region of the mRNA whereas T primers were designed to anchor the 3′ (polyA) region of the mRNA. DDRT-PCR reactions were generated at 4 different time points (3, 12, 24, and 96 hai (hours after infection)) together with controls at the same time points (mock infections). For reliable comparisons of differentially expressed band samples from each time points were loaded on the gel separately. Figure 3.3 and Figure 3.4 represent DDRT- PCR profiles

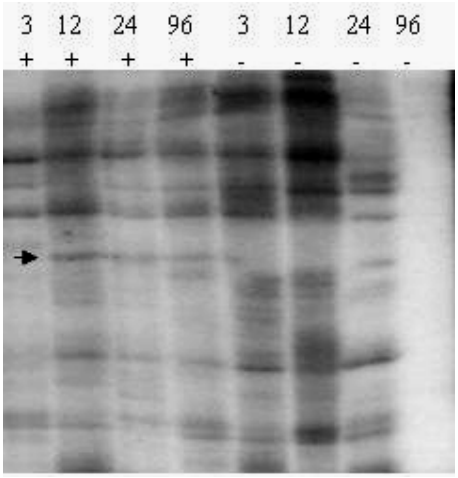

Figure 3.3 DDRT-PCR autoradiograph profile (P2/T8 primer set). **+/-** infected and mock infected time points (hai), respectively. The arrow indicates the fragment

obtained by avirulent Pst45 infection of Avocet-Yr1 plant (only present in infected samples), cloned as DDYr1-10, sequenced and found to be homologous to the *Arabidopsis thaliana* UBX domain-containing protein (NP_192817.1).

As it is clearly observed in the Figure 3.3, they DDYr1-10 band is only apparent in the lanes with samples +12 hai, +24 hai, and +96 hai, corresponding time points of mock infections lacking the band.

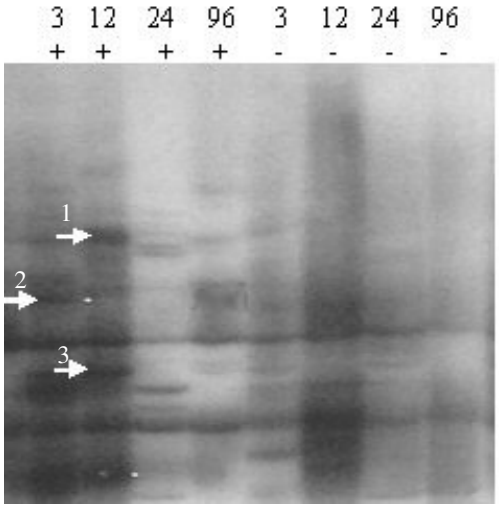

Figure 3.4 DDRT-PCR autogradigraph profile (P1/T8 primer set). **+/-** infected and mock infected time points (hai), respectively. The arrows indicate the fragments obtained by avirulent Pst17 infection of Avocet-Yr10 plant (only present in infected samples), cloned as 1. DDYr10-1 (homologous to the Rad6 gene of *Oryza sativa*), 2. DDYr10-2 (homologous to the F-box gene of A*rabidopsis thaliana*) and 3. DDYr10-3 (homologous to the Syntaxin gene of A*rabidopsis thaliana*) from top to bottom

In Figure 3.4 the bands 1, 2, and 3, available in the varying time points for infected samples, and they are absent in the mock infections.

3.4 Evaluation of results obtained from DD analysis.

The PCR products of DD were analyzed as described in the Materials and Methods section. Total of 90 primer combinations were used. A total number of 60 differentially expressed bands were identified and cut from sequencing gels for further analysis. Among them, 50 were re-amplified and 39 of them were selected to be cloned and sequenced since other 11 were too small in size. The selected differentially expressed fragments were precisely cut from DNA denaturing gels and reamplified with PCR. An example to reamplified fragments is presented in Figure3.5. The figure does not have molecular weight marker, since it was considered to be not very critical to verify the lengths of the PCR products. The only purpose was just to detect the presence of the PCR products.

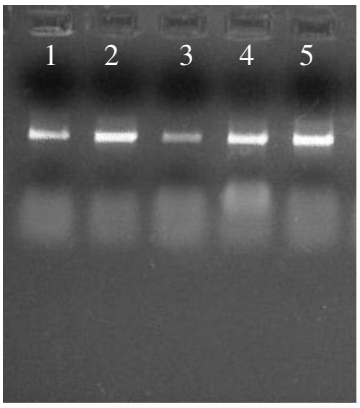

Figure.3.5. PCR re-amplified band-isolated DD fragments from DNA denaturing polyacrylamide gel separated on 1.5% agarose gel. Lanes from 1-5 are reamplified clones that belong to, DDYR10-2, DDYr1-10, DDYr10-3, DDYr10-4, and DDYr1-2, respectively.

3.5 Cloning and sequencing of differentially expressed fragments

Cloning of the fragments was achieved using pGEM-T Easy vector and competent *E.coli Dh5-α* cells. PCR amplification from the selected colonies was performed and they were observed to be carrying the expected sized inserts. Plasmids were isolated from these colonies and prepared for sequencing. In order to confirm the isolation of plasmids carrying the inserts, plasmids were digested with EcoRI restriction enzyme and electrophoresed on 1.5% agarose gel.

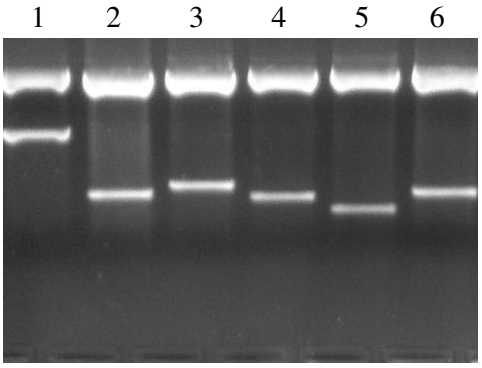

Figure 3.6 EcoRI digestion of plasmids from the selected colonies. Lanes from 1 to 6 are clones DDYr1-1, DDYr1-3, DDYr10-2, DDYr1-8, DDYr1-9, and DDYr10-4, respectively.

3.6 Results of the sequences and identities of gene fragments

Obtained 33 sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available genes and DNA sequences. The result of Blast search for sequence similarities is shown in Table 3.1. DNA sequences of differentially expressed fragments were translated to corresponding amino acid sequences (without any stop codons) using Editseq 4.0

(DNA Star Inc.) expert sequence analysis software. Amino acid sequences were searched in the NCBI (National Center for Biotechnology Information) database for comparison with available protein sequence data.

Based on the sequences, 5 of the clones were primer dimers and 1 was empty vector with no inserts. Most of the clones of the 33 showed homologies to the 3' ends and the 3' untranslated regions of the genes in the databank. However, clone DD YR1-1 showed homology to 5' untranslated region along with the coding region of the receptor like kinase, TAK33 gene of *Triticum aestivum*. Of the clones, two DDYr10-1 (RAD6) and DDYr1-9 (Cyclophilin like) contained full coding regions together with 5' and 3' end un-translated regions. The clones DD3Yr10-31 and DD3Yr10-73 showed homology only to the 3'untranslated regions of *Triticum aestivum* endochitinase and geranylgeranyl transferase mRNAs respectively. Three clone sequences showed no significant homologies, probably due to being short in length or having cloned the 3' UTRs. 33 of DDRT-PCR fragments were selected to be confirmed with QRT-PCR analysis.

Table 3.1 Homologous sequences found in Genbank, using Blast algorithm v2.2.3. The clones with the bold characters (15 clones) were confirmed by QRT-PCR. Those with Yr1- and Yr10- are of the samples produced from Avocet-Yr1 and Avocet-Yr10 differential lines, respectively.

Fragments	bp	Homology	Blast
DD Yr1-1	974	TAK33 [*T. aestivum*] AAK20741.1	3e-134
DD Yr1-2	567	csAtPR5 [*A. tauschii*] AAM81209.1	5e-12
DD Yr1-3	808	Elongation factor 1-alpha 1; EF-1-alpha1 [*Lilium longiflorum*] AAD27590.1	e-113
DD Yr1-5	546	Dihydroflavonol 4-reductase [*Perilla frutescens*] BAA19658.1	0.079
DD Yr1-6	394	Unknown protein [*O. sativa* (japonica cultivar-group)] XP_477264.1	9e-68
DD Yr1-8	486	Leucine rich repeat containing protein kinase [*O. sativa*] AAF34426.1	2e-34
DD Yr1-9	459	Peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein [*A. thaliana*] NP_187319.1	8e-07
DD Yr1-10	540	UBX domain-containing protein [*A. thaliana*] NP_192817.1	1e-28
DD Yr1-11	617	F-box family protein / LOV kelch protein 1 (LKP1) [*A. thaliana*] NP_568855.1	4e-62
DD3 Yr1-50	219	*T. aestivum* PST19 (Pst19), LRR19 (Lrr19), TAK19-1 (Tak19-1), and LRK19	4e-115

		(Lrk19) genes AF325196	
DD3 Yr1-74	268	28 S ribosomal RNA gene AY049041.1	e-108
DD Yr10-1	459	OsRad6 [*O. sativa* (japonica cultivar-group)] BAC79758.1	5e-78
DD Yr10-2	618	F-box family protein / LOV kelch protein 1 (LKP1) [*A. thaliana*] NP_568855.1	7e-56
DD Yr10-4	624	Hypothetical protein MG04541.4 [M. grisea 70-15] EAA50782.1	5e-06
DD Yr10-5A	560	Putative syntaxin of plants 41 [*O. sativa* (japonica cultivar-group)]XP_550516.1|	2e-07
DD Yr10-5B	520	NAD-dep. epimerase/dehydratase family protein [*A. thaliana*] NP_177978.1	1e-82
DD Yr10-6	361	Putative disease resistance protein [*A. thaliana*] BAC41834.1	4.8
DD Yr10-8	345	yr10-8 Rust resistance protein AAF34426.1	9.2
DD3 Yr10-36	204	Putative Mla1 [*O.sativa* (japonica cultivar-group)] NP_917546.1	3e-16
DD3 YR10-21	489	Calcium-dependent protein kinase [*A. thaliana*] BAA04830.1	4e-32
DD3 Yr10-31	355	*T.aestivum* (Chinese spring) chi gene for endochitinase X76041.1	3e-17
DD3 Yr10-37	394	csAtPR5 [A. tauschii] AAM81209.1	2e-18
DD3 Yr10-48	339	calmodulin binding [*A. thaliana*] NP_850399.1|	2.0
DD3 Yr10-49	391	hypothetical protein [*O. sativa*] BAD87844.1|	4.4
DD3 Yr10-55	239	Photosystem I reaction center subunit IV, chloroplast precursor (PSI-E) (Photosystem I 10.8 kDa polypeptide PSAE HORVU P13194	2e-05
DD3 Yr10-73	200	*T. aestivum* clone wlsu2.pk0001.h3:fis, full insert mRNA sequence (gb|BT009458.1| (Rab geranylgeranyltransferase, beta subunit)	2e-08
DD3 Yr10-85	243	No-hit	-
DD3 Yr10-87	346	Hypothetical protein [*O. sativa* (japonica cultivar-group)] XP_480023.1	0.043
DD3 Yr10-88	253	*H. vulgare* pot. psaE mRNA HVPSA2	4e-74
DD3 Yr10-90	383	*H. vulgare* part. mRNA for α-tubulin 5 (atub5 gene) AJ276013.1 HVU276013	2e-30
DDYr10-9	465	Arginine tRNA synthetase (AAT0765.1)	5e-61
DD3 Yr10-50a	887	No-hit	-
DD3Yr0-26	200	No-hit	-

3.7 Confirmation of the results obtained from DD analysis

In order to identify the false positive results from DD analysis, differential expression of the fragments identified were further analyzed using QRT-PCR method

3.7.1 QRT-PCR analysis

Currently, some housekeeping genes are described for the normalization of expression signals. The most common ones are actin, glyceraldehyde-3-phosphate

dehydrogenase, ribosomal RNA genes, ubiquitin, cyclophilin, and elongation factor 1-α (ef1α) (Stürzenbaum *et.al*. 2001**;** Bezier *et.al*. 2002). We used Actin-1 gene and 18S rRNA for normalizations. Since, in most of the similar studies (Bezier *et.al*. 2002; Brunner *et.al*. 2004), *Actin-1* gene expression levels were used for normalization, we have used *Actin-1*, too. All QRT PCR analysis was performed for both avocet Yr-1 and Yr-10 plants. Examples to QRT-PCR confirmations can be seen in Figures 3.7 to 3.11. Relative expression levels of the genes induced upon avirulent infections on both plant lines can be seen in figure 3.12.

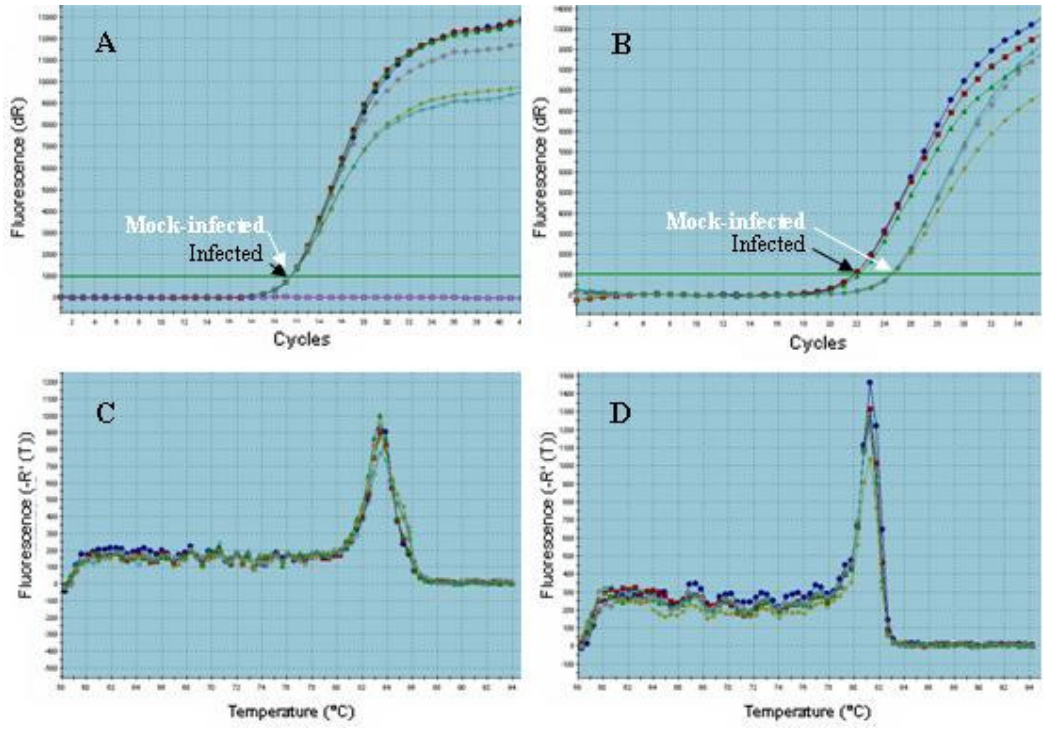

Figure 3.7 Real-time PCR (QRT-PCR) profiles for clone DDYr1-8. The infections were performed using 169E136 pathogen race on Avocet-Yr10 seedlings. RNA samples are from 48th hour of post infection. **A**: Normalization of mRNA levels using *Actin-1* gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for the LRR clone (DDYr1-8) target between the infected and mock-infected samples, indicating 2.49 cycles

corresponding to 4.95 fold expression level difference. **C**: Disassociation curve for *Actin-1* gene amplification **D**: Disassociation curve for LRR clone (DDYr1-8) target.

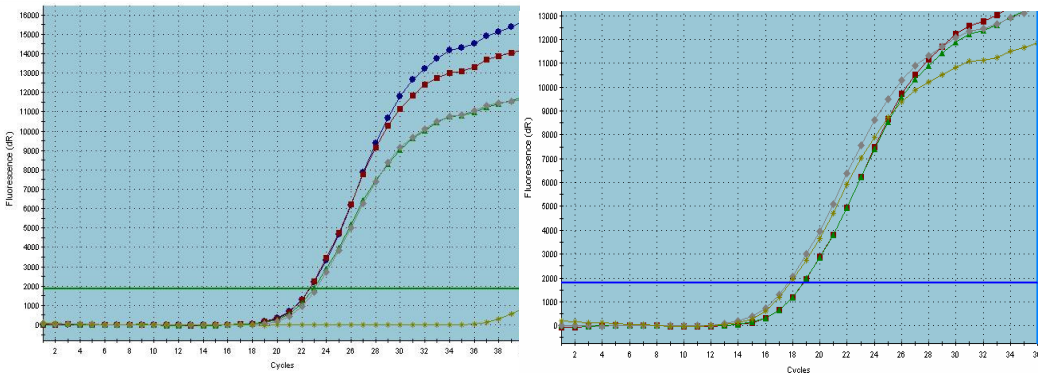

Figure 3.8 Real-time PCR (QRT-PCR) profiles for clone DDYr10-1. The infections were performed using 169E136 pathogen race on Avocet-Yr10 seedlings. RNA samples are from 48th hour of post infection. **A**: Normalization of mRNA levels using *Actin-1* gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for the RAD6 clone (DDYr10-1) target between the infected and mock-infected samples, indicating 0.9 cycles corresponding to 1.8 fold expression level difference.

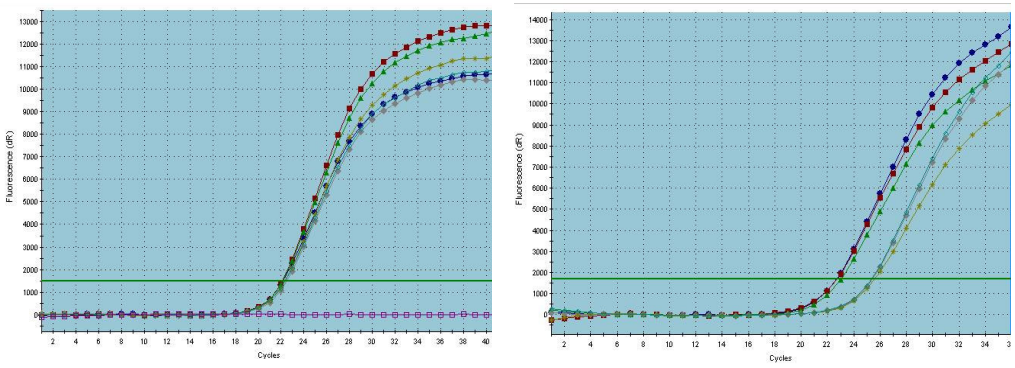

Figure 3.9 Real-time PCR (QRT-PCR) profiles for clone DDYr1-10. The infections were performed using 169E136 pathogen race on Avocet-Yr10 seedlings. **A**: Normalization of mRNA levels using *Actin-1* gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for

the UBX clone (DDYr1-10) target between the infected and mock-infected samples, indicating 1.5 cycles corresponding to 2.61 fold expression level difference.

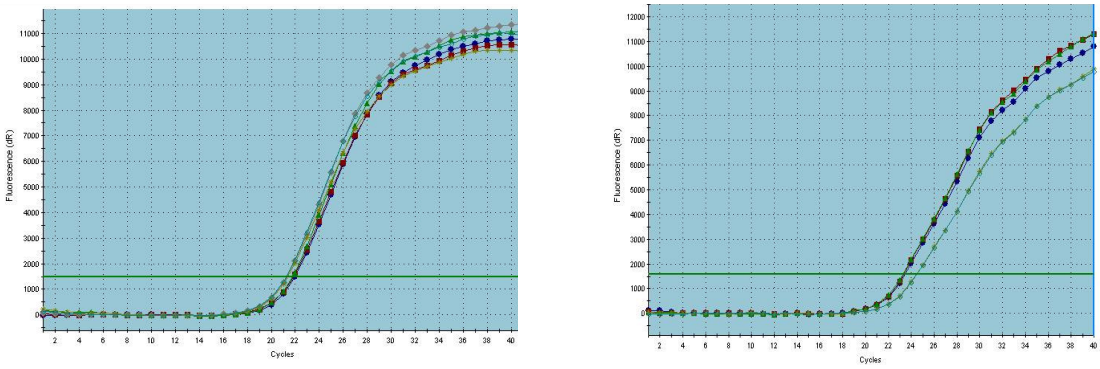

Figure 3.10 Real-time PCR (QRT-PCR) profiles for clone DD Yr1-9. The infections were performed using 232E137 pathogen race on Avocet-Yr1 seedlings. **A**: Normalization of mRNA levels using *Actin-1* gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for the Cyclophilin clone (DD Yr1-9) target between the infected and mock-infected samples, indicating 1 cycle corresponding to 1.9 fold expression level difference.

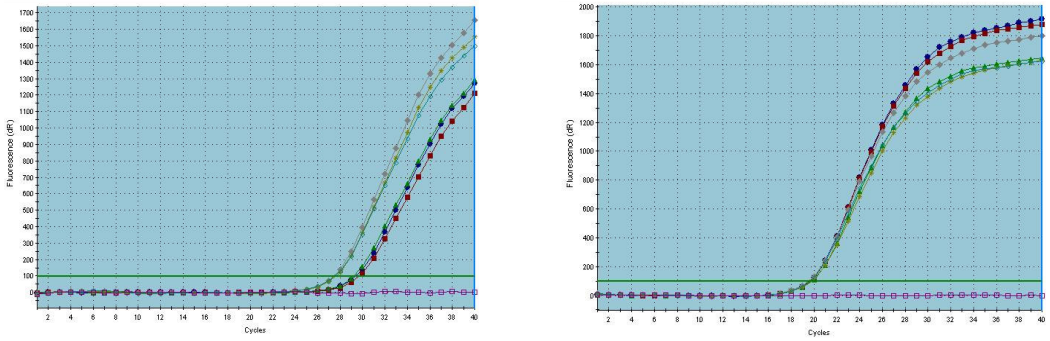

Figure 3.11 Real-time PCR (QRT-PCR) profiles for clone DD3 Yr10-37. The infections were performed using 169E136 pathogen race on Avocet-Yr10 seedlings. **A**: Normalization of mRNA levels using *Actin-1* gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for

the Pr5 clone (DD3 Yr10-37) target between the infected and mock-infected samples, indicating 1.2 cycles corresponding to 2.16 fold expression level difference.

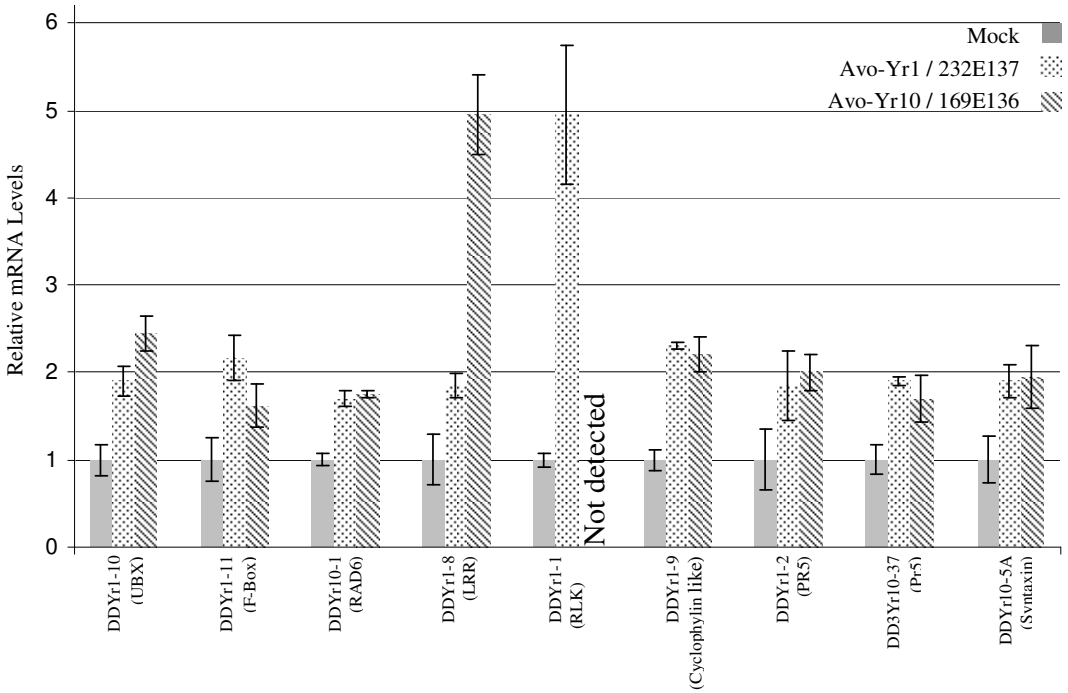

Figure 3.12 Relative expression levels of the genes induced upon avirulent infections on both plant lines. The experiments were performed 3 times for each clone and error bars calculated. The error bars are result of differences among three measurements on the identical samples. DDYr1-1 clone data on the 169E136 infection of Avocer Yr10 plant was not obtained because there was no change on the expression level of this gene fragment when compared to the mock infection (Table 3.2)

3.8 Differentially expressed genes

All of 14 the genes, except two, DD3Yr10-85 and DD3Yr10-73, were detected with no changes in expression levels on the control compatible infections, in other words when infection is causing disease formation (Table 3). They only gave PCR amplifications in infected samples not in the mock inoculated samples.

Thus, suggesting that they are likely to be involved only in the "gene for gene" mediated yellow rust disease resistance mechanism.

The first set of three clones DDYr1-11, DDYr1-10 and DDYr10-1, were found to be involved in ubiquitin mediated protein degradation and controlled cell death, F-Box, UBX, and RAD6, respectively. Although, they are all up regulated, there are differences in the induction levels on different genotypes; this may represent the varying changes of infection efficiency and responses as represented in Figure 3.12. Second set of three clones having homologies to the domains present in disease resistance related genes, namely are DDYr1-8, DDYr1-1, DD3Yr10-36, which are LRR containing protein (*O. sativa*), Receptor Like Kinase (RLK) (*T. aestivum*), and NB-LRR-Mla like (*O. sativa*), respectively. The clone DDYr1-1 (RLK) induction was only detected in incompatible interaction between 232E137 and Avocet-Yr1 (Figure 4). However, this gene was found not to be induced on Avocet-Yr10. Since, it was originally cloned from Avocet-Yr1 line; it is possible to detect unchanged expression level in another genotype background. Therefore, highly induced (5 fold) putative RLK may be a specifically involved gene in the R-gene mediated resistance in Avocet-Yr1. The clones DDYr1-2 and DD3Yr10-37 showed different levels of homologies to the different parts of the same protein, *Arabidopsis* PR5 protein. Therefore, it may be likely that they are fragments of Pr5 or they belong to PR family genes. DDYr10-5A, Syntaxin, clone is involved in vesicle trafficking of non-host and host resistance in plants. DDYr1-9 is a cyclophilin type putative anti-fungal protein encoding gene. Of the clones listed, DD3Yr1-74 (28S rRNA) was confirmed to be silenced in incompatible interaction of Avocet-Yr10; however similar outcome was not observed in the Avocet-Yr1 line. Thus, the resistance mechanism may be varying in these two plants. It was shown that ribosomal RNA was a target for pro-apoptotic proteins during apoptosis initiated by death receptor engagement (Nadano and Sato, 2000) in human cells in which, the degradation of 28S rRNA was simultaneous with protein synthesis inhibition. Transcripts of the clones DD3Yr10-85 and DD3Yr10-73 (unknown, no hit, respectively) were only detected in incompatible Avocet-Yr10 infections. DD3Yr10-87 clone has a homology to an unknown protein; also its expression is almost two fold when compared to mock inoculated samples.

Table 3.2 Real-time RT-PCR analyzed differentially expressed genes upon virulent and avirulent infections. Fold changes indicate the expression level difference between the infected plant and its mock infected sample. ∞: Expression was observed only in infected samples, not in mock infections. **+**:up regulated, **-**:down regulated. **NC**: no change; there is no expression level difference compared to mock inoculated controls. **NP**: No PCR product obtained in both infected and mock infected samples. All of the QRT-PCR amplifications were performed on the samples of 48 hours after infections or mock infections.

			Expression level fold changes			
			Avirulent infections Expression levels were compared to Mock infections		Virulent infections Expression levels compared were to Mock infections	
DD clones	**Homologies to**	**Accession numbers**	**Avocet-Yr1** 232E137	**Avocet-Yr10** 169E136	**Avocet-Yr1** 169E136	**Avocet-Yr10** brown rust
DD Yr1-10	UBX	(NP_192817.1)	+1.90	+2.61	NC	NC
DD Yr1-11	F-box	(NP_568855.1)	+2.17	+1.62	NC	NC
DD Yr10-1	RAD6	(BAC79758.1)	+1.85	+1.80	NC	NC
DD Yr1-8	LRR	(AAF34426.1)	+1.85	+4.95	NC	NC
DD Yr1-1	RLK	(AAK20741.1)	+4.95	NC	NC	NC
DD3 Yr10-36	MLA like	(NP_917546.1)	NC	∞	NC	NC
DD Yr1-2	Pr5	(AAM81209.1)	+2.30	+2.20	NC	NC
DD3 Yr10-37	Pr5	(AAM81209.1)	+1.85	+2.16	NC	NC
DD Yr10-5A	Syntaxin	(NP_850519.1)	+1.90	+1.7	NC	NC
DD Yr1-9	Cyclophilin like	(NP_187319.1)	+1.90	+1.95	NC	NC
DD3 Yr1-74	28S rRNA	(AY049041.1)	NC	-10.0	NC	NC
DD3 Yr10-85	No hit	-	NP	∞	NP	NP
DD3 Yr10-73	Unknown clone	(BT009458.1)	NP	∞	NP	NP
DD3 Yr10-87	Unknown prot.	(XP_480023.1)	+1.90	NC	NC	NC

3.9 Microarray results

In order to identify the mechanisms involved against pathogen attack and determine the changes in the gene expression during the pathogen invasion on the susceptible plant, we have designed a microarray experiment by using currently released Affymetrix wheat chip wich has 60,000 tags on it. We have used the wheat line "Avocet Yr1" which carries the Yr1 disease resistance gene for our experiments. We have done three different inoculations, first was inoculations of the *Puccinia striiformis* (yellow rust pathogen) 169E136 strain which causes disease on Avocet Yr1 plant, and second was inoculations of 232E137 strain which causes a hypersensitive response and activates the defense responses of the Avocet Yr1 plant. The third inoculation was mock inoculation performed using talc powder without spores. We have used 3 biological replicates for each inoculations corresponding to 9 wheat microarray chips in total. By this way we aimed to identify the genes up regulated or down regulated during the resistance and susceptibility mechanisms.

Because microarray is an expansive method, before going on array hybridization, integrity of all the RNAs and cDNAs were checked by fast electrophoresis Bioanalyser 2100. Electroperograms are presented in Figure 3.13 for RNA isolations and 3.14 for cDNA construction.

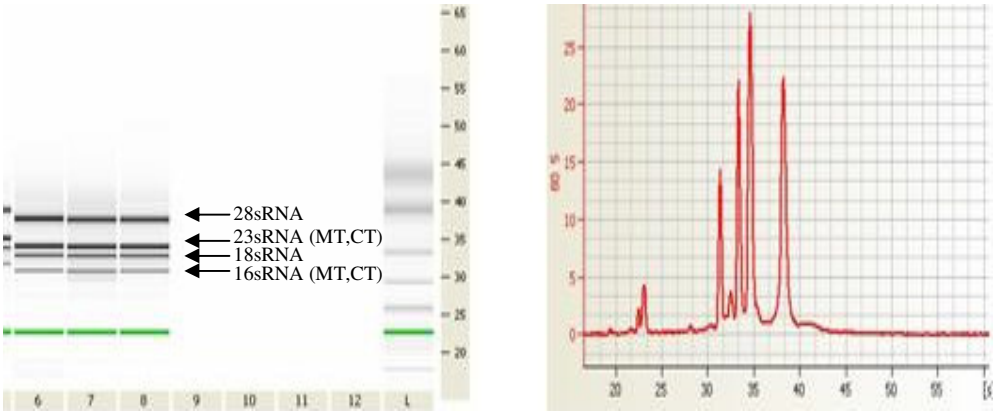

Figure 3.13 Total RNA samples loaded on Agilent Bioanalyzer. The bands on the gel reperesent the 28S and 18S genomic ribosomal RNAs and chloroplast ribosomal RNAs. The peaks on the electropherogram represent all the ribosomal RNAs on the gel with ratios approaching 2:1 for the 28S and 18 S bands.

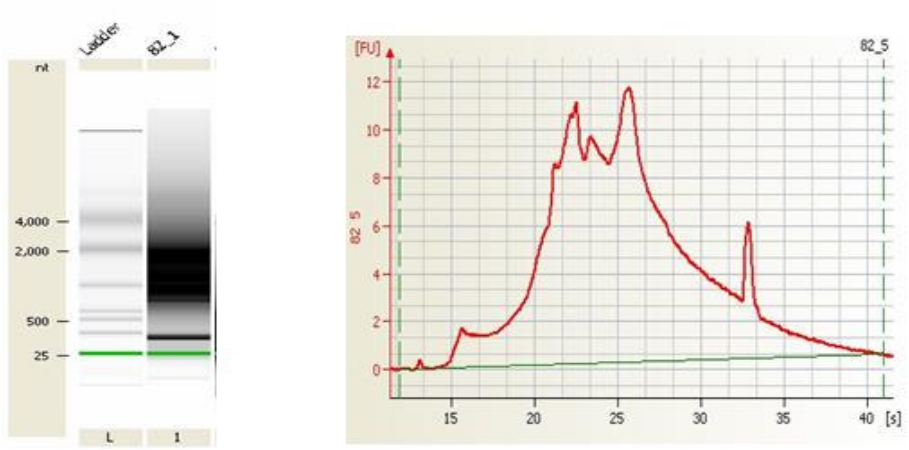

Figure 3.14 cDNA profile on Agilent Bioanalyser. The smear on the gel indicates the successful transcription of the ds cleaned up cDNAs. The peaks on the graph also confirm the success of transcription. The electroperogram displays the nucleotide size distribution for 400 ng of labeled cDNA resulting from one round of amplification.

3.10 Microaray data analysis

The data collected from the microarray experiment was analyzed using GeneSpring program of Agilent. In Figure 3.15 is the normalization and quality conrol of the collected data is presented. Scatter plot graph of 169E136 infected Avocet-Yr1 sample can be seen in figure 3.16. All the array hybridizations seem to be uniform and consistent with each other. Thus, there is no requirement for further normalizations. For the initial filtering of the genes, raw signal >50 were chosen. The signals below this threshold are not very reliable and may give false results. Genes below that threshold were discarded.

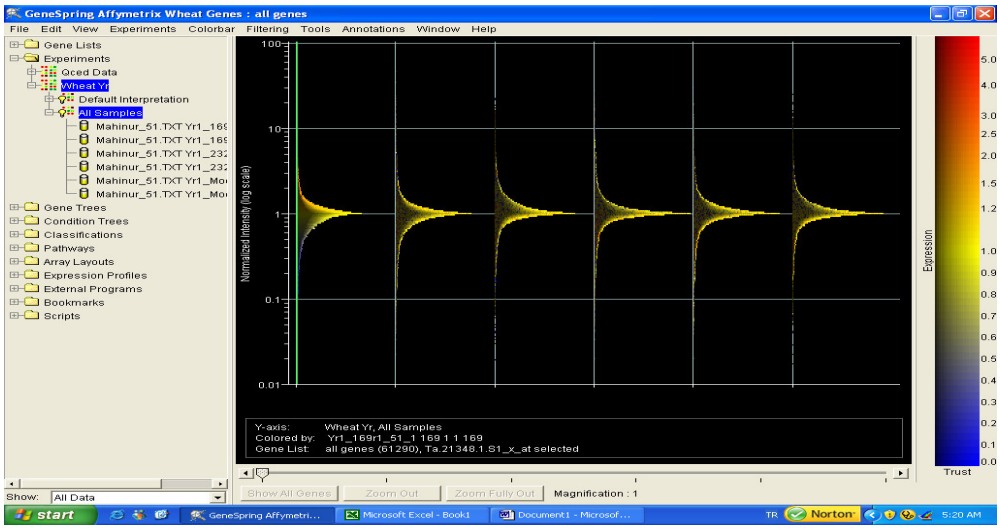

Figure 3.15 Expression level distributions after initial normalizations. Samples lined as virulent infection (169E136), avirulent infection (232E137), mock infection of YR1 plants, and other 3 are different biological replicates.

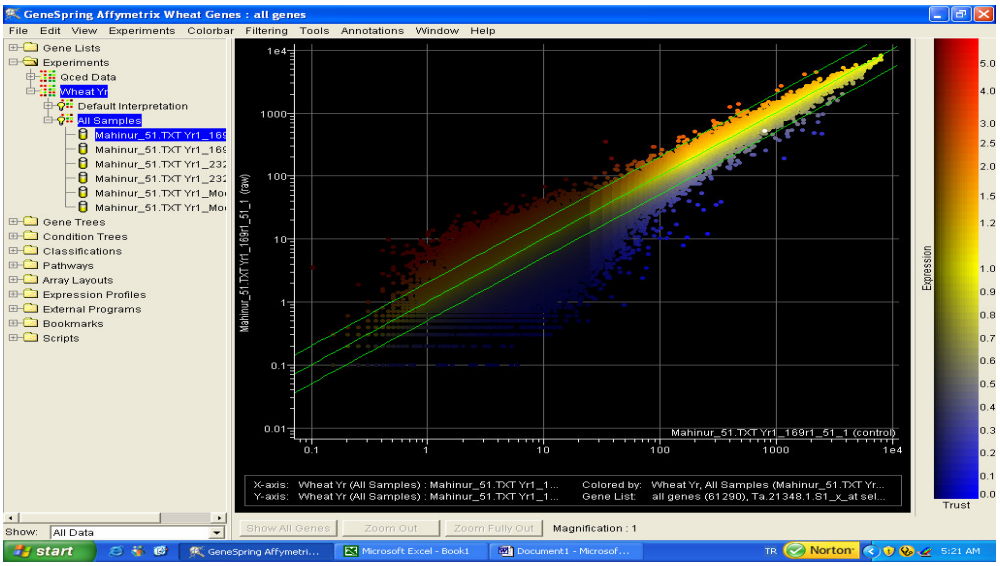

Figure 3.16 Scatter plot graph of the hybridization performed using the probe obtained from 169E136 infected Avocet Yr1 cDNA. Majority of genes are scattered trough out median as expected.

Analyses were performed for the tags that give reliable signal and which are 2 fold greater or lower than the control samples. Analyses were performed using filters as indicated in Table 3.3. Genes differentially regulated in virulent infections were filtered for only those genes which have greater than and less than 2 fold signals when compared to both mock and avirulent infections, respectively. Same filtering is also applied for avirulent infections.

Table 3.3 Gene filtering used in microarray data analysis.

Infection type	Filters used for data analysis (>2X)	Filters used for data analysis (<2X)
169E136 (virulent)	232E137 (virulent) + Mock	232E137 (virulent) + Mock
232E137 (virulent)	169E137 (virulent) + Mock	169E137 (virulent) + Mock
Mock	169E137 (virulent) + 232E137 (virulent)	169E137 (virulent) + 232E137 (virulent)

We have found that 93 tags differentially expressed during the avirulent infections. 47 of them were up regulated and 46 of them are down regulated. Among those 24 of them are related to previously identified genes that are involved in plant disease resistance mechanisms and some were confirmed to be differentially regulated by other researchers. 26 of the tags in avirulent infections were found to be undefined ESTs.

Total number of 75 was found to be differentially regulated during virulent infections. There are 12 of up regulated and 63 of down regulated tags in virulent infections. 41 of them are undefined (unknown genes) tags.

We have also found that there are differentially expressed tags in both virulent and avirulent infections. Those tags are possibly representing the genes that are differentially regulated due to direct effect of the pathogen irresponsible of the reistance or susceptibility of the plant. Differential expression profiles and identities of those tags are presented in Table 3.5 and 3.6. Tags that are differentially expressed in avirulent and virulent infections can be seen in Table 3.3 and 3.4. A simple example of distribution of the genes which were expressed in virulent infected (232E137) samples that were filtered to be 2 fold greater than the genes expressed in mock infected and virulent (169E136) infected samples can be observed in Figure 3.17. Relative expression levels of Sterol desaturase gene and an unknown EST in both samples can be seen in Figure 3.18 and 3.19.

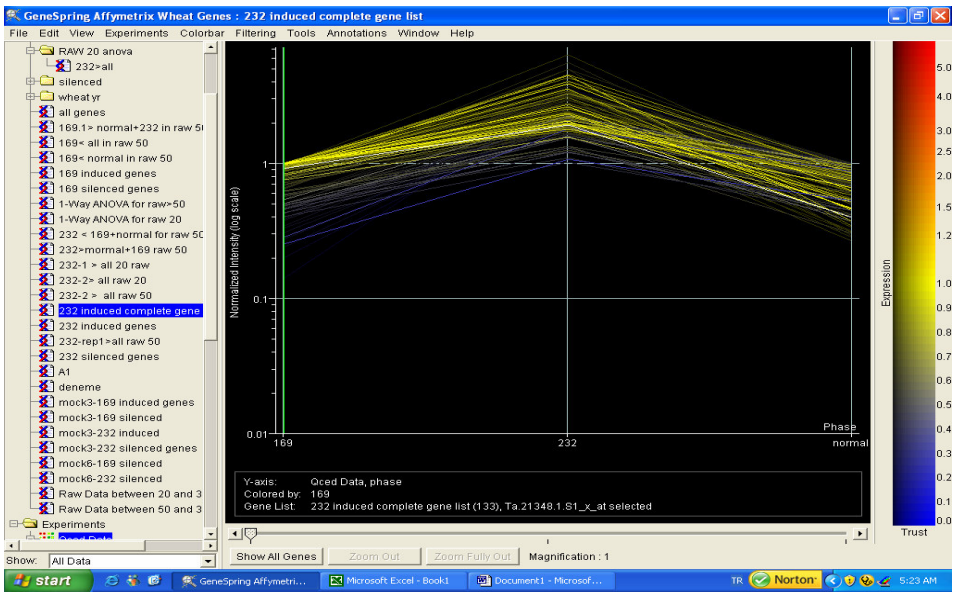

Figure 3.17 Filtered genes that are greater than 2 fold in 232E137 infected Avocet Yr1 plant sample when compared to uninfected and 169E136 infected Avocet Yr1 plant samples.

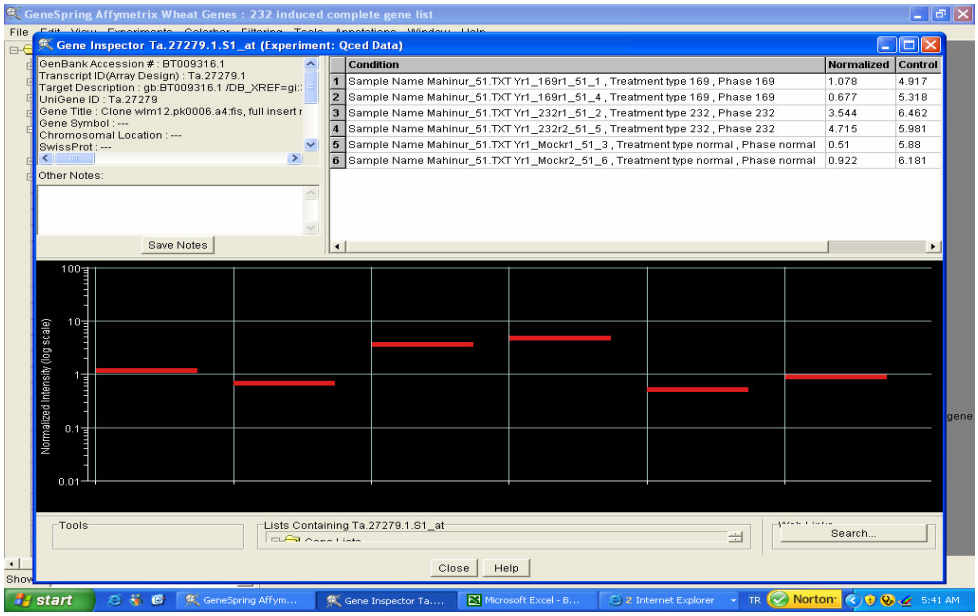

Figure 3.18 Sterol desaturase gene expression levels in wheat samples 169E136 infected (first two samples) 232E137 induced (mid 2 samples) and mock infected (last 2 samples).

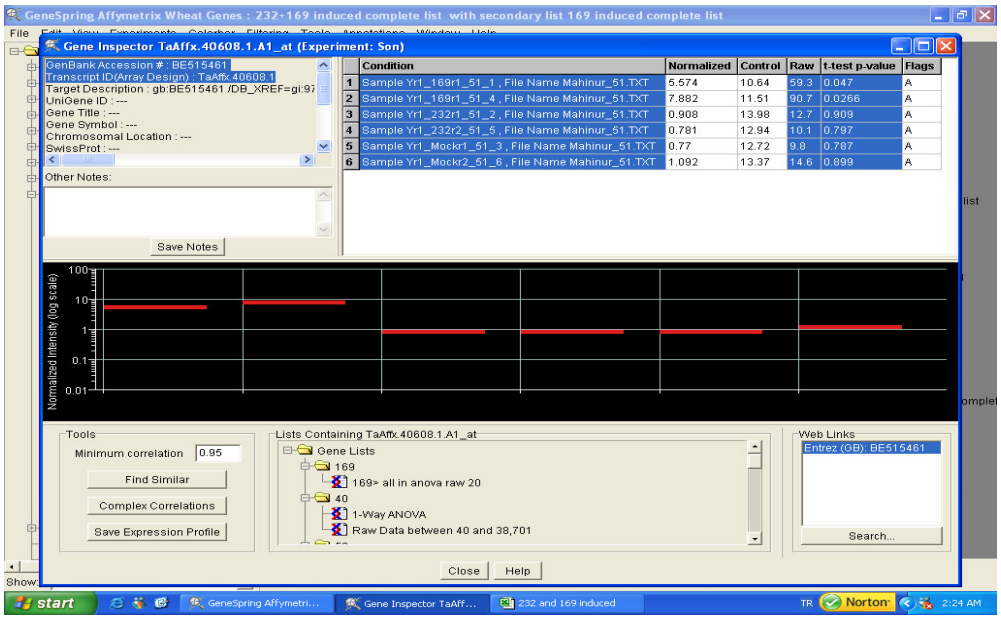

Figure 3.19 Unknown EST (BE515461) expression level in wheat samples 169E136 infected (first two samples) 232E137 induced (mid 2 samples) and mock infected (last 2 samples).

Table 3.4 Differential expression levels and their Homologous sequences found in GenBank, using Blast algorithm v2.2.3 in avirulent infected (232E137) Avocet Yr1 plants. ↑ indicates upregulation while ↓ indicates down regulation

Affy Tag	EST	Blast	Expressional fold changes	
Metabolism and Energy				
Ta.8447.1	CA669038	putative cytochrome P450 monooxygenase [Oryza sativa] BAD53446	2.80	↑
Ta.28.1	Y18212.1	beta-1.3-endoglucanase [Triticum aestivum] Y18212.1	7.60	↑

TaAffx.78404.1	CA733083	copper amine oxidase-like protein [Oryza sativa] XM_471486.1	2.86	↑
TaAffx.59356.1	CA602401	weak smilarity to NADH dehydrogenase [Boehmeria nivea] AF500382.1	2.13	↑
TaAffx.107979.1	CA692409	putative cytochrome P450 [Oryza sativa] AC108875.2	2.96	↑
TaAffx.104739.1	CA745456	CoA -3-o methyl transferase [Arachis hypogaea] AY725194	2.65	↑
Ta.15072.1	CK216153	glycosyltransferase [Triticum aestivum] AJ969052.1	3.75	↑
Ta.447.3	BE213575	putative fructose-bisphosphate aldolase [Oryza sativa] AP004279.1	2.00	↑
TaAffx.15327.1	AJ610775	glucan endo-1.3-beta-D-glucosidase [Triticum aestivum] Y18212.1	11.7	↑
TaAffx.110196.1	CA698011	beta-1.3-glucanase precursor [Triticum aestivum] AF112965.1	2.70	↑
Ta.21354.1	CA673898	Triticum aestivum beta-1.3-glucanase precursor (Glb3) mRNA AF112965	5.20	↑
TaAffx.110196.1	CA698011	beta-1.3-glucanase precursor [Triticum aestivum] AF112965.1	2.70	↑
Ta.23031.1	CA629143	putative heme-binding cytochrome P450 [Artemisia annua] DQ370065.1	4.10	↓
Ta.4274.1	BJ266714	Bambusa oldhamii sucrose synthase mRNA AF412037	5.20	↓
Ta.10088.1	CK213077	putative Riboflavin biosynthesis protein ribF [Oryza sativa] NM_195506.1	2.40	↓
Ta.10849.1	BQ166690	putative lipase [Oryza sativa] AY194234.1	2.40	↓
Ta.18225.1	BQ905539	acetohydroxyacid synthase 1 [Helianthus annuus] AY541453.1	4.50	↓
Signal Transduction				
Ta.10746.1	BQ166746	plasmodesmal receptor [Oryza sativa] XM_466392.1	3.90	↑
Ta.1357.2	CA689677	putative protein kinase [Oryza sativa] XM_466505.1	2.82	↑
TaAffx.50893.1	CA733686	serine/threonine protein kinase [Triticum aestivum]AY924304.1	2.70	↑
Transport chain				
Ta.29546.1	BE213430	Putative chlorophyll a/b-binding protein [Triticum aestivum] M10144	2.53	↑
Ta.23348.1	BJ229076	putative chloroplast nucleoid DNA b.pro [Oryza sativa] XM_467513.1	4.36	↑
Ta.28136.1	CK160740	photosystem I P700 apoprotein A1 [Anthoceros punctatus] AB013664.1	3.80	↓
Defense Related				
Ta.22619.1	CA687670	pathogenesis-related protein 10 [Hordeum vulgare] AY220734.1	8.96	↑
Ta.278.1	AF384143.1	Pr-1 [Hordeum vulgare] X74939	17.50	↑
Ta.12127.1	AJ611109	putative protein kinase Xa21[Oryza sativa] XM_466740.1	3.20	↑
TaAffx.82859.1	CA698593	wheat WIR1A [Triticum aestivum] Q01482	3.87	↑
TaAffx.52897.1	CA678785	Avena sativa clone OP14 receptor kinase gene cluster AY083681	2.00	↑
Ta.97.1	M94959.1	WIR1 mRNA [Triticum aestivum] Q01482	4.46	↑
TaAffx.108556.1	CA692789	pathogenesis-related protein 4 [Triticum monococcum] AY650053.1	3.90	↑
Ta.221.1	AF112963.1	Triticum aestivum chitinase II precursor AF112963.1	2.95	↑
TaAffx.108556.1	CA692789	Triticum monococcum pathogenesis-related protein 4 (PR-4) AY650053	5.89	↑
Ta.23322.3	CA694741	putative antifungal zeamatin-like protein [Oryza sativa] XM_469149.1	3.57	↑
Ta.97.2	CK169277	Wheat WIR1 mRNA M94959	5.45	↑
Ta.15082.1	CA689233	Avena sativa clone OP14 receptor kinase gene cluster AY083681	2.33	↑
Ta.23322.1	CA668995	Hordeum vulgare thaumatin-like protein TLP8 mRNA AF355458	2.60	↑
Ta.21556.1	CA684533	WIR1 mRNA [Triticum aestivum] Q01482	5.20	↑
Ta.5518.1	CA666657	WIR1 [Triticum aestivum] X87686.1	3.90	↑
Ta.21556.1	CA684533	T.aestivum mRNA for WIR1. pathogen defense protein X87686	5.02	↑

Ta.27762.1	AF384146.1	Triticum aestivum thaumatin-like protein mRNA AF384146	7.60	↑
Ta.24501.1	CD863039	T.aestivum mRNA for a thaumatin-like protein X58394	9.34	↑
Ta.62.1	BM136002	Triticum aestivum mRNA for PR-1.1 protein AJ007348	6.52	↑
Ta.13.1	BJ256268	WIR1 [Triticum aestivum] X87686.1	2.50	↑
Ta.21348.2	AY253444.1	sulfur-rich/thionin-like protein mRNA [Triticum aestivum] AY253444	4.82	↑
Ta.21348.1	U32429.1	sulfur-rich/thionin-like protein mRNA Triticum aestivum] AY253444	3.80	↑
Ta.15082.1	CA689233	integral membrane protein containing protein [Oryza sativa] DP000010.2	2.16	↑
Ta.30501.1	CK205943	chitinase II [Hordeum vulgare] AJ276226.1	2.70	↑
Stress Related				
Ta.21281.1	BQ162027	ABC transporter(induced) [Oryza sativa] CAD59574	2.46	↑
Ta.5358.3	CA595165	glutathione peroxidase-like protein [Hordeum vulgare] AJ238697.1	2.20	↑
Ta.9110.1	CK214493	one helix protein [Deschampsia antarctica] AY090544.1	2.70	↑
Ta.5257.3	CA656373	low temperature and salt responsive [Pennisetum glaucum] AY823550.1	3.70	↓
Ta.23376.2	CA603621	putative peroxidase [Oryza sativa] AAT94047	7.30	↓
Ta.22548.1	CA684451	zinc transporter protein ZIP7 [Hordeum vulgare] AM182059.1	2.70	↓
TaAffx.124239.1	BE213663	KED [Nicotiana tabacum] AB009883.1 wound response	3.40	↓
Ta.5557.1	CD869243	germin F [Hordeum vulgare] AF250935.1	2.40	↓
Ta.9599.1	BJ229788	glutathione transferase [Hordeum vulgare] AF430069.1	3.60	↓
Protein Degradation and Ubiquinylation				
TaAffx.81638.1	BE217029	cysteine proteinase [Hordeum vulgare Z97022	5.30	↑
Ta.191.1	U32430.1	Triticum aestivum thiol protease mRNA U32430.1	2.82	↑
TaAffx.64053.1	BJ317396	F-box domain. putative [Oryza sativa] ABA91344	3.00	↓
Transcription and Translation				
TaAffx.22878.1	CA743315	weakly similar to r EF-1a [Branchiostoma floridae] AB070234.1	2.20	↑
Ta.6388.2	BJ265803	putative 40S ribosomal protein S24 [Oryza sativa] AP003613.3	3.90	↓
Ta.28770.3	BJ225774	putative fibrillarin [Oryza sativa] XM_468448.1	2.50	↓
TaAffx.85782.1	CA632147	susceptibility homeodomain transciption factor [Oryza sativa] AF466285.1	5.00	↓
Ta.27657.4	BJ251672	Histone H2A.2.1 [Triticum aestivum] P02276	3.00	↓
Cellular Organization				
Ta.28889.1	CK195001	proline-rich glycoprotein [Chlamydomonas incerta] AY795084.1	4.50	↓
TaAffx.19523.1	BJ210974	Rad1-like protein [Oryza sativa] XM_476292.2	3.90	↓
Ta.2927.1	BJ207394	gibberellin-stimulated protein [Oryza sativa] AY604180.1	4.00	↓
Unknown				
Ta.3133.1	CA669705	unknown	9.17	↑
Ta.20605.1	CA486163	unknown	2.06	↑
TaAffx.86049.1	CA618787	unknown	2.10	↑
Ta.22662.1	CA669059	unknown	3.23	↑
Ta.23340.1	CA669161	unknown	3.20	↑
Ta.23348.3	CA675884	unknown	2.90	↑
Ta.23307.3	CD863703	unknown	3.04	↑
Ta.23340.2	CA673525	unknown	2.70	↑
Ta.30765.1	CN011347	unknown	2.06	↑
Ta.23327.1	CD862505	unknown	3.63	↑

Ta.18022.1	CA627409	unknown	2.15	↑
TaAffx.24481.1	CA718852	unknown	2.30	↑
Ta.8866.1	CA644219	unknown	4.80	↓
TaAffx.84542.1	CA634683	unknown	6.40	↓
Ta.9151.2	CK213201	unknown	4.20	↓
Ta.22802.3	CA631134	unknown	5.00	↓
TaAffx.9233.1	CA606815	unknown	6.60	↓
TaAffx.110060.1	CA598468	unknown	2.80	↓
Ta.453.1	CA608888	unknown	3.50	↓
Ta.14237.1	BQ838879	unknown	2.30	↓
TaAffx.54748.1	CA677488	unknown	3.00	↓
Ta.7398.1	BJ319340	unknown	3.20	↓
Ta.20191.1	CA673329	unknown	3.30	↓
Ta.12102.1	CK166990	unknown	2.60	↓
Ta.8129.1	BQ170051	unknown	4.70	↓
Ta.30144.1	BQ166180	unknown	5.80	↓

Table 3.5 Differential expression levels and their Homologous sequences found in Genbank, using Blast algorithm v2.2.3 in virulent infected (169E136) Avocet Yr1 plants. ↑ indicates upregulation while ↓ indicates down regulation

Affy Tag	**EST**	**BLAST**	***Expressional fold changes***	
Metabolism and Energy				
Ta.29951.1	CD903633	putative pyruvate dehydrogenase [Oryza sativa] AP004027.3	2,30	↑
Ta.17255.1	CA619137	phytochelatin synthetase-like protein 2 [Sorghum bicolor] AY188330.1	4,00	↓
Ta.937.1	BE443499	putative hydrolase [Oryza sativa] AC096687.5	7,50	↓
TaAffx.82999.1	CA663586	nodulin-like-like protein [Triticum monococum] AF326781	2,30	↓
TaAffx.84875.1	CA627620	alpha-hydroxynitrile lyase [Linum usitatissimum] Y09084.1	2,70	↓
Ta.24730.3	CK211162	protochlorophyilide reductase [Triticum aestivum] X76532.1	3,00	↓
Ta.952.1	CK151774	putative peroxidase [Oryza sativa] AP004797.3	3,60	↓
TaAffx.70516.1	CA721880	similar to Triticum aestivum endo-xyloglucan transferase U15964.1	3,00	↓
Signal Transduction				
Ta.30798.3	CK193564	legumain-like protease [Zea mays] AJ131719.1	2,20	↓
Ta.29433.1	AJ611892	Triticum turgidum protein kinase AY494981	2,70	↓
TaAffx.2000.1	CD939052	putative protein phosphatase 2C [Oryza sativa] AP003228.3	2,85	↓
Ta.14422.1	BQ905540	weakly similar to zinc finger family protein-like [Oryza sativa] AP003252.4	2,50	↓
TaAffx.53536.1	CA691435	weakly similar to Putative calcium-binding protein [Oryza sativa] NM_195147.1	3,40	↓
Stress-related				

TaAffx.124239.1	BE213663	KED [Nicotiana tabacum] AB009883.1 wound response	7,40	↓
TaAffx.90055.1	BQ609014	putative extensin [Oryza sativa] AP003761.3	4,80	↓
Ta.6304.2	CA638686	putative Avr9/Cf-9 rapidly elicited protein 231 [Oryza sativa] XP_467694.1	3,80	↓
Ta.22444.1	CA745804	Triticum aestivum Lr21 gene AY139587	2,60	↓
TaAffx.28024.1	CA666313	CBF-like protein [Secale cereale] AF370729.1	3,80	↓
TaAffx.79292.1	CA718677	extensin-like protein [Lycopersicon esculentum] AF159296.1	2,80	↓
Ta.952.1	CK151774	putative peroxidase [Oryza sativa] AP004797.3	3,60	↓
Ta.16517.1	CA607672	H.vulgare ids-1 mRNA X58540.1	2,70	↓
Translation-transcription				
TaAffx.112051.1	CA627432	Zea mays acidic ribosomal protein P1a (rpp1a) mRNA U62752.1	6,70	↓
TaAffx.58310.1	CA620766	40S ribosomal protein S16 [Oryza sativa] P46294	5,10	↓
TaAffx.62829.1	BQ606050	histone 3 [Rheum australe] DQ078122.1	6,00	↓
Protein Degredation				
TaAffx.64053.1	BJ317396	F-box domain, putative [Oryza sativa] ABA91344	5,50	↓
TaAffx.51419.1	CA688917	putative zinc metalloproteinase [Oryza sativa] XM_467714.1	3,40	↓
Ta.17896.1	CA625385	aspartic protease [Oryza sativa] D32165.1	3,70	↓
Ta.8245.2	CA680416	putative proteasome 26S non-ATPase subunit [Oryza sativa] XM_480766.1	2,70	↓
Transport chain				
TaAffx.37833.1	BJ257411	electron carrier/ oxidoreductase [Arabidopsis thaliana] NM_118204.2	2,60	↓
TaAffx.128414.40	BE213418	rib-1,5-bisphs carboxylase/oxygenase [Stenostachys laevis] AY691640.1	2,50	↓
Protein Destination				
TaAffx.58864.1	CA613989	putative peptide transporter [Oryza sativa] NM_197912.1	4,70	↓
Cellular organisation and biogenesis				
Ta.2927.1	BJ207394	gibberellin-stimulated protein [Oryza sativa] AY604180.1	4,30	↓
Unknown				
TaAffx.40608.1	BE515461	unknown	6,00	↑
Ta.9401.1	CA615658	unknown	4,90	↑
TaAffx.12939.1	BJ223852	unknown	2,10	↑
Ta.9140.1	CA656847	unknown (Zea mays heat shock protein HSP82) S59780	5,30	↑
Ta.6744.1	BJ264395	unknown	2,30	↑
TaAffx.119913.1	BJ252419	unknown	4,00	↑
TaAffx.27625.1	CA673092	unknown	2,20	↑
Ta.1866.2	CA675087	unknown	2,30	↑
Ta.5109.1	CA713571	unknown	2,00	↑
Ta.21750.1	CA700361	unknown	2,60	↑
TaAffx.32119.1	CA600549	unknown	3,80	↓
Ta.12925.1	CA486890	unknown	4,60	↓
Ta.30814.1	CN008170	unknown	4,70	↓
Ta.11160.1	BQ169530	unknown	3,80	↓
TaAffx.59111.1	CA607597	unknown	2,80	↓
Ta.21025.1	CA605280	unknown	3,00	↓
Ta.3909.2	CA605883	unknown	2,10	↓
TaAffx.106225.1	CA722935	unknown	2,30	↓

TaAffx.31560.1	CA613122	unknown	9,10	↓
TaAffx.30473.1	CA624972	unknown	3,30	↓
TaAffx.113624.2	CA637683	unknown	3,60	↓
Ta.28370.1	CA728072	unknown	3,30	↓
TaAffx.218.1	AJ610876	unknown	3,50	↓
TaAffx.106801.1	CA711278	unknown	4,40	↓
TaAffx.70742.1	BQ800902	unknown	2,80	↓
TaAffx.113599.1	CA608829	unknown	3,50	↓
TaAffx.109058.1	CA680701	unknown	3,30	↓
TaAffx.114295.1	CK206308	unknown	3,00	↓
TaAffx.24414.1	CA719235	unknown	2,40	↓
TaAffx.132656.3	CA681847	mTERF, putative [Oryza sativa] AC145321.3	2,40	↓
TaAffx.120245.1	BJ234869	unknown	2,40	↓
TaAffx.107477.1	CA699255	unknown	2,80	↓
Ta.15986.1	CA502718	unknown	3,00	↓
TaAffx.138508.1	CA621609	unknown	4,00	↓
Ta.14097.1	BQ807228	unknown	4,10	↓
TaAffx.71168.1	BE492770	unknown	2,50	↓
Ta.3830.3	CA608434	unknown	2,60	↓
Ta.28077.1	BJ254936	unknown	2,80	↓
TaAffx.81381.1	CA686786	unknown	4,60	↓
Ta.3914.1	BE420282	unknown	3,50	↓
TaAffx.85303.1	CA623871	unknown	3,10	↓
TaAffx.6520.1	CA684026	unknown	3,40	↓
TaAffx.80608.1	CA694804	unknown	2,60	↓

Table 3.6 ESTs differenrialy downregulated in both avirulent and virulent infections when compared to mock infections

EST	BLAST	Fold change (virulent infec.)	Fold changes (avirulent infec.)
BE213663	KED [Nicotiana tabacum] AB009883.1	7,40	3,40
BJ317396	F-box domain, putative [Oryza sativa] ABA91344	5,50	3,00
BJ207394	gibberellin-stimulated protein [Oryza sativa] AY604180.1	4,30	4,00

Table 3.7 ESTs differentially upregulated in both avirulent and virulent infections when compared to mock infections

EST	BLAST	Fold changes (virulent infect.)	Fold changes (avirulent infect.)
CA664784	unknown protein [Oryza sativa	5,56	7,55

CA625136	extensin-like protein [Citrus junos	3	6
CA721750	unknown	5,7	5,25
CA610276	putative acetyl transferase [Oryza sativa	6,42	5,92
CA635238	putative uroporphyrinogen decarboxylase [Oryza sativa	3	3,9
CA667670	putative phosphogluconate dehydrogenase	2,5	4,33
CA681450	putative carboxymethylenebutenolidase [Oryza sativa	2,34	2,34

3.11 Confirmation of microarray results using QRT-PCR

Genes identified to be differentially expressed (176) from microarray data were decided to be confirmed using QRT-PCR. For the normalization of realtime results, 18S rRNA was used. Before going on expression comparisons standard curve analysis was performed and efficiency of the PCR amplification for primer pairs was calculated. It has been found that it is nearly 90% for most of the sets. A total number of 21 tags were selected to be confirmed by realtime PCR. 15 of them were confirmed to be differentially regulated. 6 of the primer sets did not give any amplification. This may be due to the presence of ESTs on the chip that belong to other *Triticum* species or bad primer design. Examples to realtime PCR confirmations can be seen in figure 3.20. Table 3.5 and 3.6 show the data collected from all of the realtime experiments.

Table 3.8 Differential expression levels of tags selected to be confirmed by realtime PCR in avirulent (232E137) infections and definitions of tags.

GenBank accession	Description	Avirulent infection (232E137)	
BQ162027	Abc transporter	1.90	↑
BJ3202685	Protein kinase	2.60	↑
BJ266714	Sucrose synthase	3.60	↓
AJ611109	Putative protein kinase Xa21	3.60	↑
Y18212.1	Beta-1.3-endoglucanase	6.20	↑
BJ229788	Glutathione transferase	1.90	↓
BJ265803	Putative 40S ribosomal protein S24	5.00	↓
CA669038	Putative cytochrome P450 monooxygenase	1.70	↑
CA603621	Putative peroxidase	2.00	↑
AB029936.1	Chitinase 3	3,2	↑

Table 3.9 Differential expression levels of tags selected to be confirmed by realtime PCR in virulent (169E136) infections and definitions of tags.

GenBank accession	Description	Virulent infection (169E136)		
BJ3202685	Protein kinase	2.00		↑
CA638686	Putative Avr9/Cf-9 rapidly elicited protein	4,00	↓	
BQ609014	Putative extensin	2.00	↓	
CA656847	Unknown	2.45		↑
CD906333	Putative RAN binding protein	2.00		↑
AB029936.1	Chitinase 3	2.20		↑
CA721880	Endo-xyloglucan transferase	1.80	↓	

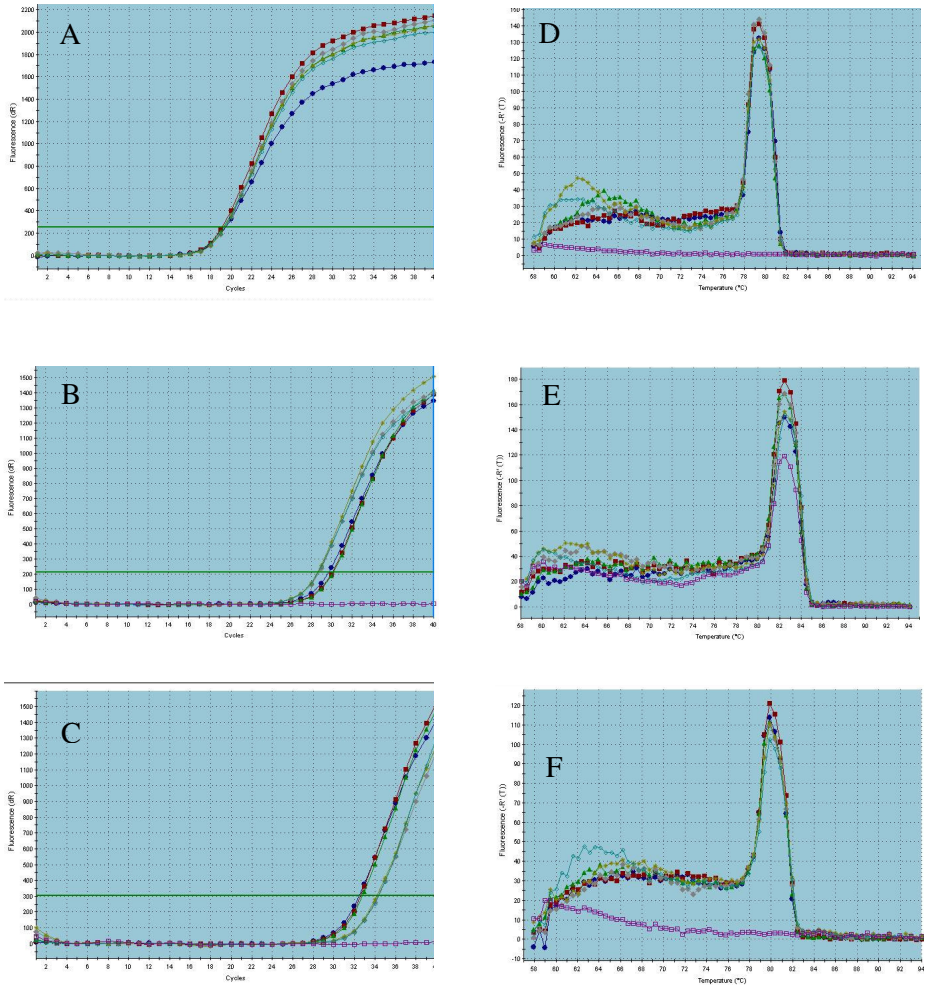

Figure3.20 Realtime PCR results for the confirmation of Microarray results **A**: Normalization of mRNA levels using 18S rRNA gene expression with triplicates for infected and mock-infected samples. **B**: Expression level differences observed for the BJ3202685 target between the infected and mock-infected samples, indicating 1.1 cycles corresponding to 2.0 fold expression level difference **C:** Expression level differences observed for the AJ611109 target between the infected and mock-infected samples, indicating 2 cycles corresponding to 3.6 fold expression level difference **D, E,** and **F** represent the disassociation curves of the amplifications respectively

3.12 Vigs results

BSMV vectors carrying the *PDS* gene fragment in sense orientation which was designed to silence the endogenous wheat *PDS* and *GFP* as a control of viral propagation in host cells were amplified in *E.coli* DH5-α cells successfully and linearized by restriction digestion using appropriate enzymes. Linearized vectors were checked on agarose gel. Vectors were then used for *in vitro* transcription and success of transcription was assessed on the agorose gel. Plants were inoculated as described in materials and methods section. At our first attempt, we have seen that plants got too much mechanical damage on the applied leaves and they were unhealthy. Therefore silencing failed. Next attempts plants were infected gently by applying little pressure. Photo-bleaching started to be appearing on the 8th day of the post infection and was obvious on the 12th day (Figure 3.21-3.23)

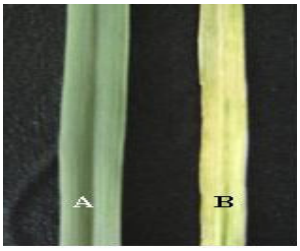

Figure 3.21 A) Mock-inoculated plant leaf **B)** PDS silenced plants 12 dpi

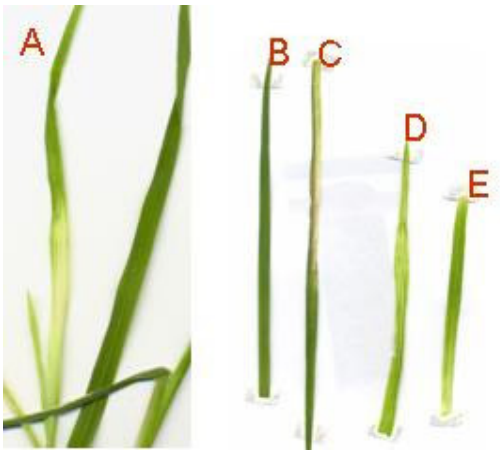

Figure 3.22 A) PDS silenced plant 12 dpi **B)** Mock-inoculated plant leaf **C)**Inoculated 2nd leaf at 12 dpi **D)** Inoculated 4th leaf at 12 dpi **E)** Inoculated 3rd leaf at 12 dpi

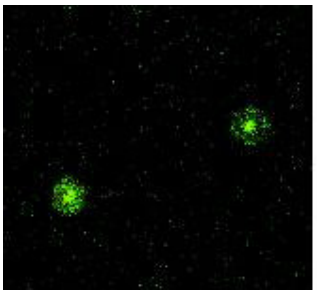

Figure 3.23. GFP expression. GFP expression was observed by flourescence microscopy in infected and newly growing leaves after 6-7 days of infection**.**

3.13 QRT-PCR analyses for determination of PDS silencing at molecular level

For QRT-PCR confirmations, primers that amplified short region of Actin or region of *PDS* excluded from BSMV vector were used. The relative quantification of the genes was calculated according to Pfaffl MW (2001). Realtime PCR analysis of *PDS* gene is presented in Figure 3.24.

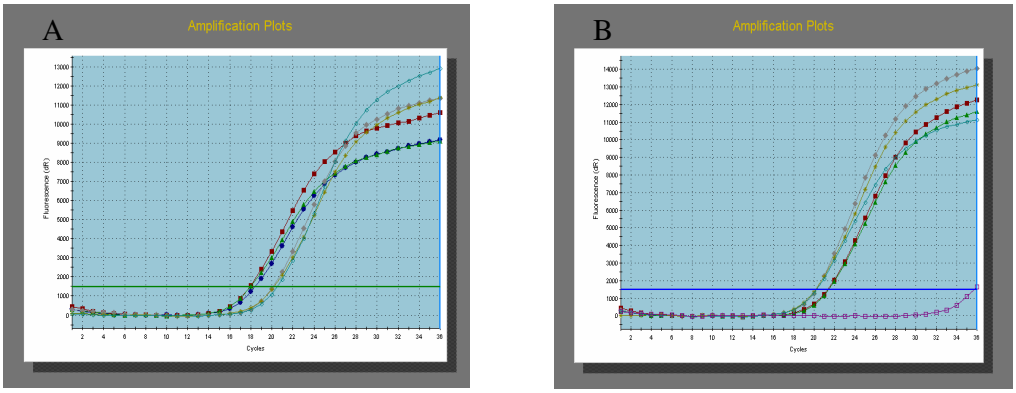

Figure 3.24 **A)** Using actin primer PDS silenced and control plant cDNA levels were normalized **B)** when the cDNA levels were normalized by checking the actin gene expression, PDS silencing was found to be 10 fold (3.6 cycles of CT difference). Note: reactions repeated three times for each primer set.

3.14 Discussion

3.14.1 Differential display

The genes we identified allowed us to speculate on the possible roles in the R-mediated resistance mechanism based on the available literature information as presented here.

DDYr10-1 clone shows very high homology to RAD6 ubiquitin-conjugating enzyme (E2) of *Oryza sativa* at the protein level (98%). Dinesh-Kumar's group (Liu *et.al*. 2002) showed the importance of SCF-type E3 ligases (ubiquitin ligases) in pathogen responses. They used combination of genetic screens and gene-silencing technologies and observed that *N. benthamina* plants with reduced amounts of SKP1 are compromised for *R* gene-mediated resistance to tobacco mosaic virus (TMV). Additionally, it is known that R proteins in plants require either RAR1 or SGT1 proteins or both in order to be functional. Previously, RAR1 and SGT1 were reported to be interacting with 26S proteasome subunits. RAR1 was found to be interacting with COP9 signalosome which acts as a lid for proteasome complex, whereas SGT1 was found to be interacting with SKP1 which is involved in E3 complex. Selection of target protein to be ubiquitinylated is provided cooperatively by E2 in conjunction with an appropriate E3 (Chen and Pickart, 1990). Previously, SGT1 playing an important role in the plant disease resistance was reported to be interacting with proteins in the E3 enzyme complex. Additionally, in a paper of its context unrelated to the plant defense, Sakaguchi's group recently reported that SGT1 also interacts with RAD6 which is an E2 enzyme. They also showed that RAD6 mRNA level is induced with H_2O_2 treatment. H_2O_2 is also accumulated during the HR response of plant defense. (Yamamoto *et.al*. 2004). All these findings strengthen the putative role of our clone DDYr10-1, RAD6 encoding gene, in plant defense response. Although, it was known that RAD6 was involved in repair, post-replication repair (with Rad18p), DNA damage (with Ubr1p) (Jentsch *et.al*. 1987), sporulation, telomere silencing, and ubiquitin-mediated N-end rule protein degradation, its role in plant defense mechanism was not reported previously. Our

result showing that RAD6 is induced upon incompatible pathogen attack indicates that it maybe the E2 involved in ubiquitin mediated protein degradation in disease resistance mechanism. Based on the recent reports of antagonistic roles of SGT1 and RAR1 in regulation of R protein levels in plants in which SGT1 functions to negatively regulate R protein accumulation, while RAR1 functions to positively control R protein accumulation (Bieri *et.al*. 2004; Holt et. al. 2005), and the fact that SGT1 also has a role in the regulation of programmed cell death during infection in a RAR1-independent manner (Holt *et.al*. 2005), the SGT1 interacting RAD6 protein (Yamamoto *et.al*. 2004) may also have a role in the regulation of R protein level. Unlike polyubiquitinylation in which the targeted proteins are degraded, monoubiquitinylation, in which the target proteins are activated or stabilized, is also achieved by involvement of E2 enzymes. It was previously reported that RAD6 functions to monoubiquitinylate histones. Therefore, monoubiquitinylation is another function of Rad6. In *Phaseolus vulgaris*, it was shown that upon pathogen bacterial and fungal infections, Actin-1 is monoubiqutinylated and this induction was not observed, when virus or other stresses are applied (Dantan-Gonzalez *et.al*. 2001). However, H_2O_2, which is an inducer of Rad6 gene expression, also induces monoubiquitinylation of Actin-1 protein. Thus, it is possible that RAD6 may have a role in Actin-1 monoubiquitinylation during pathogen attack.

One of our DD clones, DDYR1-11, shows high (84%) homology to the F-box LOV kelch protein 1 (LKP1) of *Arabidopsis* at the protein level. Previously, 700 F-Box motif encoding genes in *Arabidopsis* genome have been identified (Gagne *et.al*.; 2001, Weissman 2001). Wide array of F-box encoding genes suggests that F-box proteins have different functions and targeting distinct types of proteins or substrates. Therefore, it is important to identify unique F-box proteins playing specific roles in plant disease resistance. Two F-box motif containing proteins in plants, COL1 and SON1, were previously demonstrated to be involved in plant defense. “Coronatine insensitive1” (COI1) protein controls response signaling molecules, jasmonic acid (JA), that regulate defense pathways (Xie *et.al*. 1998). The *coi1* mutant plants can not express the JA inducible gene PLANT DEFENSIN1.2 (PDF1.2) and are susceptible to insect herbivores, to fungal and bacterial pathogens

(Thomma *et.al*., 1998). The other F-box protein "Suppressor of nim1-1" (SON1) acts negatively to regulate plant defense responses. *Son1* mutant plants shows resistance to infection of both fungal and bacterial pathogens. This mutant displays a novel form of plant defense response. SON1 seems to be targeting some of the positive regulators of plant disease resistance for degradation. Therefore, some of the SCF complex (Skp1-Cullin-F-box-Rbx1 protein) proteins may play a negative role in regulation of plant resistance (Kim and Delaney, 2002). One possible role of our clone Yr1-11 (F-box) might be targeting negative regulators of plant disease responses, which may be taking place by degrading the apoptosis inhibitors via ubiquitinylation, thereby stimulating the apoptosis. Such assumption is supported by a study, showing that a human overexpression of F-box protein, Fbxo7, interacting with a member of apoptosis inhibitor proteins, cIAP1, promotes the ubiqutinylation of cIAP1 (Chang *et.al*. 2006).

Clone DDYr1-10 shows homology to UBX domain of human FAF1 (FAS associated factor1), which is previously identified as an essential component of FAS associated death signaling complex (Ryu *et.al*., 2003) and has been reported to enhance but not initiate apoptosis in murine L cells when overexpresed (Chu *et.al*. 1995). Lately, it was shown to have the ability to initiate apoptosis in BOSC23 cells when overexpressed (Ryu *et.al*. 1999). However, FAF1 does not have a death signal region, unlike the other proteins in the of FAS associated death signaling complex. But it possesses 2 ubiquitin homologous domains and one UBX (80 aa) domain which shows homology to proteins involved in ubiquitin mediated protein degradation pathway. Unfortunately, its role in apoptosis and ubiquitinylation pathway is not clear. Recently, it has been identified that when a novel N-terminal UBA domain (81 aa) of FAF1 is overexpresed, it leads to induction of apoptosis. UBA domain of FAF1 was shown to be interacting with multiubiquinylated proteins rather than mono-ubiquitinylated proteins and its transient overexpression leads to the accumulation of multi-ubiquitinated proteins *via* inhibiting their degradation by proteasome. It was also reported that hFAF1 interacts with VCP (Valosin containing protein), an AAA ATPase type protein which is involved in recruitment and substantial degradation of ubiquitinated proteins by proteasome, *via* its C-terminal

UBX domain (Song *et.al*. 2005). Results obtained by Song *et.al*. suggests that VCP binding to hFAF1 inhibits the role of hFAF1 and they seem to be working antagonistically in which VCP induces the degradation of polyubiqutinylated proteins, whereas hFAF1 inhibits their degradation through binding with its C-terminal UBA domain and leading to accumulation of polyubiquitinylated proteins. Through this perspective, one possible role of FAF1 in plant disease resistance might be the induction of plant cell death by inhibiting the degradation of proteins by proteasome and leading to accumulation of proteins involved in apoptosis and hypersensitive response. One such target protein may be R protein itself, because in some cases it was proven that overexpression of the R genes leads to HR in the absence of the corresponding Avr product. (Tao *et.al*. 2000; Tang *et.al*. 1999; Frost *et.al*. 2004). Additionally, Jones group showed that transient expression of RPS4 gene induces an HR like phenotype in the absence of the corresponding avrRPS4 avirulence protein and that phenotype is dependent on the accumulation of the RPS4 (Zhang *et.al*. 2004). Another role of hFAF1 in apoptosis is related to its inhibitory role on HSP70. Recently, hFAF1 was reported to be directly interacting with HSP70 protein with its N–terminal 82-181 amino acid region and inhibiting its chaperone activity (Kim *et.al*. 2005). HSP70 is an antiapoptotic protein that exerts its activity by inhibiting BAX translocation to mitochondria and subsequent release of proapoptotic factors in heat stressed cells (Stankiewicz *et.al*. 2005). Thus, our clone Yr1-10 with UBX homology, may promote cell death during HR by binding HSP70 and inhibiting its antiapoptotic role.

Most of the pathogens cannot introduce the disease on entire plant species since majority of plants are immune against a broad range of pathogens which is defined as non-host plant disease resistance. Non-host resistance is the most common form of disease resistance (Thordal-Christensen 2003). PEN1 encoding syntaxin SYP121 was cloned by map-based cloning strategy from *Arabidopsis*. It has been shown that mutations in the PENETRATION1 (PEN1) gene reduces the non-host resistance of *Arabidopsis* plants against barley powdery mildew pathogen Bgh-conidia (*Blumeria graminis* f. sp. *hordei*) to about seven fold of that of wild

type plants. So PEN1 represents the importance of vesicle trafficking in penetration resistance. ROR2, encoding a functional homologue of syntaxin, was identified in a mutant search and it is required for full mlo resistance. Collins *et.al*. showed that SNAP-25 (synaptosome-associated protein, molecular mass 25 kDa) homologue (HvSNAP34) is capable of forming a binary SNAP receptor (SNARE) complex, which is required for penetration resistance in barley. ROR2 being a syntaxin family member interacts with HvSNAP34 and mutant ror2-1 plants are susceptible to Bgh penetration. Our Yr10-5A clone shows homology to a putative syntaxin of *Arabidopsis*. It might have a similar function as ROR2 and form a SNARE complex to inhibit pathogen penetration. Possible link between host and non-host plant disease resistance could be concluded from ROR2 and PEN1 homologues because of the fact that elements of vesicle trafficking are conserved both in host and non-host resistance (Collins *et.al*. 2003). Thus, we have detected the involvement of vesicle trafficking element in the host dependent yellow rust disease resistance mechanism in wheat.

The DDYr1-1 clone shows homology to a type of receptor like kinase (RLK), Tak33, in wheat. Plant RLK proteins contain a signal sequence, a transmembrane region, and a C-terminal eukaryotic protein kinase signatures. RLKs have distinct roles in plant development, defense responses and growth. *Arabidopsis* genome contains more than 600 genes and the *Oryza sativa* has nearly twice as many (Shiu *et.al*. 2004). RLK takes signal and propagates *via* its kinase domain. *Xa21* from *Oryza sativa* is a serine/theonine RLK type protein involved in plant–pathogen interactions (Song *et.al*., 1995). We also found that the DDYr1-1 fragment expression is up-regulated, when Avocet-Yr1 plant is infected with avirulent strain of *Puccinia striiformis* 232E137, but the expression of that gene is not induced when Avocet-Yr10 plant is infected with avirulant race, 169E136. This means that the fragment is the induced level of transcripts found in Avocet-Yr1 plants, may be a functional disease resistance gene.

Our clone DDYr1-11 shows homology to N-terminal region of cyclophilin type proteins. A few number of similar proteins having that kind of homology were

previously reported to posses antifungal activities. Mungin, an 18-kDa protein isolated from mungbeans was demonstrated to be antifungal against *Rhizoctonia solani, Coprinus comatus, Mycosphaerella arachidicola, Botrytis cinerea,* and *Fusarium oxysporum*. Its inhibitory role on the activity of A and B glucanases was determined (Ye and Ng. 2000). Additionally, ungulin a similar protein having antimitogenic, antiviral and antifungal activities, was also identified from black-eyed pea (Ye and Ng. 2001). Ungulin was also reported to be an inhibitor of HIV (Human immunodeficiency Virus) reverse transcriptase activity. No cyclophilin like protein was previously identified in cereals. Therefore, it would be interesting to study the possible antifungal and antiviral roles of our clone Yr1-11.

Pathogenesis related (PR) proteins are defined as proteins that are induced upon pathogen attacks or treatment of chemicals mimicking pathogen infection effect encoded by host plant. Several homologues of the dicot PR genes have been identified in monocots. PR1 and PR5 homologues were characterized in barley and maize. (Bryngelsson *et.al*.,1994). These genes have been cloned from wheat and published in NCBI database. Antifungal activity of PR-1 gene was shown that PR-1 proteins inhibit zoospore germination of *Phytophthora infestans.* (Niederman *et.al*., 1995). PR-5 proteins have also antifungal activity these are sometimes called thaumatin like proteins because their amino acid sequences are highly similar to those of thaumatin. Transgenic rice and orange plants overexpressing thaumatin-like PR-5 possessed increased tolerance to *Rhizoctonia solani* and *Phytophthora citrophthora*, respectively (Datta *et.al*. 1999; Fagoaga *et.al*. 2001). Our two clones (DDYr1-2 and DD3Yr10-37) show homology to Pr-5 gene.

3.14.2 Microarray

Microarray experiments very successfully completed and analysis were performed accurately. When we compare the results between compatible and incompatible infections, it is obvious that in former one, genes tend to be rather downregulated than upregulated. One explanation may be that due to the tags that are present on the chip. Another explanation might be that the pathogen tries to mediate host cell expression by downregulating some genes so that plant can feed itself or suppress host defense mechanisms, thereby it can propagate. When we compare results from virulent and avirulent infections we see that no defense related gene is upregulated in avirulent infections. It is also obvious that genes involved in stress response and signaling pathways are also mostly down regulated in virulent infections. That is probably due to pathogens attempt to suppress host surveillance system and make it defenseless in order to propagate efficiently. This indicates that pathogen tries to block signaling pathways that can trigger defense responses in the plant. When we look at the incompatible infections it is no surprise that lots of defense related genes like chitinases, Pr genes, and endo-beta glucanases and genes related to signal transduction were upregulated. Those genes seem to be highly up regulated when compared to other genes identified. That is probably the reason which made them easier to be discovered in earlier studies. Genes involved in metabolism and energy are down regulated in avirulent infections. This might be due to plant's strategy to minimize the all metabolic actions and to focus on stopping the pathogen infection. There are also a number of ESTs that were differentially regulated in both virulet and avirulent infections when compared to mock infections. Those ESTs might be differentially expressed as a result of direct effect of the pathogen on host to feed itself or to provide a suitable environment for its propagation. However, better conclusions using microarray data can be acquired as more genes identified in wheat and presented in future wheat microarray chips.

3.14.3 VIGS

For the future studies using the accumulated data from DD and microarray experiments, we decided to use BSMV mediated VIGS method in order to reveal possible roles of the genes that we identified. To begin with, we needed to see if BSMV mediated VIGS works in wheat and if so optimize the system. Our results showed that BSMV-based vector replicates sufficiently in wheat to trigger posttranscriptional gene silencing of host sequences as demonstrated by quantifying the silencing of phytone desaturase (*PDS)* mRNA. Unfortunately, as we finished our first experiments while we were preparing to repeat the experiments Scoffields group published the BSMV mediated silencing of genes in wheat. Therefore, it is possible to silence those genes that we have identified to be differentially regulated. After confirmation of silencing, plants can be infected with the avirulent pathogens and genes could be scored whether or not are essential for disease responses.

CHAPTER IV

CONCLUSION

In this study, we have identified novel genes which may putatively play important roles in plant disease resistance. Among those, two were identified as full ORF including 5' and 3' end un-translated regions (UTR). Our findings indicate that genes involved in ubiquitin mediated protein degradation are induced in wheat in response to yellow rust incompatible pathogen infection and suggest that ubiquitinylation and protein degradation, a very generic pathway in the cell, is an important event for wheat yellow rust disease resistance. There is accumulating data indicating the possible roles of ubiquitinylation in plant disease responses. Unfortunately, those genes belong to model organisms such as *Arabidopsis* There are a number of recently identified genes encoding F-box type or E3 type ubiquitin ligases which were confirmed to be involved in disease response mechanism of several plants. However, there is no report of an E2 (ubiquitin conjugating enzyme) conjugating enzyme which is responsible for the disease resistance mechanisms in plants. Our clone encoding RAD6 gene (E2) might be one of the regulators of the disease resistance in cereals.

We have used differential display method in combination with microarray technology. However, none of the genes we identified in DD analysis were found in the microarray data. This was no surprise to us, as none of our genes were present on

the latest released Affymetrix wheat chip. Therefore, for the organisms whose genomes that have not been sequenced yet, application of microarray technology alone could give limited information. However, it allows a wide view of the differential expression profiles of the transcriptome for the applied treatment.

As a future goal, those genes we have identified need to be further characterized using functional studies and their possible roles in disease resistance should be investigated. For this purpose we have successfully managed to silence the wheat *PDS* gene using BSMV mediated VIGS. Studies using Barley Stripe Mosaic Virus (BSMV) to identify the possible roles and functions of those genes are ongoing in our laboratory.

Results in this thesis will contribute to the understanding the molecular basis of disease resistance in plants. One of the major goals of plant science is engineering plants to produce resistance proteins that can recognize essential pathogen molecules. Therefore, our work is expected to contribute to the aim of engineering durable disease resistance in agricultural crops using biotechnological approaches.

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APPENDIX A

DNA sequences of the genes identified from DD analysis

DD3-31

ACAAGCGAATAATGGTGCGATCCACGCAAGGTGCGTGTGTAGTCGATTATAATGCAATA
AATGGTGTGTTCCTATCCAAGTTTTTTTTAAATGAAATGCATTTAGCCCGACTTTAAATTA
ATGAAACCACCAACCGGGTAGGATACAAAATGCAGATTGATACAACGGGTGCGCATGAG
AAAAAAAAAGACTGCAAAATTATAGACACTAGTACAATGCCCGTGCGTTGCCACGGGCT
TTTGAAATATTTTGCTAGAGTTATATAAATATAATGCATGCTAAATTGATATATTAAAAA
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AAGTGGTAGAACTCCAAAATTAATATAATTGCAAGGAAAGGGAAAAAATACTAGAAATA
GATCACAATAATCGATATTAATAGTCATATTGAATACATACATGCATCAAGAAAGTCATA
GATACATAGCAGAAACAAATACTATAAACTTCACCCCATTGCAGTCAAGCTCCATATCCC
CAGTGCTATTGCGTACTAGTATAATGCCCGTGCGTTGCCACGGGCTTTTGAAATATTTTGC
TGAAATTATGTAAACATAATGCATATTATATTTCACATGAAAAAAAAAGATATCACTCAG
CA

DD3-51

CTGACTGGAAAAGTACAACCTATAAGTCCAGCATGGGGTGTGGAAGGTTTTGATCCTTTT
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CTATTCCATCTTAGTGTCCGTCCGCCCCAACGTCTATACAAAGGATTACGTATGGGCAAT
ATTGAAACCGTTCTTTCTAGTAGTATCGCTGCTGTCTTTTTTGCAGCTTTTGTTGTTGCCGG
AACTATGTGGTATGGTTCAGCAACGACTCCGATCGAATTATTTGGCCCCACCCGTTATCA
ATGGGATCAAGGATACTTTCAGCAAGAAATATACCGAAGAGTAAGTGCTGGGCTAGCTG
AAAAAAAAAGATATCACT

DD3-90

TCGCGAATGCATCTAGATTGGGGGGGTGGGGAAGACGACGAGGACGAAGGCGATGAGT
ACTGAGTTGATCAAGCATGGGTACGTGCTTAATTGGGTGGCAGTGTTGTGCACTTCATGC
CGTATGTTGTTCTTCTCTGTCCCTGTTTGCTTGCCGTTGCATCTGTGATGTTCGGTGGTTAG
CAGAGTACAAAAATTGTCAGATTGGTTCGGGTGCACTGACGGCTCGGTGCCACATAGTCT
GTGAGGAGGTGGTATCGATACATCATAATAATGAACGAAGATTTTTTGTACTGCAAGTTG

CAGGGGTATGTCTTCCTTTGTTTTGTTATGAAAGCAAAGCATGTTAGACCATACGANGTCTGT
TAAAAAAAAAGATATCACTCAGCATAANGAATCGGATCCNGGCCCGT

DD3-55

CGAGCTCGGTACCTCGCGAATGCATCTAGATTGCATTGGAACAAGGTGAAGAAGATGGCATT
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TGATTGATACTGACAATAGTGGGACAATAACTTATGAAGAACTGAAGGATGGCTTGAAAAG
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AGGGAGGAAAATTTGGTGTCAGCATTCGCATTCTTTGATAAAGATGGAAGTGGCTTTATAAC
GATTGATGAGCTCTCGCAAGCATGCGAGAAATTTGGCCTTTCTGACGTTCATCTTGAGGATAT
GATCAAGGATGTGGACCAAAATAATGATGGACAAATTGATTACAGTGAGTTTG

DD3-73

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GTAAATTAACCGAAGAACCCTAGCTAGCAGAGAGAGAGGGGGGTTTACGGACGGTGCTTTA
GTGAGGGTTAATA

DD3-48

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GGTTGTCAACGCGTCGGACTTACCGCTTACCTTTAACATTCGCAATTATAAAACAATTTTAAG
CGCAATTTAAAAACAATTTAGTCGAGTAAAAAATTGGTTATCCGGCTTTAGCCGTTTTAGGG
AATATTTAGTTTTCTTATCTGGCTCTATCCCAACTCACAACAAGGTCAAACCTTGTTCTCAGG
TGATAATTTCTTGCACCTGAGGTTGCA

DD3-37 CSATPR5

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TTTCAAATCATCGTTGTTGATTATACGAGTAATGGACTCGTCGGATTCATTATGGTGTAGCCT
CGTTTGTACTACTTAGTTCATGTTGTCCATCCCTCCATGGTTCCGTACTGTTATATCTATGTAT
TTATCAAGTTTGCCGAATCGACCCGTTTGGTGCCCATTCCTACCGGTTTGTCTATTCGAACCT
AAAATTTGGGCTATCGTTCTTTGTTCCCTGGTTCGTTGACATTGATCGTAAAACGTCTTTTGGT
CTAGTGTGCACGCTCATGATGAAGGGGAACCGTGAAACTAGGGCGATTTGTTAGATCTACGT
AAGCGCTCCATGGCT

DD3-74

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AAATGTTGCAGCACTGACCCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAG
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GCGTCGGACTTACCGCTTACCTTTAACATTCGCAATTATAAAACAATTTTAAGCGCAATTTAA
AAACAATTTAGTCGAGTAA

DD3-50

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AAAGCGGATGATAACCGCAATGTATTACACTGAAATCTTTTGGGTCAGCTCTCACTGGTCGA
TGGTGAACTCAGCCACTCAGGCAGTGCCGGTTATCAATCTCTCTTGTTGGTCCAGTTTCTTCA
CCTTGTTCCAATGCAATCTAGATGCATTCGCGAG

DD1-YR10-2

GGGAATTCNATTTGAGCGGCCGCAGTCTAAGANTNNNTCCTCTCCGNCTACGATCTAGTGAT
GTGTTCACAATGGACCTAAGCGAAGAAGAGCCCTGTTGGCGGTGCCTAACCGGGAGTGGAA
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AGTTTGCCAGGGGGAAGAGTGTTGATATTTGGTGGATCAGTGGCAGGCCTCCACTCGGCGTC
ACAGCTGCATCTCTTGGATCCGACTGAAGAGAAACCTACGTGGAGGNTNNTNAATGTTCCCG

GGCGACCTCCGCGGTTTGCGTGGGGCCACAGTACCTGTGTCGTTGGAGGTACAAAAGCGATA
GTGCTTGGAGACAAACTGGAGAAGAGTGGATGCTCACCGAAGTACATGAGCTCTCTCTGGCT
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TTAGACCTTTCAAGTGGCGTTAAGCTTGATTGTCTTCACCACCGGGCCAGAGCACTGAATGG
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CGGCCGCTC

DD1-YR105-A

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AAGCTGCTGCTCATTTGCACCATATGCAATACGATTTAGCGATCTCAAAAGATGGTTCATCTG
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ACAGTTCACACCTGCCTGCCTGTCGTGGAGCGACCAGGTCTGGTTTTGTATGATCTGTATAAA
GTTGTACCATCATCCAGTTATTAGAAATTGAAAAAAAAAGATATCACTCAGCATAATG

DD1-YR1-1

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CCTTCTCGACAGCAATTTCGTCCCGAAGGTCGCCGATTTCGGACTCGCCAAACTGTACCCAA
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DD1-YR1-2

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CATAA

DD1-YR10-1

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ATAATG

DD1-YR1-3

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DD1-YR1-8

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DD1-YR1-9

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DD1-YR1-10

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DD1-YR10-8

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DD1-YR10-9

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DD1-YR10-4

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DD1-YR10-6

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APPENDIX B

List of primers used in microarray QRT-PCR confirmations

Primer ID	Primer Sequence	BP
AB0299361 F	ATGATGCGAGAAGACATGCAAAA	23
AB0299361 R	ATGATGCGAGAAGACATGCAAAA	23
AJ611109 F	ATGATGCGAGAAGACATGCAAAA	23
AJ611109 R	ATGATGCGAGAAGACATGCAAAA	23
BE213663 F	ATGATGCGAGAAGACATGCAAAA	23
BE213663 R	ATGATGCGAGAAGACATGCAAAA	23
BE443499 F	ATGATGCGAGAAGACATGCAAAA	23
BE443499 R	ATGATGCGAGAAGACATGCAAAA	23
BE515461 F	ATGATGCGAGAAGACATGCAAAA	23
BE515461 R	ATGATGCGAGAAGACATGCAAAA	24
BJ265803 F	ATGATGCGAGAAGACATGCAAAA	23
BJ265803 R	ATGATGCGAGAAGACATGCAAAA	23
BJ266714 F	ATGATGCGAGAAGACATGCAAAA	23
BJ266714 R	ATGATGCGAGAAGACATGCAAAA	23
BJ320268 F	ATGATGCGAGAAGACATGCAAAA	23
BJ320268 R	ATGATGCGAGAAGACATGCAAAA	23
BQ166180 F	ATGATGCGAGAAGACATGCAAAA	23
BQ166180 R	ATGATGCGAGAAGACATGCAAAA	24
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BQ166746 R	ATGATGCGAGAAGACATGCAAAA	23
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CA606815 R	ATGATGCGAGAAGACATGCAAAA	22
CA610276 F	ATGATGCGAGAAGACATGCAAAA	19
CA610276 R	ATGATGCGAGAAGACATGCAAAA	21
CA627432 F	ATGATGCGAGAAGACATGCAAAA	23
CA627432 R	ATGATGCGAGAAGACATGCAAAA	23
CA634683 F	ATGATGCGAGAAGACATGCAAAA	20

CA634683 R	ATGATGCGAGAAGACATGCAAAA	23
CA656847 F	ATGATGCGAGAAGACATGCAAAA	23
CA656847 R	ATGATGCGAGAAGACATGCAAAA	23
CA664784 F	ATGATGCGAGAAGACATGCAAAA	23
CA664784 R	ATGATGCGAGAAGACATGCAAAA	23
CA669038 F	ATGATGCGAGAAGACATGCAAAA	23
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CA721750 F	ATGATGCGAGAAGACATGCAAAA	22
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CA745804 R	ATGATGCGAGAAGACATGCAAAA	24
CD903633 F	ATGATGCGAGAAGACATGCAAAA	23
CD903633 R	ATGATGCGAGAAGACATGCAAAA	23
Y182121 F	ATGATGCGAGAAGACATGCAAAA	23
Y182121 R	ATGATGCGAGAAGACATGCAAAA	23

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EDUCATION

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MS	METU Biotechnology	2002
BS	Hacettepe Univ. Biology	1999
High School	Cankaya High school	1995

WORK EXPERIENCE

Year	**Place**	**Enrollment**
2001-Present	METU Graduate School of Natural & Applied Sceinces	Research assistant

FOREIGN LANGUAGES

Advanced English, Fluent Turkish

PUBLICATIONS

- **Bozkurt, O.**, Hakki, E.E., AKKAYA, M.S**.**, "Isolation and sequence analysis of wheat disease resistance gene analogs using degenerate PCR primers" Submitted to *Biochemical Genetics*. accepted

Meeting Abstracts:

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