DETECTION AND CHARACTERIZATION OF PLANT GENES INVOLVED IN VARIOUS BIOTIC AND ABIOTIC STRESS CONDITIONS USING DDRT-PCR AND ISOLATION OF INTERACTING PROTEINS

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Approval of the thesis

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ABSTRACT

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The main objective of this thesis dissertation is functionally characterizing the genes involved in biotic and abiotic stresses of plants at molecular level. Previously, upon pathogen attack *Rad6* gene expression was found to be changed in wheat and barley plants. To functionally characterize the *Rad6* gene, VIGS (Virus induced gene silencing) system was used. HR (Hypersensitive response) like symptoms was detected in every silenced barley and wheat plants. To figure out, transcriptomes and proteomes of *Rad6* silenced plants were analyzed. 2-D PAGE analysis was also performed on silenced and control wheat plants. No pathogen growth was observed in *Rad6* silenced barley lines. Additionally, the susceptible wild type *Arabidopsis* plants showed resistant phenotype when any of the *Rad6* gene copies is mutated. This suggests that *Rad6* gene has a negative regulatory role in plant disease resistance which was proved for the first time. Yeast two hybrid protein interaction study suggests that RAD6 carrying out its function by interacting with SGT1 protein and regulating resistance related genes. It has been first time reported in this thesis that E2 (Ubiquitin conjugating enzyme) takes role in plant disease resistance.

Boron which is the other consideration in the scope of thesis as an abiotic stress factor at a very limited amount is necessary for the normal development of plants. This study is conducted on highly boron tolerant *Gypsophila perfoliata* L. collected from a location in the boron mining area. The plant samples were tested in the presence of high boron (35 mg/kg) concentrations. The transcriptomes of the plant samples treated with the excess levels of boron to that of the samples grown under normal concentration were compared using differential display PCR method. Thirty bands showing differential expression levels at varying time points were analyzed. 18 of them were confirmed *via* qRT-PCR.

Key Words: Differential display (DD) technique, qRT-PCR, *Rad6*, E2 Ubiquitin conjugating enzyme, barley, wheat, boron, *Gypsophila perfoliata,* hypersensitive response, plant disease resistance, VIGS, 2-D PAGE, gene silencing, *Rar1*, *Sgt1*, *Hsp90*, *Arabidopsis thaliana*.

ÖZ

ÇEŞİTLİ BİYOTİK VE ABİYOTİK STRES KOŞULLARINDA BİTKİLERDE ROL ALAN GENLERİN FARKLILIK GÖSTERİM YÖNTEMİYLE BULUNMASI, FONKSİYONLARININ BELİRLENMESİ VE İLİŞKİDE BULUNAN PROTEİNLERİN BELİRLENİLMESİ

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Bu doktora tezinin ana hedefi biyotik ve abiyotik stres koşullarında bitkilerde görev alan genlerin fonksiyonel olarak moleküler seviyede karakterize edilmesidir. Bitki patojen ilişkileri ve bor toksikliği dayanıklılığı tez kapsamında ele alınmıştır.

Rad6 gen ifadesinin buğday ve arpa bitkilerinde patojen ataklarıyla birlikte nasıl değiştiği saptanılmıştır. Bu baskılanmanın sebebi ise bitkinin patojen enfeksiyonuna karşı kendini koruma mekanizmalarıyla ilgili olabilir. Bu durumu daha iyi anlamak için virüs indüklemesiyle gen susturma (VİGS) uygulaması yapılıp *Rad6* geninin ifade edilmesi engellenilmiştir. Bitkilerde *Rad6* geni susturulmasıyla bir şekilde aşırı duyarlılık reaksiyonları gözlemlenilmiştir. Bu durumu daha iyi anlamak üzere *Rad6* geni susturulmuş olan bitkilerde transkriptom ve proteom analizleri yapılmıştır. Yapılan bu araştırma *Rad6* geni susturulması sonucunda oluşan aşırı duyarlılık durumunu RNA ve prtoein seviyesinde kanıtlamaktadır.

Ayrıca, *Rad6* genine ait olan iki kopya ayrı ayrı mutasyona uğratılmış olan model bitki *Arabidopsis'* ler bulunarak normalde patojen hassas olan mutant *Arabidopsis* bitkilerinde artırılmış dirençlilik saptanmıştır. Bu tez kapsamında ilk

defa E2 yani Ubikutin bağlayıcı enzimin bitki dirençlilik mekanizmasında rol oynadığı gösterilmektedir. RAD6 bitkilerde dirençlilik mekanizmasında negatif regulator olarak rol oynamaktadır.

Son zamanlarda yapılan çalışmalarda bazı *Gypsophila* türlerinin aşırı borik asit içeren koşullarda yaşayabildiği gösterilmiştir. Borik asitin yoğun olduğu maden bölgesinde yaşayan *G. perfoliata* bitkisi Kirka'dan toplanılıp laboratuarımıza getirilmiştir. Aşırı miktarda borik asit içeren ortamda 35 mg/kg (500 μM) bekletilen bitkilerden RNA izole edilerek farklılık gösterim yöntemi uygulanılmıştır. Otuz tane bant klonlanılarak ilk etapta sekans analizleri yapılmıştır. Daha sonra otuz banta ait uygun primerler dizayn edilerek gerçek zamanlı kantitatif PZR ile doğrulama deneyleri uygulanmıştır. Bu 30 genin 18 tanesinin farklı olarak ifade edilmekte olduğu saptanılmıştır.

Anahtar kelimeler: farklılık gösterim tekniği, hastalık dirençlilik, VİGS, *Gypsophyla perfoliata, Rad6,* E2 Ubikutin bağlayıcı enzim, bor, buğday, arpa, gerçek zamanlı kantitatif PZR, 2 boyutlu PAGE, *Rar1*, *Sgt1*, *Hsp90*, aşırı duyarlılık reaksiyonu, gen susturma, *Arabidopsis thaliana*.

to my wife Seval
and
my parents (Mediha, Rıdvan)

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LIST OF ABBREVIATIONS

μg	: Microgram
μL	: Microliter
μM	: Micromolar
[α-^{32}P]-dATP	: [α-^{32}P]-deoxyadenosinetriphosphate
2D	: 2 dimensional
ACRE	: Avr/Cf Rapid Elicidated
ADP	: Adenosine Di phosphate
APS	: Ammoniumpersulfate
As	: Anti sense
At	: *Arabidopsis thaliana*
Avo	: Avocet
Avr	: Avirulence
Bgh	: *Blumeria graminis* f. sp. *hordei*
B	: Boron
BSA	: Bovine Serum Albumin
BSMV	: Barley Stripe Mosaic Virus
CC	: Coiled Coil
Cf	: *Clodisporium fulvum*
DD	: Differential Display
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotidetriphosphate
dpi	: Day post inoculation

ds : Double stranded

DTT : Dithiothretiol solution

DUB : Deubiquitinase

EDTA : Ethylenediaminetetraacetic acid

ELF : Early Flowering

ETI : Effector Triggered Immunity

ETS : Effector Triggered Susceptibility

GFP : Green Flourescent Protein

GLP : Germine-Like Protein

GST : Gutathione –transferase

h : Hour

Hai : Hour after inoculation

HR : Hypersensitive response

HSP90 : Heat Shock Protein 90

kb : Kilobase

LC/MS/MS : Liquid chromatography/Mass /Mass spectrometry

LiCl : Lithium Chloride

LRR : Leucine rich repeat

M : Molar

mCi : Milicurie

mg : Miligram

min. : Minute

mL : Mililiter

Mla : Mildew Locus A

mM : Milimolar

NB : Nucleotide binding

NC : Not Change

ND : Not Determined

NEB : New England Biolabs

ng : Nanogram

nm : Nanometer

NTC : No Template Control

Os : *Oryza sativa*

p : Plasmid

PAGE : Poly-acrylamide gel electrophoresis

PAMP : Pathogen Associated Molecular Pattern

PCD : Programmed cell death

PCR : Polymerase chain reaction

PDS : Phytoene desaturase

pmol : Picomole

PR : Pathogenesis related

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

PTGS : Post Transcriptional Gene Silencing

PTI : PAMP Triggered Immunity

PVX : Potato Virus X

qRT-PCR : quantitative Reverse transcriptase PCR

R : Resistance

RAR1 : Required for Mildew LocusA Resistance 1

RISC : RNA Induced Silencing Complex

RNA : Ribonucleic acid

RNAi : RNA interference

ROS : Reactive oxygen species

rpm : Rotation per minute

RT-PCR : Reverse transcriptase polymerase chain reaction

SCF : Skp1-Cullin-Fbox

SGS : SGT1 Specific

SGT1 : Suppressor of G2 allele

SiRNA : Small interfering RNA

SOD : Superoxide dismutase

Spl : Spotted leaf

Ss : Single stranded

Ta : *Triticum aestivum*

Taq : *Thermus aquaticus*

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

TIR : Toll Like Interleukine

U : Unit

Ub : Ubiquitin

UBC : Ubiquitin conjugating enzyme**,**

UV : Ultraviolet

v/v : Volume per volume

VIGS : Virus Induced Gene Silencing

CHAPTER 1

INTRODUCTION

1.1 Stress factors for plants

Plants are continually challenged by abiotic and biotic factors. Biotic stresses come from potential pathogens, bacteria, fungi, viruses, insects, and nematodes. How does the plant respond to these external factors is a matter of concern to many laboratories in the world. Plants have developed strong strategies to adapt the environmental stressors by using constitutive or inducible biochemical and molecular mechanisms. They generally use receptors at the cell surface to detect environmental changes and translate this information into an adaptive response. Abiotic stresses are those that are generated from environmental physicochemical factors such as salinity, extreme temperature, drought, metal toxicity and deficiency. Those stressors not only affect the plant growth and productivity but also cause serial direct or indirect morphological and physiological changes in plants. Therefore, plants are under multiple stresses during their lifespan and this is the main reason of huge amounts of annual yield loss reported worldwide. To date, physiological and molecular knowledge of the processes of abiotic and biotic stress tolerance/resistance in plants is still progressing. In this study, molecular mechanisms of disease resistance and boron toxicity tolerance in plants were investigated.

1.2 Plant disease resistance

It is important to understand the plant defense mechanisms in order to establish better strategies in minimizing losses due to diseases. Over the years, plant sciences have passed through some progress especially in molecular level studies towards elucidation of disease susceptibility and disease resistance mechanisms despite the difficulties such as studying crops and their interactions with pathogens. Pathogens

such as fungi, viruses, bacteria, and nematodes attack plants and cause significant yield losses in crops. It must be emphasized that plants have not got any mobile defender cells and adaptive immune system as animals have. Instead plants have their innate immunity in each cell and the systemic signal transduction resourced from infection sites (Dangl and Jones, 2001).

Plants have very complex recognition systems to detect pathogen effectors such as avirulence factors and pathogen associated molecular patterns (PAMP). Primary role of plant receptors is the detection of pathogen effectors or PAMPs and the transduction of the signal to downstream components for effective response mechanisms. In plants, it is R (Resistance) proteins that are found to be responsible for resistance against pathogen attacks. These R proteins recognize the pathogen effector molecules directly or indirectly and activate a common disease resistance mechanism called a hypersensitive response (HR). Subsequent defense responses include 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. These responses are accompanied by localized cell death or programmed cell death (PCD) at the site of infection (Hammond-Kosack and Jones, 1996; Shirasu and Schulze-Lefert, 2000). Two main hypotheses are presented to explain plant disease resistance mechanisms, one is gene-for-gene and the other is the guard hypothesis. Gene for gene hypothesis was firstly coined by Flor for flax-flax rust interaction (Flor, 1971). Each *R* gene product is assumed to recognize only a single pathogenic avirulence (*avr*) gene product and this surveillance system is required for efficient resistance. The second hypothesis suggests indirect interactions between R and AVR proteins where the R protein recognizes AVR induced plant factors by which guard protein so that the interaction will take place indirectly (Dangl and Jones, 2001).

To date on the light of current knowledge scientists have represented a zigzag model for unrevealing of plant disease resistance mechanism. The model provides us with a better understanding of immunity in plants. PAMP triggered immunity (PTI) and effector triggered immunity (ETI) should be differentiated between each other. Non-host and host specific disease resistance mechanisms also can be mentioned in

this model. Jones and Dangl shared their ideas on plant disease immunity with us at Nature journal in 2006 as pictured in Figure 1.1. Up until now the pathogen at the site of infection was not given as much importance as the responses occurring at the infected plant cell. This is why for a better understanding of the plant disease resistance mechanisms should be focused on pathogen as well as on molecular biology of the plant cell.

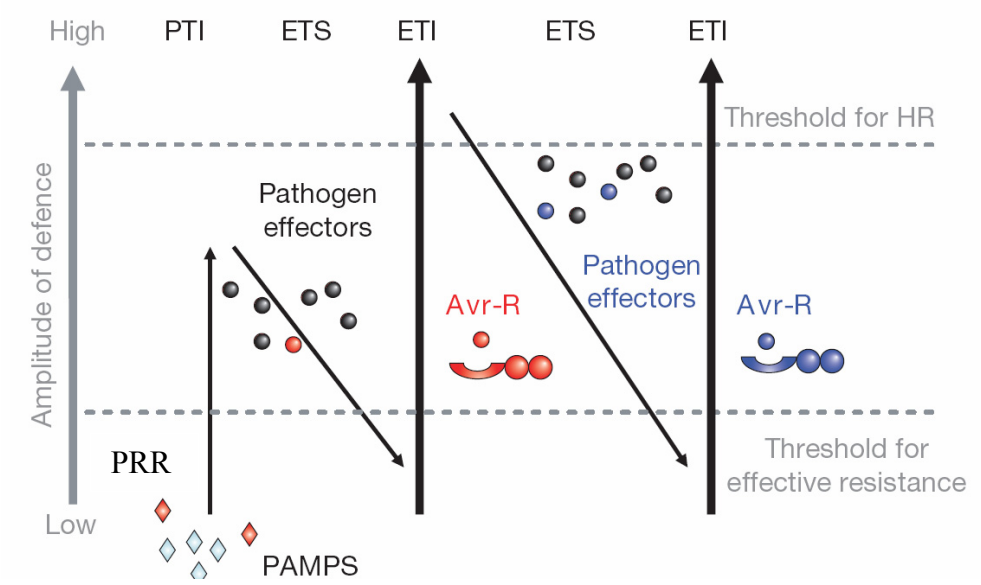


Figure 1.1 Jones and Dangl's zigzag model for illustration of plant immunity (Jones and Dangl, 2006). PTI: PAMP triggered immunity, ETS: Effector triggered susceptibility, ETI: Effector triggered immunity PAMP: Pathogen associated molecular pattern, PRR: Pattern recognition receptor.

Pathogens have the ability to infect, colonize and grow on plants. They especially use their PAMPs and effector molecules for a successful infection while the plants have their arsenal to combat with pathogens. There are two main type of in plant immunity to respond. First, plant cells use their PRR (pattern recognition receptor) to recognize the PAMPs (Zipfel and Felix, 2005) and the plant cell responses PAMP triggered immunity (PTI) which is not a very effective immunity. Second, happens in cytoplasma even in nucleus, *R* gene encoded protein products

are used by plant cells to produce very effective immunity, effector triggered immunity, ETI against invading pathogens.

Figure 1.1 represents plant-pathogen interaction by for-step "zigzag" model. In step 1 the PAMPs are recognized by several types of PRRs then PTI takes place which is not a strong immunity. In step 2, pathogen uses its effector molecules (AVR proteins) to modify and alter the host immunity PTI, to prevent resistance of plant cell against pathogen which is named as the effector triggered susceptibility (ETS). In step 3, if the plant cell has its specific R protein (mainly NB-LRR types) recognizing its effector molecules, it will trigger the HR immunity resulting in PCD, which is very effective response (ETI). Then in step 4 the pathogen alters its effector molecule structure or gains different type of effectors to suppress the ETI and generate ETS. In order to generate effector triggered immunity (ETI); R protein should gain its specific activity for effector's virulent factors.

1.2.1 Pathogen AVR proteins-effector diversity

Avirulence and virulence genes are defined in the literature: AVR proteins are products of virulence genes that are expressed by the pathogen and recognized by host plant R proteins during the infection resulting incompatible interaction thereby it is referred as avirulence factors (or AVR protein). A virulent protein can cause disease and become avirulent protein when an R protein found in the host recognizes it. These terms were first mentioned by Flor, during his pioneer genetic studies of the flax- flax rust interaction explaining the "gene-for-gene" hypothesis (Flor, 1955). *Avr* genes promote virulence in the host plant cell that does not express the corresponding *R* gene. Following terminology included in gene-for-gene resistance when the plant is resistant, the pathogen is called to be avirulent, and the interaction is said to be incompatible; when the plant is susceptible the pathogen is said to be virulent and interaction is called as compatible.

Understanding the effector molecules may provide clues to investigate the resistance mechanisms and functions of R proteins. A single aminoacid change in

the AVR proteins may result pathogen escaping from the recognition by R protein. Pathogen effectors are highly diverse in terms of their sequences. Many effectors from species and different races have been identified with more than one in any particular species. Up to date many *Avr* genes have been cloned from different types of pathogens and some of them were subjected to be functional analysis. Avr of fungal pathogen such as Avr-Pita from *Magnophorte grisea* having a putative metalloprotease activity (Orbach et al., 2000) and tomato pathogen *Clodosporum fulvum* (Luderer et al., 2002) were structurally identified. Both of AVR proteins are rich in cystine bridges which stabilize their structure and help recognition by the related R protein. Bacterial Avrs have been focused in much more detail, over 40 diverse effectors that are available in the literature (Petnicki-Ocwieja et al., 2002) delivered by the type III secretion system (TTSS) relaying on their characteristic hrp box promoter element or their secretion signal (Fouts et al., 2002). A number of bacterial AVR proteins were reviewed by Grant et al., (2006). Also all known of the Avr effectors were cataloged by (Birch et al., 2005 and Kamoun, 2006). Effector proteins of rust, powdery mildew and smut fungi have been listed by Ellis et al., (2007).

Fungal AVR proteins are also being studied; such as some rusts, powdery mildews and downy mildews. Dodds et al. identified AvrL567 from *Melompsora lini* flax rust having 150 amino acids including a signal peptide (Dodds et al., 2004). Ridout et al. has identified Avr-a10 from *B. graminis*, powdery mildew (Ridout et al., 2006). Allen et al. identified AVR protein ATR13, 187 amino acids with signal peptide from *Hyaloperonospora parasitica* oomycetes (Allen et al., 2004). Oomycete AVR proteins have a conserved motif which is not observed in flax rust AVR proteins. This motif is located just in the downstream part of the signal peptide called as RxLR motif (Birch et al., 2005). It has been proven that RxLR motif acts as host targeting signal (Rehmany et al., 2005). Avr3a is an effector molecule identified (Armstrong et al., 2005) from *Phytophtora infestans*, a necrotrophic oomycete, and it was shown that Avr3a inhibits the necrosis in tobacco plant by targeting another *P. infestans* elicitor INF1 which causes the necrosis (Bos et al., 2006). This is a suggestion for suppression of plant response mechanism.

1.2.2 *R* gene diversity

A number of disease resistance (*R*) genes have been cloned from diverse plant species. *R* genes encode R proteins to detect the presence of disease caused by bacteria, viruses, nematodes or fungi by specifically recognizing the pathogen effector molecules. Their protein products share common structural modules such as nucleotide binding sites (NB) and leucine rich repeat (LRR) domains which are conserved domains. Some R proteins have other domains than NB-LRR such as transmembrane protein, serin/threonine kinase and WRKY domains. R proteins may act as primary receptors for pathogen recognition. The general functions of R proteins are phosphorylation, protein degradation, ubiquitination or specific localization within host cells (Martin et al., 2003, Jones 2001).

There are five common different types of R proteins. The most common R protein type is NB-LRR. NB sites are more conserved and are required for ATP binding. As mentioned above LRR parts are interaction parts with AVR and other pathogen associated molecular patterns. Their functions are also related with the oligomerization of R proteins. The NB-LRR proteins can be subdivided into two different groups according to their N terminal regions. CC-NB-LRR and TIR-NB-LRR. CC: coiled coil, TIR: Toll like interleukin IL-1 receptor. The second class of R proteins does not have any NB region but has extracellular LRR region with transmembrane domain such as *Cf-9* in tomato (Dixon, 1996). Third class is only a serine/threonine kinase domain containing R protein. *Pto* is an example to serine/threonine kinase type R protein from tomato by Martin et al. (Martin et al., 1993). The members of the forth class of R proteins 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 et al., 1995). Transmembrane domain and CC domain containing RPW8 (*Arabidopsis*) type R protein is one of the fifth class R proteins. Figure 1.2 shows the types of R proteins.

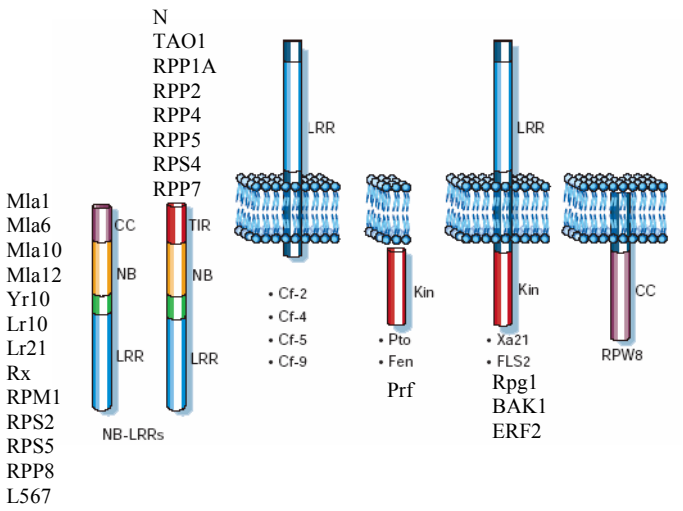


Figure 1.2 R protein classification and examples (Dangl and Jones Nature 2001).

Each of NB-LRR type R protein domains should be functional to confer disease resistance. Tameling et al. showed that I-2 NB-LRR protein hydrolyzes ATP domain and trigger defense resistance in its ATP bound active state rather than ADP bound state (Tameling et al., 2006). NB-ARC (A: apoptosis, R: resistance, C: CED4 homolog) domain molecular states are regulated by LRR domain. Pathogen effector recognition triggers the conformational changes and causes oligomerization of R protein. Conformational changes and interactions deploy between NB and LRR parts of R proteins and activate downstream signaling components. (Takken et al., 2006, Bent and Mackey, 2007). NB-LRR proteins not only contain nucleotide binding site and LRRs but also include conserved domains called NB-ARC homologs of human Apaf1 and CED4 (Van Der Biezen and Jones, 1998; Jones and Dangl, 2006). Rx is one of CC-NB-LRR type R protein and Moffett and his colleagues showed that domains of that R protein physically interact and effector AVR protein deploys some of these interaction. After then R protein returns in its active form and disease resistance is conferred (Moffett et al., 2002).

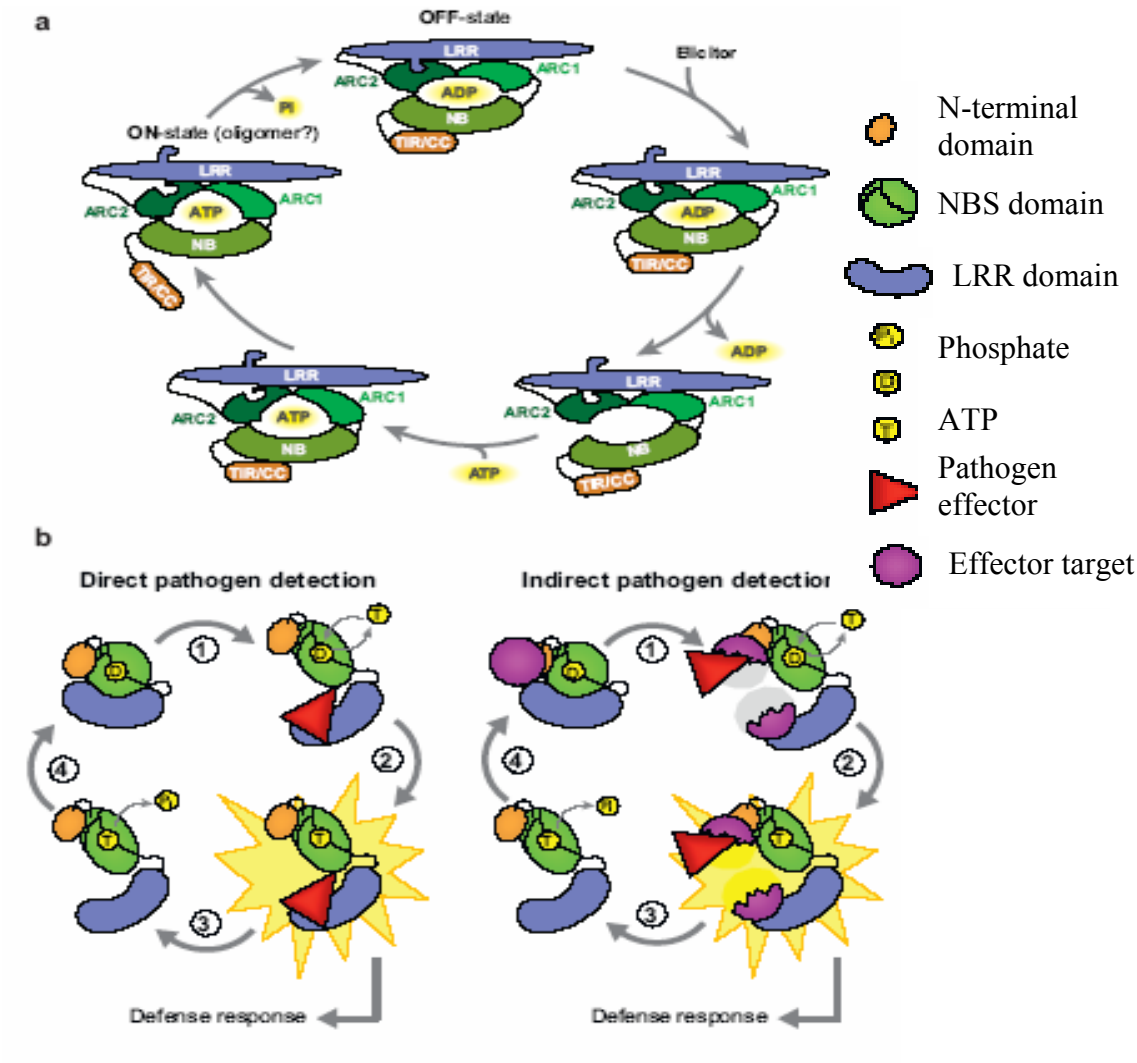


Figure 1.3 NB-LRR type R protein interaction states. **a**: ON and OFF states of R protein activation. In the presence of AVR protein, elicited from pathogen the interaction between LRR and NB domains is deployed and conformational change is seen then ADP release is allowed. ATP binding provides second conformational change and signal is transduced for effective disease resistance. **b**: Gene-for-gene (left) and guard hypotheses (right). (Bent and Mackey, 2007).

1.2.3 Ubiquitylation/26S protosome pathway and plant disease resistance

Ubiquitylation is a post-translational modification that plants use in their lifecycles. Proteins are continually synthesized, modified and proteolized all over the life span of plant cells. This is the protein cycle that in every week 50% of all plant cell proteins is replaced by new ones. Proteins generally are poly-ubiquitylated for selective turn-over. Ubiquitin (Ub) is used as a recognition signal and is covalently attached to targeted proteins for proteolysis. Ub/26S proteosome protein degredation pathway is highly specific and regulated system. It provides degradation of individual proteins and this mechanism is crucial for survival of all organisms (Sullivan et al., 2003). The system plays important roles for plants such as growth, defense response, flowering and development. Considerable number of genes is involved in the system; in *Arabidopsis* genome there are more than 1400 participant genes for Ub/26S proteosome system that covers nearly 5% of all *Arabidopsis* proteome (Hershko et al, 1998; Bachmair et al., 2002; Kosarev et al., 2002; Yan et al., 2000).

Ub is the 76 amino acids re-usable moiety that is “ubiquitously” present in eukaryotic organisms, with the ATP dependent series of reactions where poly-ubiquitins are attached to the target for specific proteolysis. Targets for poly ubiquitylation may be localized in cytoplasm, nucleus and cell membrane. Initial reaction is started by the E1 ubiquitin activating enzyme, E1 provides formation for E1-Ub intermediates with ATP hydrolysis (Hatfield et al., 1997). Activated Ub is then transferred to E2 ubiquitin conjugating enzyme. Thioesther linkage is the conjugation of E2 and Ub. E2 moves the Ubs to the target proteins to initiate the 26S proteosome mediated degradation. Individual E2 proteins have different functions in yeast and animals such as cell cycle regulation and DNA repair (Hershko et al., 1998; Pickart 2001). E3 ubiquitin ligases are responsible for identification of target proteins. E3 binds or ligates the Ubs to the target protein. *Arabidopsis* genome contains more than 1300 genes encoding E3 proteins or E3 complexes (Gange et al., 2002). This situation shows to specify the selection of targets for proteolysis (Smalle and Vierstra, 2004). To date four types of E3 proteins or complexes were identified based on their component homologies and mechanism

of actions. HECT, RING/Ubox, (Skp1-Cullin-FBox) SCF, APC are four different types of E3 ubiquitin ligases.

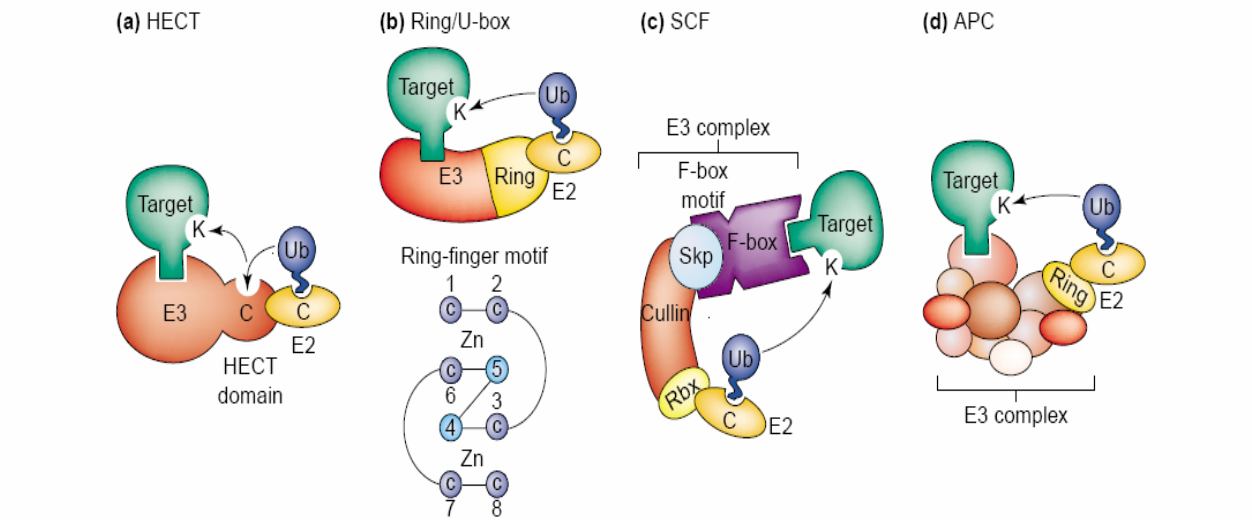


Figure 1.4 Structures of four different types of E3 ubiquitin ligase complexes (Vierstra, 2003).

SCF type E3 complexes consist of at least four components. SKP1, Cullin, F-box and the forth subunit is RBX (Deshaies, 1999). SCF complex specificity is conferred by the F-box subunit and F-box binds to SKP1 (Gagne et al., 2002). The Cullin-RBX-SKP1 subcomplex provides ubiquitin ligase activity (Deshaies, 1999). SGT1 is a disease resistance related protein which associates with SCF type E3 (Kitagawa et al., 1999). C: Cystein, Ub: Ubiquitin, K: Lysine, APC: Anaphase-promoting complex, HECT: Homology to E6-AP C-Terminus, Ring/U-box E3: Ring finger motif containing U-box E3.

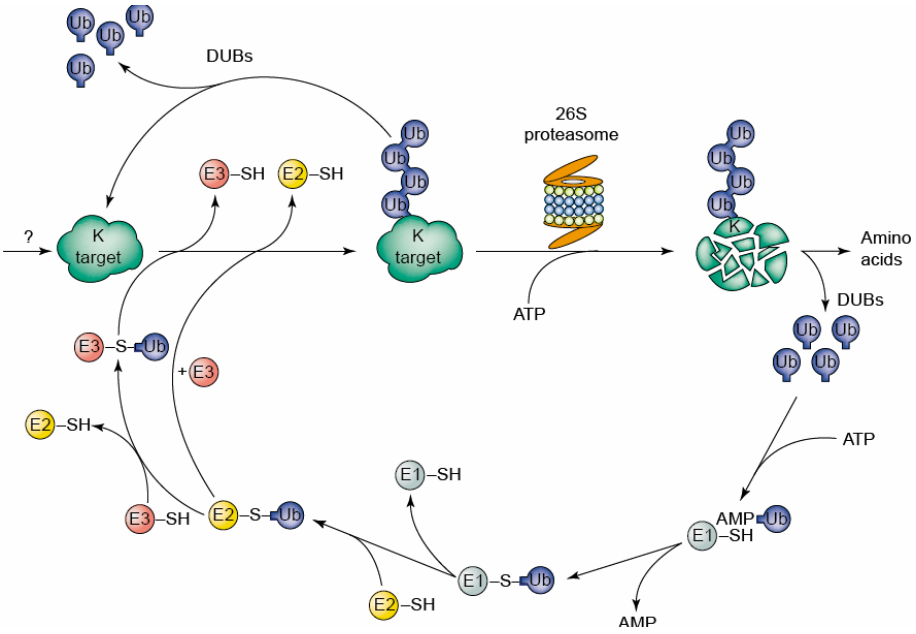


Figure 1.5 The ubiquitin 26S proteosome pathway: Ubs are attached to targets for proteolysis by an adenosine triphosphate (ATP) dependent manner E1 → E2 → E3 conjugation cascade serves for ubiquitination of individually selected proteins. Poly ubiquitinated target is recognized by 26S proteosome complex for protein degradation. K is lysine; DUBs are deubiquitylation enzymes (Vierstra, 2003).

The 26S proteosome complex is a 2-MDa ATP-dependent proteolytic complex that degrades poly ubiquitylated proteins (Shibahara et al., 2002). 26S proteosome complex consists of two big subcomplexes where one is a 20S core protease (CP) and the other 19S regulatory particle (RP). RP assists the recognition of ubiquitinylated target proteins, removing of Ubs and unfolding the target proteins. Finally CP degrades the unfolded and de-ubiquitinylated targets (Hartmann-Petersen et al., 2003; Voges et al., 1999).

Ubiquitylation 26S proteosome pathway is also related with plant defense mechanisms. The first clue was the SGT1 association with SCF type E3. In 1999 Kitagawa et al., showed the association of SGT1 with SKP1 *in vivo* and *in vitro* for yeast proteins (Kitagawa et al., 1999). Plant SGT1 proteins are necessary for defense response against a variety of pathogens where they participate in resistance mechanisms by the various R proteins recognition and signaling (Tor et al., 2002; Austin et al., 2002; Peart et al., 2002). Then the *Sgt1* gene was cloned by Tor et al.,

and its roles in plant disease resistance was confirmed by the other related works. The plant SGT1 association with SKP1 was also detected (Austin et al., 2002; Azevedo et al., 2002; Peart et al., 2002). Many putative E3s in plant appeared as disease resistance mechanism regulators (Devoto et al., 2003).

F-box is a subunit of SCF type E3 ligase and the 694 potential F-box protein in *Arabidopsis* data has been published (Gagne et al., 2002). Two different F-box proteins were shown that they are important for disease resistance where one is CORONATINE INSENSITIVE1 (COI1) (Xie et al., 1998). The coi1 mutant *Arabidopsis* plants were susceptible to insect, fungal and bacterial pathogens (Mc Conn et al., 1997, Thomma et al., 1998) COI1 protein has the F-box motif and LRR domain to regulate disease responses *via* Jasmonic acid (Devoto et al., 2002). The other F-box protein is SON1. *Arabidopsis* son1 mutant has been identified as resistant to fungal and bacterial pathogens. The son1 mutant genetic screen showed that SON1 is a negative regulator of plant disease resistance (Kim and Delaney 2002). Not only F-box subunit of E3 complexes is related with regulation of plant disease resistance but also RBX1 protein has been determined as regulator of plant response to pathogens. Silencing of RBX1 gene in Arabidopsis gives same phenotype as coi1 mutants (Xu et al., 2002). RING finger/Ubox type of E3 ubiquitin ligases also plays important roles for plant disease resistance. CMPG1 is ACRE-74 rapidly elicited Ubox domain containing protein in tomato and tobacco which has been shown that it is a positive regulator of PCD, HR (Gonzalez-Lamothe et al., 2006). As mentioned above some pathogen effectors mimic E3 in its activity. Such an AVR protein from bacteria AvrPtoB has E3 ligase activity as Avr3a has (Janjusevic et al., 2006, Bos et al., 2006).

1.2.4 RAR1, SGT1 and HSP90 are disease resistance related proteins

Proteins such as RAR1, SGT1 and HSP90, play essential roles in enabling the function of NB-LRR type R proteins (Holt et al., 2005, Jones and Dangl 2006). HSP90 is a heat shock protein and is required for activation of many signaling proteins. It is a highly conserved molecular chaperone for correct folding of proteins. Maturation and folding of protein needs direct interaction of HSP90 (Pratt

and Toft 2003). HSP90 has been shown as required protein for many R proteins to be functional. Silencing of *Hsp90* gene in monocotyledons and dicotyledons showed the crucial requirement of HSP90 for disease resistance in plants. Direct physical interaction of RPM1 R protein and HSP90 protein has been proven (Hubert et al., 2003, Kanzaki et al., 2003; Takahashi et al., 2003, Liu et al., 2004). HSP90 protein may take role in stabilization of R proteins. SGT1 and RAR1 which are highly conserved eukaryotic proteins also interact with each other. They are two proteins crucial for disease resistance regulation and they take roles in R protein accumulation, stabilization and function (Tornero et al., 2002, Bieri et al., 2004, Azevedo et al., 2006). HSP90 protein physically interacts with two major disease resistant regulatory components SGT1 and RAR1 and they form a triple complex (Shirasu et al., 1999, Azevedo et al., 2002, Takahashi et al., 2003).

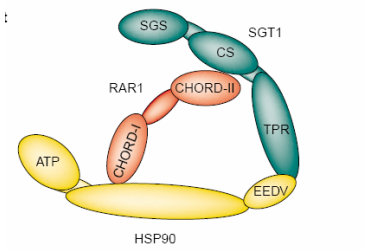


Figure 1.6 HSP90, RAR1 and SGT1 interact with each other (Shirasu and Lefert 2003).

RAR1 has two homologous zinc binding domains, CHORD I and CHORD II (Shirasu et al., 1999). SGT1 is composed of three conserved regions: an N-terminal TPR (for tetratricopeptide repeat) domain, a central CS (for CHORD-SGT1) domain, and a C-terminal SGS (for SGT1-specific) domain (Azevedo et al., 2002). SGT1 CS domain interacts with CHORD II domain of RAR1 protein (Marta Bote¨ r et al., 2007) SGT1 associates with SCF ubiquitin ligase component (Azevedo et al., 2002). In recessive single sgt1 and rar1 mutant plants R protein level is decreased. (Holt et al., 2005) But the partial recovery of R protein levels is provided in Arabidopsis rar1 single and rar1 sgt1b double mutants which was explained by Azevedo et al., in 2006 EMBO journal article and this was because the control of R protein stability is conferred by RAR1 and SGT1, a compensatory SGT1b-like activity supplied by the closely related SGT1a homolog (Holt et al., 2005, Azevedo et al., 2006).

1.2.5 VIGS as a post transcriptional gene silencing mechanism in plants

Post transcriptional gene silencing (PTGS) is an RNA-based silencing used to suppress the expression of targeted genes in plants. It is also described as quelling in fungi (Cogoni et al., 1996) and RNA interference in animals (Fire et al., 1998). Several different techniques were developed to use PTGS machinery for functional analysis of genes. One of the developed methods is Virus Induced Gene Silencing (VIGS) (Baulcombe, 1999, Dinesh-Kumar et al., 2003) VIGS is an RNA-induced gene silencing system as a transient, fast and effective method. This method involves cloning of a short sequence of the plant gene to be silenced into viral delivery vector. Virus mediated gene silencing provides sequence specific gene knock out to determine the targeted gene function as a reverse genetic approach. General mechanism for RNA interference is also acting in VIGS (Figure 1.7). Firstly large amounts of double-stranded (dsRNA) RNAs are produced and the plant defense system cleaves those dsRNAs into 21-25 base pairs to form short interfering RNAs (siRNA). siRNAs are incorporated into RNA induced silencing complex (RISC) and the targeted RNAs with sequence similarity are degraded (Denli and Hannon 2003, see figure 1.7). To silence the plant genes some viruses were previously modified and used. One of the viruses modified for effective gene silencing is tobacco mosaic virus TMV which was used to silence the *Pds* gene in *Nicotiana benthamiana* plants (Kumagai et al., 1995.) Tobacco rattle virus (TRV) was also used as a silencing tool in *N. benthamiana* (Liu et al., 2002-1.) and in tomato by Liu et al., (Liu et al., 2002-2) The significant advantages of TRV-based VIGS in solanaceous species are the ease of introduction of the VIGS vector into plants. This is usually mediated by *Agrobacterium tumefaciens* with the VIGS vector placed between RB and LB sites of T-DNA (Ratcliff et al., 2001, Liu et al., 2002-2). Potato virus X (PVX) mediated gene silencing was also developed and used in *N. benthamiana* plant (Lu et al., 2003). Previously Holzberg et al., developed a virus system for efficient silencing of monocot plants. They have silenced the *Pds* gene in barley by using barley stripe mosaic virus (BSMV) (Holzberg et al., 2002). Later Scofield et al., used this BSMV system to silence the wheat genes (Scofield et al., 2005). BSMV is a positive sense RNA virus and contains tripartite genome with α, β, γ transcripts. The BSMV γ DNA vector used for plant gene cloning and β

genome was also modified for coat protein deletion. Each of modified DNAs used to synthesize RNAs by *in vitro* transcription.

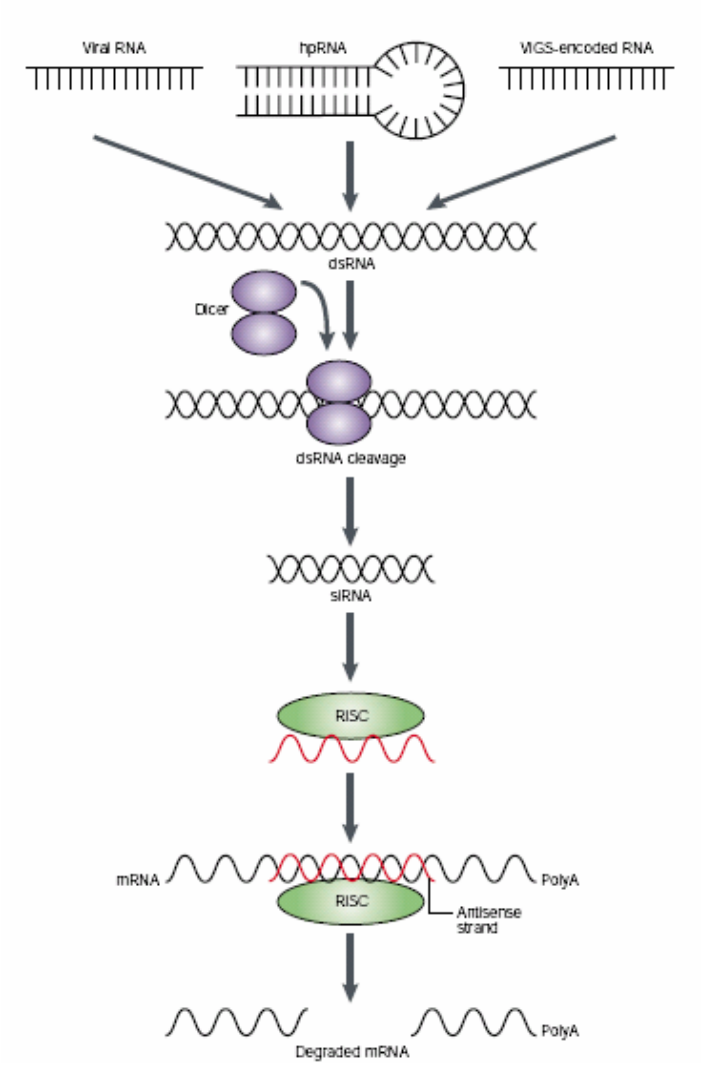


Figure 1.7 The RNA-mediated gene silencing model. Double stranded RNA (ds RNA) is generated from replicating viral RNA. In our case VIGS vectors (viral-vector-derived BSMV vectors) are used to produce viral ds RNA by *in vitro* transcription (Waterhouse and Helliwell, 2003).

1.3 Boron: Being an essential micronutrient and abiotic stress factor for plants

Abiotic stresses include excess or lack of nutrition such as metal ions, along with, drought, light, humidity etc. Like boron many metal ions are utilized in very minute amounts as essential nutrients. However, toxicity is shown when the excess concentration is present, resulting in abnormal symptoms together with abiotic stress responses. Both low and excess amount of micronutrients can suppress the growth, cell cycle, development, production, and survival. As a micronutrient, boron is one of the essential trace elements (Marschner 1995, Warington, 1923). It is found largely (96%) in the form of boric acid $B(OH)_3$ in biological fluids and in small amounts as borate ion. It is detected that the deficiency of boron lowers the productivity of many different crop species all over the world (Shorrocks, 1997). Both low and excess amount of micronutrients can suppress the growth of plants. Boric acid and borate can react with many kinds of biological compounds. Boric acid makes complexes with mono, di, poly-hydroxy compounds. Soluble boron polyol complexes also have been isolated (Hu et al., 1997) and rhamnogalacturonan-II boron (RG-II-B) complexes were isolated and characterized as well. Boron as a micronutrient is required primarily for plant cell wall integrity (O'Neill et al., 2001) and the deficiency of it hampers the productivity of 132 crops all over the world (more than 80 countries) (Shorrocks, 1997). The requirement of boron for plants was firstly detected in 1923 (Warington, 1923). Boron is involved in cell wall integrity maintenance as a borate ester rhamnogalacturonan II (RG-II), a cell wall pectic polysaccharide, and this cross-linking has been shown to be important for plant growth (O'Neill et al., 2001). Boron plays a role also in plant reproductive growth and development. Male sterility was detected in wheat (Rawson 1996), changes in sex ratio in mango (Singh and Dhillan, 1989) and poor seed quality (Brown et al., 2005) were detected upon deficiency of boron. It has been proposed that boron also is involved in many plant metabolisms. (Lukaszewski and Blevins, 1996) Boron has a role in membrane function and structure where membrane transport is affected by its deficiency and toxicity (Pollard et al., 1977; Ferrol et al., 1993).

The transport mechanism and uptake of boron is not well understood and in but has been shown to play a role (Dordas et al., 2000, Stangoulis et al., 2001). The

involvement of channel proteins for transport of boron was shown by Wasserman's group (Barone et al., 1997). These channel mediated and passive diffusion of boron transport were detected under adequate and greater boron supply. Moreover, the membrane permeability for boron uptake differs among the species, membrane types and membrane composition. Boron uptake is energy dependent as shown in a detailed study Dannel et al., showed the active transport of boron uptake using stable isotopes and different boron concentrations. An active-energy dependent transport of boron uptake was depending on limited boron concentrations (Dannel et al., 2002).

According to Takano et al. (2002), natural abundance of boron in soil is 30 μM and there is a critical very small range between deficiency and toxicity levels. Miwa et al., (2007) noted that the boron concentration of 15 mg/kg (~ 200 μM) is above the threshold for normal plant growth. Different kinds of plant species have different range of boron level requirements for effective growth and tolerance. For example bean is sensitive to low amounts of boron (Maas, 1987), however, boron toxicity is an important problem causing crop yield loss (Cartwright et al., 1986; Nable, 1988).

There are many genes and proteins known to be related with abiotic stress tolerance and deficiency that are involved in the mechanisms that cope with these unfavorable micronutrient conditions (Grover et al., 2001; Alves et al., 2006). Transport mechanism and uptake of boron are yet heavily researched and as a result, valuable data have been accumulated. *BOR1* gene was the first gene identified as a boron efflux type transporter in *Arabidopsis*. However, this gene was found to be providing tolerance to boron deficiency and not to excess boron, which was confirmed by *bor1-1* mutant and over-expression of *BOR1*. BOR1 is responsible for the transport of boron from root to shoot under low level of boron concentration (The *Arabidopsis* Information Resource code: At2g47160; Takano et al., 2002), but in higher levels of boron supply, this protein is degraded *via* endocytosis (Takano et al., 2005). *BOR1* overexpressed transgenic *Arabidopsis* plants provide improved plant growth and seed development under boron (B) limited medium but the transgenic plants do not improve the plant growth in toxic level of B conditions (Miwa et al., 2006). *AtBOR4* (The Arabidopsis Information Resource code:

At1g15460) a paralog of *BOR1,* was shown to be accumulated under very high toxic level (3000 μM) of B in transgenic plants. *BOR4* overexpressing plants which are tolerant to high toxic level (10 mM) showed improved growth. Lower level of B was detected in root and shoots of *BOR4* overexpressing plants than wild type plants (Miwa et al., 2007). One of the *BOR1* orthologs, *Bot1* in barley (landrace Sahara), has been detected as the gene responsible for boron toxicity tolerance. It has been proven that *Bot1* mRNA level is nearly 18 fold upregulated in tolerant barley roots and shoots compared to that of intolerant barley. The Bot1 protein has a role in limiting the entry of boron into root and in the disposal of boron from leaves *via* hydathode guttation (Sutton et al., 2007). Therefore, the *Bot1* of barley and *BOR4* of Arabidopsis share similar roles of boron efflux transporter. Another important gene product is an aquaporin protein (NIP5); it is a major B uptake channel (in influx transport) under low B condition in *Arabidopsis*. *NIP5* was detected as the gene which is up regulated in roots of *Arabidopsis* under limited B conditions. *NIP5* overexpressed *Xenopus laevis* oocytes showed the facilitated B transport. Additionally nip5 mutants showed lower boric acid uptake from soil, lower biomass production and increased susceptibility to boron deficiency, decreased plant growth and development under lower level of boron supply (Takano et al., 2006). Thus to this date, these genes are the most important ones found with roles in boron tolerance and deficiency.

Metal hyper-accumulator plants collect high amount of metal ions found in their natural habitats (Baker, 1981). The hyper-accumulator plants on the other hand can even be used to clean up the soil contaminants; the process is referred as phytoremediation (Yang X et al., 2005). Nevertheless my study is conducted in a plant species reported for **the first time as a hyperaccumulater of boron**, *Gypsophila perfoliata*. Not only it was observed that these species are growing successfully under 8900 mg/kg^{-} high total boron, but also it was shown that their leaves accumulate 1490$\pm$172 mg/kg (dry matter) boron (Babaoglu et al., 2004). Interestingly, the B content in the root and the stem of the plants contained only ~1/25th of the leaf B content. High level accumulation of B in leaves classifies *Gypsohila perfoliata* as a hyperaccumulater based on the criterion of Baker et al., (1981). The flora of gypsum areas in Turkey was revised in 2005 (Akpulat and

Celik, 2005). The only molecular study on *Gypsophila* genus to this date was DNA fingerprinting characterization of different species collected from various natural habitats (Hakki et al., 2006). The aim of preliminary study was to identify the genes expressed differentially in a high boron level in leaves. Although the results presented maybe premature, the genes identified in this study will not only provide a basis for further studies of these gene homologous in other plants, but also the study will direct the attention to *Gypsophila* species to identify new genes or to take advantage of *Gypsophila* species for phytoremediation (Yang X et al., 2005). Thus, it will stimulate the identification of boron transporters in roots.

Tolerance to the excess levels of boron in some plant species, which can be used for phytoremediation, is still not well understood. I need more detailed information about boron uptake and transport mechanisms at the molecular level to cope with the problems raised from both boron deficiency and toxicity. To this end, I attempted to identify genes expressing differentially on a high boron tolerant and accumulator plant, *Gypsophila perfoliata* (Figure 2.5).

1.4 Aim of the study

Functional genomics and proteomics have been widely used to understand the gene functions and the roles of proteins in cellular pathways. How do plants resist or tolerate the extreme conditions at the molecular level? To address that BSMV mediated gene silencing was performed in different plant-pathogen systems. Before the silencing of the gene of interest the VIGS in wheat was optimized using BSMV:PDS4As inoculation. This inoculation was resulted with efficient *Pds* gene silencing in different wheat plants. GFP expression was also successful by BSMV:GFP inoculations. Then using own-designed constructs BSMV:RAD6.2As the *Rad6* (gene of interest) gene was efficiently and reproducibly silenced in different lines of wheat and barley plants. All the silenced plants resulted in an unexpected phenotype: HR. Additionally, the level of some disease resistance regulatory components (*Rar1, Sgt,* and *Hsp90* genes) were analyzed in *Rad6* silenced wheat and barley plants by qRT-PCR. Using BSMV mediated silencing,

Rar1 and *Sgt1* genes were effectively silenced in wheat then the expression level changes of *Rad6* gene were measured by qRT-PCR.

I reached valuable data after silencing of *Rad6* in wheat and barley plants. To confirm the PCD/HR response in silenced plants with proteomics approach so 2-D PAG Electrophoresis was applied. Mass analysis results confirmed HR since HR related proteins were observed to be differentiated. These exciting results directed our interests to protein interaction studies to further investigate the hypothesis. Yeast two hybrid (Y2H) protein-protein interaction studies were performed to see if the RAD6 protein interacts with other disease resistance related components such as RAR1 and SGT1.

In conclusion, as a model organism, *Arabidopsis thaliana*, has been searched for *Rad6* gene mutants. Those mutants were tested for race specific pathogen resistance or susceptibility. On the other hand, hyperaccumulation or abiotic stress tolerance of boron was the other study of this thesis, as well. Due to the lack of information about the boron tolerance in literature at molecular level, by studying boron hyperaccumulator *Gypsophila perfoliata* plant, clues were tried to be found out. By addressing a question of genes are up or down regulated at high level of boron containing medium, which genes are differentially expressed in excess but tolerable and toxic level of boron containing growth medium. The differentially expressed genes under high level of boron using DDRT-PCR were performed then the results were confirmed with qRT-PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fungal inoculations:

2.1.1 Plant and pathogen materials

The powdery mildew resistant and susceptible barley plants Mla1, Mla6 HA- and Myc- tagged Golden promise seeds were provided by Dr. Paul Schulze Lefert from Max-Planct Institute. Powdery mildew pathogen of barley *Blumeria graminis hordei* Bgh95(53/01) and Bgh103(64/01) were obtained from Dr. Mogens Hovmoller of Danish Institute of Agricultural Sciences. They were amplified in our laboratory and selected on the differential barley lines 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 of yellow rust agent *Puccinia striiformis tritici* Pst169E136 / Pst232E137 set were provided by Dr. Lesley A. Boyd of John Innes Center, UK.

2.1.2 Plant growth and infection conditions

2.1.2.1 Yellow rust inoculations on wheat

The Pst169E136 and Pst 232E137 infected Avo-Yr1 (having *Yr1* gene) 15 day old seedlings were prepared for qRT-PCR analysis. Two weeks old Avocet-Yr1 seedlings were infected with 169E136 (virulent) and 232E137 (avirulent) using freshly generated spores. Both plants were mock inoculated without yellow rust spores. 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 24 hai and stored at -80 ^{o}C.

2.1.2.2 Powdery mildew inoculations on barley

Pallas 01 *Mla1 R* gene containing barley and Bulbul (susceptible) barley plants were inoculated with powdery mildew fungus spores Bgh95(53/01) (virulent) and Bgh103(64/01) (avirulent) on 10 day old Pallas-01 (*Mla1* gene having) barley plants. Bgh103(64/01) inoculation was done on susceptible 10 day old Bülbül cultivar. Infected seedlings were incubated at 17 ^{o}C at extreme high humidity for 24 hrs for the 16 hrs day and 8 hrs dark periods. Following the incubation period, the normal growth conditions were set to 17 ^{o}C at the same time and night periods.

2.2 RNA isolation from plant leaf tissue

In this thesis total RNAs of all the plant materials (wheat, barley, *Gypsophila perfoliata*) were extracted from the collected leaf samples using Trizol® reagent (Invitrogen, CA, USA) according to suggested procedure by the manufacturer. Total RNAs from leaf samples that belong to specific time points (silenced, mock, BSMV control, pathogen infected, mock infected, treated and untreated control) of the plants were isolated individually. Tissue samples were powdered using a mortar in liquid nitrogen. Powdered tissues were homogenized in 1 mL of TRIzol reagent per 100 mg of tissue in eppendorf 1.5 or 2 mL sterile tubes. Homogenized samples were incubated for a few minutes at room temperature. 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 10 to 15 seconds and incubated at room temperature for 5 min. The samples were centrifuged at 15,300 rpm for 15 min at 4 ^{o}C. Following centrifugation, RNA remains exclusively in the aqueous and colorless upper phase. The upper phase was transferred to a fresh sterile tube. Isopropyl alcohol was used for precipitation of RNA from the aqueous phase by mixing with. 0.5 mL of isopropyl alcohol used per 1 mL of TRIzol reagent. Samples were incubated at room temperature for 10 min and centrifuged at 15,300

rpm for 10 min at 4 ^{o}C. The RNA precipitates were observed as pellet on the bottom of the tubes. Supernatants were removed and the RNA pellets were washed out with 75% ethanol, adding at least 0.75 mL of 75% ethanol per 1 mL of used TRIzol reagent. Samples were mixed by vigorous hand shaking and centrifuged at 10.000 rpm for 6 min at 4 ^{o}C. The RNA pellets were briefly dried (10 – 15 min) after decanting upper phase. RNAs were dissolved in sterile water and incubated for 5 to 10 min at 55 ^{o}C-60 ^{o}C and stored in -20 ^{o}C.

2.3 Concentration determination of the isolated RNA samples

RNAs were dissolved approximately 40-50 µL with sterile water. Only 1 µL of sample was used for concentration determination on NanoDrop ND-1000 spectrophotometer. The intactness of RNA was checked by running RNA samples on 2 % formaldehyde-agarose gel and for same samples and Agilent 2100 Bioanalyzer chip run system.

2.4 Synthesis of first strand cDNA

Following components were combined in a sterile PCR tube: 50 pmol oligo dT(20), total RNA (1000 ng-1500 ng), 0.1 mM dNTPs (Fermentas) mix, sterile distilled water up to 12 µl. Mixture was incubated at 65 ^{o}C for 5 min and quickly chilled on ice for 2 min. Following components were added: 4 µL 5X first strand buffer, 1 µL 0.1M DTT, 1 µL 40 U RNase inhibitor (Invitrogen CA, USA). The content of the tube was spinned briefly and incubated at 50 ^{o}C for 2 min, finally 200 U of SuperScript III^{TM} (Invitrogen CA, USA) Reverse Transcriptase enzyme was added. Reaction were carried out at 50 ^{o}C for 90 min and stopped by incubating at 70 ^{o}C for 15 min.

2.5 qRT-PCR and measurement of *Rad6* gene expression level changes

The measurements of *Rad6* transcripts of inoculated and mock inoculated barley and wheat plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs of the 24 hpi leaf samples of virulent, avirulent and mock inoculated wheat and barley silenced plants have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample per primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA* (GenBank accession number: X16077.1) 18s rRNA forward and 18S rRNA reverse primer pairs (5'TTTGACT CAACACGGGGAAA3' and 5'CAGACAAATCGCTCCACCAA3' respectively) for real-time PCR normalization. *Rad6* gene qPCR analyses were performed by the Rad6 Forward 5' GCGAGAGTACAACCGCAAAG3' and Rad6 reverse 5' GCGAGAGTACAACCGCAAAG3' primers designed using Primer3 software (Rozen and Skaletsky, 2000). 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 according to Pfaffl's model (Pfaffl, 2001). For expression analysis the mock inoculated and fungal inoculated plant *Rad6* gene transcript levels were compared. Prior to performing the qRT-PCR for the gene expression level change the standard curve has been calculated as follows; The Standard Curve is a plot of the initial template quantity in the Standard wells (X-axis), versus the Ct (threshold cycle). A standard curve consists of at 3 to 4 points, and each concentration should be run at least in triplicate. A linear standard curve provides the efficiency of qRT-PCR amplification. To perform the standard curve analysis, our initial template which was assumed to be 8X concentration then the serial dilutions have been applied to the sample as 4X, 2X and 1X. Templates were used for 18 s rRNA reactions they were expected as 2 fold, 4 fold, and 8 fold differences comparing to each other. Then the amplification plots were calculated. One hundred percent efficiency implies perfect doubling of amplicon each cycle.

2.6 Barley Stripe Mosaic Virus (BSMV) vectors for silencing

BSMV vectors pα, pβΔβa, pγ and pγ.bPDS4S (sense orientation) and pγ.bPDS4As (anti-sense orientation) (Figure 2.1) were obtained from Large Scale Biology Corporation (CA, USA). Vectors were transformed into competent *E.coli* DH5α strains as described in Section 2.6.3.3. Amplified plasmids were isolated and purified as described in Section 2.6.3.4. The pγ.bpds4As was used to construct pγRad6.2As by replacing pds4 insert with 185 bp Rad6.2 fragment by restriction digestion.

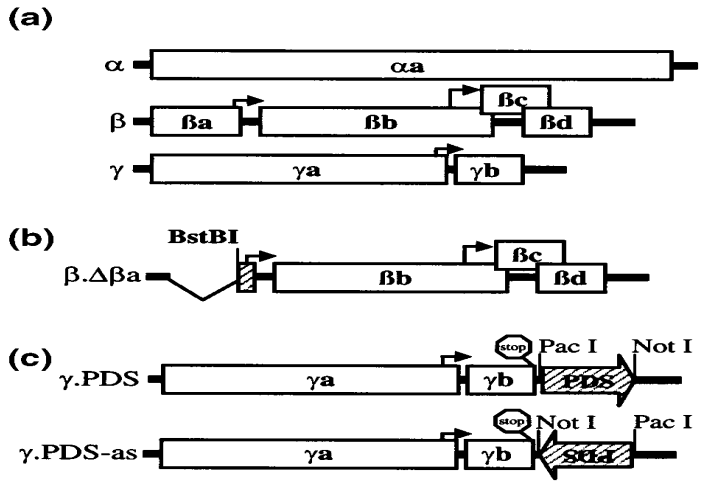


Figure 2.1 BSMV RNA derived vectors. **(a)** pα, pβ and pγ genomes of BSMV. **(b)** pβΔβa representing the deleted version of pβ genome. **(c)** pγ.bpds4S and pγ.bpds4As orientations The picture was taken from Holzberg et al. 2002.

pγ.bpds4As

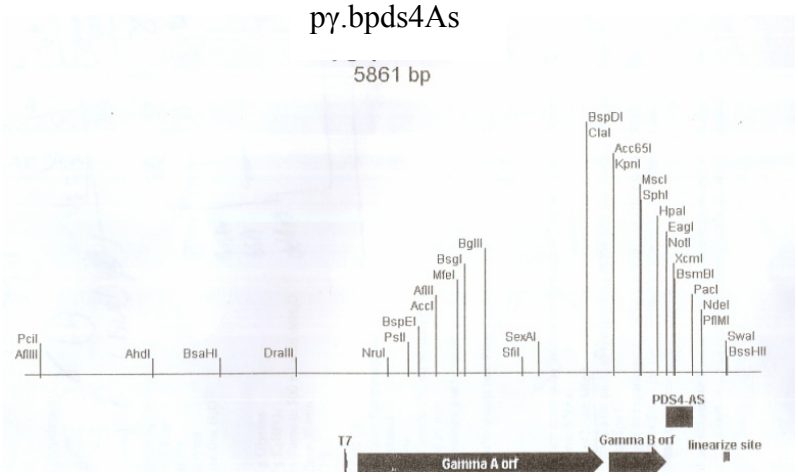


Figure 2.2 pγ.bpds4As map representing restriction cleavage sites.

2.6.1 *Pds* gene silencing of wheat and quantification of silencing levels

2.6.1.1 Linearization of plasmids

pα, pβΔβa, pγ, pγ.bpds4S and pγ.bpds4As plasmids were digested with restriction enzymes in order to be linearized. pα plasmid DNA was digested with *Mlu*I enzyme (MBI fermentas). 10 µg purified pα plasmid DNA, 1X RE buffer (MBI fermentas), 10 U *Mlu*I enzyme (MBI fermentas) and PCR water were combined in a PCR tube to a final volume of 50 µl. Mixture was incubated at 37 °C for 2 hrs. pβΔβa plasmid DNA was digested with *Bcu*I or *Spe*I enzyme (MBI fermentas). 7 µg purified pβΔβa plasmid DNA, 1X yellow enzyme buffer (MBI fermentas), 10 U *Bcu*I (MBI fermentas) and PCR water were combined in a PCR tube to a final volume of 50 µL. Mixture was incubated at 37 °C for 2 hrs. pγ plasmid DNA was digested using *BssH*II enzyme (New England Biolabs). 10 µg pγ plasmid DNA, 1X NEB3 Buffer (New England Biolabs), 10 U *BssH*II enzyme (New England Biolabs) and PCR water were combined in a PCR tube to a final volume of 50 µl. Mixture was incubated at 50 °C for 3 hrs. After the incubation samples were

observed on 1 % agarose gel. Linearized plasmids were purified. The purification was made according to PCR purification kit of QIAGEN (Cat no: 28104).

2.6.1.2 *In vitro* transcription of linearized vectors

All of the α, βΔβa and γ linearized genomes were transcribed *in vitro* according to manufacturer's procedure the mMessage mMachine T7 *in vitro* transcription kit (cat no: 1340, Ambion, Austin, TX). Following components were combined in a 500 µL sterile PCR tube: separately for each plasmids (pα-*Mlu*I, pβΔβa-*Bcu*I, pγ-*BssH*II or pγ.bpds4S-*BssH*II and pγ.bpds4As-*BssH*II) per one silencing reaction 80 ng template was used (linearized plasmid DNA), 1X Buffer (Ambion), 1X nucleotide mix with NTP Cap (Ambion), 0.15 µl of T7 RNA polymerase mix (Ambion) and sterile distilled water up to 1.5 µl. Mixture was incubated at 37 ^{o}C for 2 hrs and stored at -80 °C until use.

2.6.1.3 Inoculation of plants with BSMV transcripts

Avo-Yr1, Avo-Yr1, Myc-Mla1/Golden promise, Myc-Mla6/Golden promise and Bulbul barley plants were used for BSMV mediated PTGS. The second leaves (approximately 7-10 days upon germination) were inoculated with BSMV for silencing. Transcripts of each of the BSMV transcripts were mixed in a 1:1:1 ratio (1.5 µg of each transcript concentration was determineted on NanoDrop ND-1000 spectrophotometer). Transcription mix was combined with 45 µL FES (50 mL 10X GP (18.77 g glycine, 26.13 g K_2HPO_4, ddH_20 upto 500 mL, sterilized by 20 min autoclaving, 2.5 g sodium pyrophosphate, 2.5 g bentonite, 2.5 g celite and ddH_20 up to 250 mL (Pogue et al. 1998) and directly applied, with two light strokes to the second leaf (when it is 5-7 cm long) from the bottom of leaf to the tip. A systemic spread was determined by the appearance of mosaic symptoms on leaves after 7-10 days post inoculation (dpi). Leaves from inoculated plants were collected after 7, 14, and 21 dpi in order to check *Pds* gene silencing level by qRT-PCR. RNAs were isolated then cleaned up.

	pα	pβΔβa	pγ	pγ.bpds4S	pγRad6.2As	FES
BSMV:00	1.5 µg	1.5 µg	1.5 µg	-	-	45 µL
BSMV:PDS4S	1.5 µg	1.5 µg	-	1.5 µg	-	45 µL
BSMV:RAD6.2As	1.5 µg	1.5 µg	-	-	1.5 µg	45 µL
FES	-	-	-	-	-	45 µL

Figure 2.3 Transcripts of each of the BSMV inoculation were mixed in a 1:1:1 ratio.

2.6.1.4 Clean up of total RNA for qRT-PCR analysis

Total RNAs of Pds silenced samples of the 3rd leaves from 7th and 14th dpi and 4th leaves of 21 dpi were treated with RNAse free-DNAse and precipitated with Lithium chloride. DNAse treatment was performed using 1 U of Turbo DNAse (AMBION INC. Lott 095K81). Following components were combined in a 500 µL sterile PCR tube: 10-15 µg RNA was used for treatment. Samples were incubated at 37 °C for 15 minutes and then reaction was inactivated by heating for 10 minutes at 65 °C. For further purification of RNA from the mixture, 2.5 M lithium chloride (Ambion salt, 7.5M, #9480) was used, equal volume of (DNAse treated) mixture, sterile water and 7.5 M lithium chloride (1:1:1) were added on sterile eppendorf tube. Then tubes were frozen at -20 °C for minimum of 60 minutes. Samples were mixed by and centrifuged at 15.300 rpm for 15 min at 4 ^{o}C. Following centrifugation, supernatant was removed and pellets were washed with 70% ethanol made with nuclease free water. Finally, samples were centrifuged at 10.000 rpm for 6 min at 4 ^{o}C and pellets were re-suspended in 15 µL of nuclease free water.

2.6.1.5 Measurement of *Pds* gene expression *via* qRT-PCR

All of the RealTime qRT-PCR experiments was performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Same procedure was applied in 2.5 for expression level determination of *Pds* gene we used the primers designed for *Pds* gene of wheat for

qRT-PCR determination. TaPDSqPCR F 5'CCCTGACGAGTTATCCATGCAG3' and TaPDSqPCR R primers 5'GGACCTCACCACCCAAAGACT 3'.

2.6.2 GFP expression by BSMV in wheat

The pγGFP vector was used constructed by Holzberg et al. (2002). this vector was also transformed and linearized with *BssH*II enzyme. Using mMessage mMachine T7 Ambion kit *in vitro* transcription was performed for α, βΔβa and γGFP linearized genomes. All the transcripts were applied on Avo-Yr1 and Avo-Yr10 wheat lines with FES as described in section 2.6.1.3 then the GFP expression visualized by Leica flourescent microscope DFC 280 digital camera system.

2.6.3 *Rad6* gene silencing in wheat and barley

2.6.3.1 *Rad6* gene amplification from wheat cDNA clone for VIGS

Previously the full length of *Rad6* gene had been cloned from wheat then using *Pac*I restriction cleavage site containing forward 5' ATAGCGGCCGC GCGACTGGATAGATGTAAGTAT3' and *Not*I site containing reverse primers 5' ATATTAATTAAGGCACGTTTAAGCTGACTCTCCAG 3' 185 bp length *Rad6* fragment was amplified from wheat (*Triticum aestivum*) plants. Annealing temperature for PCR amplification using primers was the 59 ^{0}C. 30 µL of final volume and following components were mixed; 2 µl diluted plasmid DNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 30 µl volume. PCR cycling conditions were 94 °C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min. Amplified PCR products were loaded on 1% agarose gel and analyzed. PCR products were purified and then ligated into pGEM-T easy vector for amplification and sequencing analysis.

CATTATGCTGAGTGATATCTTTTTTTTTCAGAATCAATGTGATATGATACAACATTACAA
ACTCACACAAGACGTTACAAAATGTTTGCGTTCCAGATACAGACAGTGTAGACACAGGCC
ATGACAGAGCAATCTCCAACCGTCCAGCCAAACGATGCAACAAAGCAAGTTGCTGTTTCA
GGTGGTAACGACAGAGTTCAGTCATTTTAGTTACAAACATTTTGGTACAGAAACCGCATG
ATAACACATGGTGAGCTCAACTTATTAGTCTGCCGTCCAGCTCTGCTCAACAATCTCCCG
CACTTTGCGGTTGTACTCTCGCTTGTTCTCACTGAACATGCGGGCAGCTTCTGAGTTAGC
AGGCGAATTTGGGTTAGGATCACACAGCAGCGACTGGATAGATGTAAGTATAGCAGCTAC
ATCATATATTGGGCTCCACTGATTCTGTAGAATATCTAAGCATATGCTTCCATCAGCATA
AATGTTAGGGTGAAACATCCGAGAAATAAACCGAACTGTTGGTGGCTTATTAGGATATTC
TTCATTAAACTGGAGAGTCAGCTTAAACGTGCCTCCATCCCACGGGCTATCGTCAGGGCC
AAAAATCACAGCATTCCACAGCATTATGTTGTTGTCCTGCGGCGCCCCGCTTATGCCCGC
AGGAGGGTCCTGCATCAGCCGCTTGAAGTCCCTCATCAGCCTCTTCCTTGAAGGAGTCGA
CATCCTCGAAATTCTCTTAGACTGCGGCCGCTC

Figure 2.4 Sequence of the clone of *Rad6* gene representing the sequence used to make silencing construct (colored region) and pGEMT-Easy vector sites (underlined).

2.6.3.2 Ligation of amplified *Rad6* gene fragments to pGEM-T-Easy vector

Amplified PCR product was ligated with pGEM-T-Easy vector (Promega) with a final volume of 10 µl in an eppendorf tube. Following components were combined; 6 µl PCR product, 5 ng pGEM-T-Easy vector (Promega), 1 x Ligase Buffer (Promega) and 2 Us T_4 DNA Ligase enzyme (Promega). Mixture was incubated at 4°C overnight (~ 18 hours).

2.6.3.3 Transformation of ligation products

Prior to transformation of plasmid into the cells, *E.coli* Dh5-α cells were made competent. A single colony of *E.coli* cells was inoculated into 2 mL LB medium without any antibiotic. Cells were let grown at 37 °C with shaking (200-250 rpm) 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 A_{590nm} of 0.375. Culture was aliquated into two 50-mL tubes and put in ice for 10 min. Cells were centrifuged for 10 min at 4000 rpm. Supernatant was poured off and each pellet was re-suspended in 5 mL ice-cold sterilized $CaCl_2$ (4mM) solution. Cells were centrifuged for 5 min at 2500 rpm and supernatant was discarded. Each pellet was re-suspended again in 2 mL of ice-cold $CaCl_2$ (4mM) solution. The centrifugation was

repeated once. Each pellet was re-suspended in 2 mL of ice-cold $CaCl_2$ (4mM) solution. Cells were suspended in fresh 2 mL 4mM $CaCl_2$ dispensed into sterile eppendorf tubes, keep their ability for efficient transformation up to two weeks at 4°C. *E.coli* Dh5-α competent cells were transformed with ligation products. In a sterile 2 mL tube following components were combined; 5 µL ligation product, and 50 µL *E.coli* Dh5-α competent cells and placed on ice for 30 min, and then heat shocked at 42 °C for 45 sec. SOC or LB liquid medium was added up to 200 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 and ampicilin (50 µg/mL) for selection, X-gal (80 µg/mL) for blue/white screening and IPTG 100 µg/mL for induction of cell growth). Plates were incubated at 37 °C overnight. After the incubation, white colonies were picked selected among grown colonies and these were transferred to 200 µl LB medium containing sterile tubes with final concentration of 40% glycerol to make stock and use for plasmid isolation. Selected colonies were PCR amplified with the specific Rad6 primers for confirmation of cloning.

2.6.3.4 Plasmid isolation of the selected colonies

Plasmids were isolated using Qiagen QIAprep Spin Miniprep Kit (Cat no: 27104) according to the kit protocol. Bacterial culture of 2 mL was harvested by centrifugation for 2 min at 14,000 rpm. Supernatant was decanted; tube was inverted and blotted to on a paper towel to remove excess media. 250 µL of Buffer P1 (RNAse added) was added on to the collected cells which were suspended by vortexing up to no visible cell clumps seen. 250 µL of Buffer P2 was added, then the tube content was mixed 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 14.000 rpm for 10 minutes. A spin column, provided by manufacturer, inserted in to collection tube. Cleared lysate, the upper phase was transferred to the spin column, centrifuged at 14.000 rpm for 1 minute. 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 14,000 rpm for 1 minute, flow-through discarded and the collection tube reinserted and to remove residual wash buffer completely additional 1 min centrifugation was involved. The spin column was transferred to a new sterile tube, the plasmid DNA was eluted by adding 50 µl of Buffer elution buffer and centrifugated at 14.000 rpm for 1 min. Spin column assembly was removed and plasmid DNA was stored at –20 °C. All the centrifugations were applied at room temperature.

2.6.3.5 Ligation of *Rad6* amplified fragment with pγ-antisense vector

*Not*I and *Pac*I engineered *Rad6* gene fragment for silencing and pγPDS4As plasmids were double digested with the *Not*I and *Pac*I restriction enzymes. Following components were combined in eppendorf tubes for digestion. *Not*I and *Pac*I (NEB) 10 U each, 1X NEB2 buffer, template (Rad6 PCR product 3 µg and 3 µg insert excised, purified pγ plasmid). The mixture was incubated at 37 °C for 2 hours. Ligation of Rad6 *NotI* and *PacI* engineered fragment and restriction digested pγAs plasmid was performed as Section 2.6.3.2. The ligation product was transformed into competent, *E.coli* Dh5-α following presented procedure in Section 2.6.3.3. Right colonies were selected and the plasmids were isolated in order for linearization later to use in *in vitro* transcription.

2.6.3.6 *In vitro* transcription of linearized vectors

All of the α, βΔβa and γ linearized genomes were *in vitro* transcribed as described in Section 2.6.1.2. BSMV:00 inoculation is the combination of α, βΔβa and γ transcripts. BSMV:RAD6.2As inoculation is the combination of α, βΔβa and pγRad6.2As transcripts. In result and discussion sections the figure 3.16 **a** and **b** show the linearized and *in vitro* transcribed α, βΔβa, γ, pγRad62As.

2.6.3.7 Inoculation of plants with BSMV transcripts

Avo-Yr1, Avo-Yr10 wheat plants, Gloden promise Mla1-Myc, Mla6-Myc and Bulbul barley lines were inoculated with BSMV:00 and BSMV:RAD6.2As as described in Section 2.6.1.3. Barley BSMV:00 and BSMV:RAD6.2As inoculated samples collected at 7 dpi for fungal infection controls. In order to be qRT-PCR and measure the level of *Rad6* gene silencing all the wheat and barley samples were collected at 15 dpi. RNA isolations and cleanings were performed then cDNA syntheses were completed as previous Section 2.4.

2.6.3.8 qRT-PCR and measurement of *Rad6* gene expression

Section 2.5 describes the level determination of *Rad6* gene expression. Using this procedure the silencing of *Rad6* levels were detected as relative quantification. BSMV:00 inoculated plant samples at same time and same conditions with BSMV:RAD6.2As have been compared with respect to *Rad6* gene expression levels.

2.6.3.9 qRT-PCR detection of expression levels in genes known to be involved in plant disease resistance upon *Rad6* gene silencing

The measurements of *Rad6* transcripts of inoculated and mock inoculated barley and wheat plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). qRT-PCR experiments were performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA* (GenBank accession number: X16077.1) the reactions were performed as Section 2.5. For *Rar1* gene expression level determination in *Rad6* gene silenced and non-silenced plants qRT-PCR primers were used as Forward 5' TGATGGCATGAAAGAGTGGA 3' and Reverse 5' TGGACTGGAGCTGACTTTGG 3'. For *Sgt1* gene expression level determination in *Rad6* gene silenced and non-silenced plants qRT-PCR primers were used as Forward 5' AGCTTTTGCCAGGCGTATTT 3' and Reverse 5' GCGTGGTTGTTGACTTTGGT 3'. For *Hsp90* gene expression level determination in *Rad6* gene silenced and non-silenced

plants qRT-PCR primers were used Forward 5' CGACCAGCACGCTCACGAT 3' and Reverse 5'GCGATGGTCCCGAGGTTGT 3'.

2.6.3.10 Trypan blue staining for silenced, unsilenced and fungal inoculated wheat and barley lines

Trypan blue staining was performed as Koch s procedure, virus inoculated (*Rad6* silenced) and fungal inoculated (Bgh95(53/01) and Bgh103(64/01)) plant leaf tissues were collected and boiled for 2 min in alcoholic lactophenol trypan blue (20 mL of ethanol, 10 mL of phenol, 10 mL of water, 10 mL of lactic acid (83%), and 10 mg of trypan blue). Stained leaves were cleared in chloral hydrate (2.5 g dissolved in 1 mL of water) overnight at room temperature (Koch and Slusarenko, 1990).

2.6.3.11 Auto-flourescence for silenced, unsilenced and fungal inoculated wheat and barley plants

Avo-Yr1, Avo-Yr10 wheat plants, Myc-Mla1/Gloden promise, Myc-Mla6/Gloden promise and Bulbul barley plants were inoculated with BSMV:00 and BSMV:RAD6.2As and fungal inoculated barley plant leaves were visualized with Leica Model DCM4000 B fluorescent microscope (Leica DFC 280 camera) at 40 X and 10X magnification.

2.6.3.12 Histochemical DAB (3,3'-diaminobenzidine) staining

Avo-Yr10 and Avo-Yr1 wheat *Rad6* gene silenced and BSMV:00 inoculated control plants were stained with DAB liquid chromogen. HR like symptoms were detected by DAB staining according to Thordal-Christensen, 1997 on the silenced plant leaves. 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.

2.6.4 *Rar1* gene silencing in wheat plants

2.6.4.1 *Rar1* gene cloning from wheat

Rar1 gene was cloned as full length from Avo-Yr10 wheat plant cDNA. The amplification was performed by using Rar1 specific forward 5' ATGTCGGCGGAGACGGAGAGG 3' and reverse 5' TCACACAGCATCAGCATTGTG 3' primers. Annealing temperature for PCR amplification of those primers was the 58 ^{o}C. 30 µl of final volume and following components were mixed; 2 µl Avo-Yr10 cDNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 30 µL volume. PCR cycling conditions were 94°C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72 °C for 2 min. Amplified PCR products were loaded on 1% agarose gel and analyzed (Figure 3.36). PCR products were purified and then ligated with pGEM-T easy vector for amplification and sequencing analysis.

2.6.4.2 *Rar1* gene amplification for VIGS construction and BSMV inoculations

Previously the full length of *Rar1* gene was cloned from wheat then using *Pac*I restriction cleavage site containing forward 5' ATAGCGGCCGCGGGT TGCCTTTGAGGAGTT 3' and *Not*I site containing reverse primers 5' ATATTAATTAAGCATGATGGCATGAAAGAG 3' 148 bp length *Rar1* fragment was amplified from wheat (*Triticum aestivum*) plant's cDNA clone. Annealing temperature for PCR amplification of those primers was the 59 ^{0}C. 30 µL of final volume and following components were mixed; 2 µl diluted plasmid DNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer

sterile distilled water up to 30 µl volume. PCR cycling conditions were 94 °C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min. Amplified PCR products were loaded on 1% agarose gel and analyzed. PCR products were purified and then ligated with pGEM-T easy vector for amplification and sequencing analysis. Using same procedures with 2.6.3.2 to 2.6.3.6 pγRar1.1As clone was constructed and linearized. α, βΔβa, γ, pγRar1.1As *in vitro* transcriptions were done and Avo-Yr10 plants were inoculated with BSMV:RAR1.1As.

2.6.4.3 qRT-PCR and measurement of *Rar1* and *Rad6* gene expression level changes in BSMV:RAR1.1As silenced wheat

The measurements of *Rar1* and *Rad6* gene expression of *Rar1* gene silenced and BSMV:00 wheat plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs of the 15 dpi leaf samples have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA* (GenBank accession no: X16077.1). *Rar1* qRT-PCR analyses were performed by the Rar1 Forward 5' TGATGGCATGAAAGAGTGGA 3' and Rar1 reverse 5' TGGACTGGAGCTGACTTTG 3' primers.

2.6.5 *Sgt1* gene silencing in wheat

2.6.5.1 *Sgt1* gene cloning from wheat

Sgt1 gene was cloned as full length from Avo-Yr10 wheat plant cDNA. The amplification was performed by using Sgt1 specific forward 5' ATGGCC GCCGCCGCCGCGTCG 3' and reverse 5' TTAATACTCCCACTTGAGCTC 3' primers. Annealing temperature for PCR amplification of those primers was the 58.5 ^{o}C. 30 µl of final volume and following components were mixed; 2 µl Avo-Yr10 cDNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 30 µL volume. PCR cycling conditions were 94 °C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min. Amplified PCR products were loaded on 1% agarose gel and analyzed (figure 3.41). PCR products were purified and then ligated with pGEM-T easy vector for amplification and sequencing analysis.

2.6.5.2 *Sgt1* gene amplification for VIGS construction and BSMV inoculations

The full length of *Sgt1* gene has been cloned from wheat then using *Pac*I restriction cleavage site containing forward 5' TATGCGGCCGCTTAATA CTCCCACTT 3' and *Not*I site containing reverse primers 5' ATATTAATT AAGGGGAAAAAGACGGTTGAA GGA 3' 83 bp length *Sgt1* fragment was amplified from wheat (*Triticum aestivum*) plant's cDNA clone. Annealing temperature for PCR amplification of those primers was the 59 ^{o}C. 30 µl of final volume and following components were mixed; 2 µl diluted plasmid DNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer sterile distilled water

up to 30 μL volume. PCR cycling conditions were 94 °C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min. Amplified bands were loaded on 1% agarose gel and analyzed. PCR products were purified and then ligated with pGEM-T easy vector for amplification and sequencing analysis. Using same procedures with 2.6.3.2 to 2.6.3.6 pγSgt1.1As clone was constructed and linearized. α, βΔβa, γ, pγSgt1.1As *in vitro* transcriptions were done and Avo-Yr10 plants were inoculated with BSMV:SGT1.1As.

2.6.5.3 qRT-PCR and measurement of *Sgt1*, *Hsp90*, *Rar1,* and *Rad6* gene expression level changes in BSMV:SGT1.1As silenced wheat

The measurements of *Sgt1*, *Hsp90*, *Rar1,* and *Rad6* gene expressions of *Sgt1* gene silenced and BSMV:00 wheat plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs of the 15 dpi leaf samples have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA*. *Sgt1* qRT-PCR analyses were performed by the Sgt1 Forward 5' AGCTTTTGCCAGGCGTATTT 3' and Sgt1 reverse 5' GCGTGGTTGTTGACTTTGGT 3' primers. Other primers were used for qRT-PCR determination of expression level changes was written in section 2.6.3.9.

2.7 2-D PAGE proteomics studies of *Rad6* gene silenced Avo-Yr10 wheat lines

BSMV:RAD6.2 As silenced Avo-Yr10 plant leave samples and BSMV:00 inoculated positive control Avo-Yr10 wheat plant leave samples were used for 2D PAGE. The differentially expressed protein determination was performed by Proteome factory (http:/www.proteomefactory.com Proteome Factory AG, Dorotheenstr. 94 D-10117 Berlin) following the procedure given in the website.

2.8 *Arabidopsis Rad6* gene mutant search and oomycet inoculation

Arabidopsis in its genome has two copies of *Rad6* gene which are called as Rad6.1 and Rad6.2. Two mutant lines of *Arabidopsis* were subjected to be found for which one is important in disease resistance. Characterization of the T-DNA Insertion Lines was performed and confirmed using the protocols described by Siebert et al. (1995). The location of the T-DNA insertions within each line was originally identified at the Salk Institute Genomic Analysis (La Jolla, CA) and Syngenta Arabidopsis Insertion Library Line Information (SAIL). Mutant plants were related with the Rad6-1 (SALK Accession no: 060994) and Rad-2 (SAIL Accession no: N622055) gene in *Arabidopsis*, respectively. All the lines were grown and their DNAs (DNA extractions were performed by following manufacturer's procedure, Red Extract-N-Amp tissue PCR kit Sigma, USA, Cat # XNATS) were isolated. PCR reactions were performed with specific primers flanking the T-DNA region and gene specific regions. Homozygote mutant plants were selected by PCR (Rad6-1 gene mutants were analyzed by SALK-060994-LP: GTCCAGCTTTGTTTGTTCGACAAC and SALK-060994-RP: GATTCAAATGGTTTGAG) (Rad6-2 gene mutants were analyzed by SAIL-N622055-LP: CGCTTGAAAAGCCAACTTAAG and SAIL-N622055-RP: GTAAAGTGTCCCTGGAGCTCC) and seeded for further analysis. Two different *Hyaloperonospora parasitica* (Oomycete) strains were used for phytopathologic tests one of them (Emoy2) was avirulent on *Arabidopsis* Col8 and the other virulent (Noks1) on *Arabidopsis* Col8. *Arabidopsis Rad6* mutants were tested for virulent and avirulent *H. parasitica* infections. *Arabidopsis* seedlings were grown on soil (10-14 day old seedlings were inoculated) in a clean growth chamber (10 h day, 14 h night at 21°C) and spray inoculated with *H. parasitica* isolates. After 7 to 8 days pathogen growth of downy mildew was observed.

2.9 Yeast-two hybrid (Y2H) studies

2.9.1 Gateway cloning of *Rad6, Rar1, Sgt1, Yr10-1506, Yr10-1562*

Avo-Yr10 wheat plant cDNA was used as template for amplification of genes and gene fragments. pDONR™/Zeo (cat no:12535-035) Invitrogen gateway vector was used for getting entry clones. The genes were amplified from wheat were *Rad6*, *Sgt1*, *Rar1,* Yr10-1506 (first 1506 bp region of the *Yr10* gene) and Yr10-1562 (last 1562 bp region of the *Yr10* gene). Total size of *Yr10* gene is 2475 bp (GenBank accession no: AF149114). The primers which are listed in Table 2.1 were used for appropriate Gateway cloning. PCR amplifications were performed as in Section 2.6.5.2 using Avo-Yr10 cDNA as a template.

Table 2.1 List of primer sequences used in Gateway cloning system to be able to perform Y2H one-to-one interaction studies.

Primer name	Primer sequence (5'-3')	T_m (^oC)
Attb1 Rad6 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCGACTCCTTCAAAGGAA	66,0
Attb2 Rad6 R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTCTGCCGTCCAGCTCTG	65,9
Attb1 Sgt1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCCGCCGCCGCCGCGTC	65,0
Attb2 Sgt1 R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAATACTCCCACTTGAGCTC	67,0
Attb1 Rar1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCGGCGGAGACGGAGAGG	66,8
Attb2 Rar1R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCACACAGCATCAGCATTGTG	67,5
Attb1 Yr10-1506 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGAGGTCGTGACCGGGGCG	67,0
Attb2 Yr10-1506 R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCGTGTACACGGACAGAGCT	68,5
Attb1 Yr10-1562 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTAGTAGAGTAATCGCAAC	68,0
Attb2 Yr10 1562 R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGCGTGGAGTTACCTTCAC	66.9

2.9.1.1 BP reactions for gateway cloning

Amplified genes and gene fragments (Figure 3.43 and 3.45). Those amplification products were purified and used for BP reaction. Gateway® BP

Clonase™ II Enzyme Mix (Cat. No. 11789-020) was used for ligation. In order to produce entry vector, 7 μL *att*B-PCR product (150 ng) was added into eppendorf tube with 1 μL pDONR/Zeo vector (150 ng) and 2 μL BP enzyme mix, then the mixture was incubated in 25 ^{o}C for 1 h. Transformation of ligated product was performed as described in Section 2.6.3.3 *ccdB* survival and kanamycin resistant cells were selected. Plasmid isolations were performed as in Section 2.6.3.4 then the sequences were analyzed with EditSeq (DNA Star Inc.).

2.9.1.2 LR reactions for gateway cloning

pDONR/Zeo-Rad6, -Rar1, -Sgt1, -Yr10-1506, -Yr10-1562 plasmids as entry clones can be used for any cloning with LR reaction. Destination vectors can be used for plant transformation, over-expression, Y2H studies. Those BP products were used for different purposes such as Y2H protein-protein interaction analysis. In other words, Rad6 BP products can be used as entry vector for over-expression and localization studies. LR reactions were performed as followings, 1 μL LR Clonase™ enzyme mix (Catalog no.11791-019), 1 μL destination vector, 1 μL entry vector, 2 μL 5X LR clonase reaction buffer and TE buffer at was added to complete 10 μL reaction volume. The mixture was incubated in 25 ^{o}C for 1 h. Transformation was performed as describe in Section 2.6.3.3. Plasmid isolations were completed as described in Section 2.6.3.4 then sequences analyzed with EditSeq (DNA Star Inc.).

2.9.2 Medium preparations for Y2H

Yeast-2-hybrid experiments were performed according to ProQuest™ Two-Hybrid System with Gateway® Technology Invitrogen (Cat.Series 10835). YPAD is a rich medium for the routine growth of yeast was prepared as 10 g bacto-yeast extract, 20 g bacto-peptone, 20 g dextrose, 100 mg adenine sulfate were added into duran glass bottle and completed with autoclaved-distilled water to 1 liter. For agar plates, 20 g bacteriological-grade agar was added per liter of non-autoclaved YPAD medium. After then, the medium was autoclaved at 121 °C for 25 min. Synthetic

Complete (SC) medium preparation: nitrogen base, a carbon source, and a "dropout" solution containing essential amino acids, nucleic acids, trace elements and vitamins. Amino acid powder mix of purine and amino acids were prepared by mixing equal amounts (for example 2-3 g for each compound) of the following: adenine sulfate, alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Depending on the auxotrophies (surviving on lack of various amino acids) to be tested with the dropout medium, the appropriate amino acids (e.g., for SC-Leu, add all except Leucine): 16 mL of 20 mM uracil, 16 mL of 100 mM histidine-HCl, 16 mL of 100 mM leucine, 16 mL of 40 mM tryptophan were added for 2 L appropriate medium.

2.9.3 Yeast transformation and one-to-one interaction assay

ProQuest yeast two hybrid kit provides us to find out the interaction of proteins not only one to one protein interactions but also single protein screening in cDNA library (whole proteome pool). To find out the interaction between our RAD6 protein with RAR1 of wheat and SGT1 of wheat, firstly the clones of interests were transformed into yeast competent cells which are Mav203 (Invitrogen catalog no. 11445-012). The MaV203 is an appropriate host yeast cell for Y2H assays because it contains *HIS3*, *URA3,* and *β galactosidase* genes under GAL4 promoter. Mav203 yeast strain is provided with the kit to serve as the host for the bait pDEST32-RAD6 and prey pDEST22-RAR1 and pDEST22-SGT1 plasmids. YPAD liquid medium (10 mL) was prepared by one loop of Mav203 using YPAD and incubated overnight at 30°C by constant shaking. Dilution was applied when the growth of yeast cells reaches to A_{600nm} 0.4 in 50 mL of YPAD and cells were subjected to additional incubation for 2 hours. Then the culture was centrifuged at 2500 rpm and the pellet was dissolved in 40 mL 1X TE. The cell suspension was re-centrifuged and collected in 2 mL of 1X LiAc/0.5X TE. The Mav203 competent cells were incubated in room temperature for 10 min. For each transformation, 1 µg plasmid DNA (e.g. pDEST22-RAR1) and 100 µg denatured sheared salmon sperm DNA were mixed in 100 µL of the yeast suspension. Then 700 µL of 1X LiAc/40% PEG-3350/1X TE were added and mixed. The mixture was incubated at 30°C for 30 min. 88 µL of DMSO was

added and heat shock was applied at 42°C for 7 minutes for the transformants. Transformed cells were centrifuged for 10 seconds and supernatant was removed, and washed in 1 mL 1X TE, then finally re-suspended in 50-100 µL TE. All the cells were spread on two selective plates. To select the recombinant colonies the SC-Leu-Trp plates were used, since the bait vector has Leu gene and prey vector has Trp gene for growth. Later, to find out the self activation of our proteins the bait vectors were co-transformed with pExpAD502 and prey vectors with pDBLeu. These cells must grow on the -Leu, -Trp selection medium however self-activation must be negative (white instead of blue). Since Mav203 including *URA3* in its genome, SC-Leu-Trp-Ura plates were prepared to test *URA*3 induction, and same experiments were repeated on SC-Leu-Trp-His plates. LacZ induction is another determination of interaction analysis. YPAD plates were prepared (YPAD a nitrocellulose or nylon membrane), for X-gal assays to test *lac*Z induction. Firstly a SC-Leu-Trp master plate was prepared to replica plate on to SC-Leu-Trp-Ura, SC-Leu-Trp-His, and YPAD containing a nitrocellulose or nylon membrane plates. Surviving colonies in -His and -Ura and blue color were selected. So that the recombinant constructs with interacting proteins are all must be grown in -Leu, -Trp, -Ura and -Leu, -Trp, -His selective medium. These colonies must also produce blue color in + X-gal YPAD - nonselective medium. Interacted and co-transformed clones were replica cleaned with velvet wrapped replica plater. Mav203 has *URA3* and *HIS3* in its genome under the promoter of *GAL4*. If the interaction takes place the GAL4 transcription factor activates the transcription then HIS3 and URA3 are synthesized. As mentioned above the third confirmation for the interaction is the X-gal assay which results in blue color since LacZ gene is under control of GAL4 promoter in the Mav203 also controlling HIS3 and URA3. YPAD plates with nitrocellulose membrane were prepared and the assay was designed: 10 mg X-gal was dissolved in 100 µL DMF. 100 µL X-gal in DMF, 60 µL 2-mercaptoethanol and 10 mL Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) were combined. 125-mm Whatman 541 filter papers were placed in a 15-cm petri dish and saturated with ~8 mL of the X-gal solution removing air bubbles were stacked two rounds. The membrane was removed from the surface of the YPAD plate and immersed the membrane in liquid nitrogen for 20-30 seconds. The plates were covered and

incubated at 37°C in X-gal solution and monitored for 2-24 h for blue color formation.

2.10 Identification of differentially expressed transcripts from leaves of the boron tolerant plant *Gypsophila perfoliata* L.

2.10.1 Plant growth and treatments

Plants were collected from the boron mine area (soil boron content is 8900 mg/kg (Babaoglu et al., 2004)) of Kirka, Eskisehir, the natural habitat, by Dr. Mehmet Babaoglu (Selcuk University, Konya, Turkiye). *Gypsophila perfoliata* plant is one of the Caryophyllaceae family species. *G. perfoliata* is perennial and 30-120 cm tall. It propagates from strong woody roots with succulent perfoliat type leaves shown in Figure 2.4. The plants maintained in Murashige Skoog (MS) medium (Murashige and Skoog 1962) upon collection from the natural habitat at which contained ~1300-1600 mg/kg B in dry leaf matter, were transported to our lab in MS as short seedlings (10-15 cm). The medium was exchanged with Hoagland solution (Hoagland and Arnon, 1950). Seedlings were grown with natural abundance concentration of boric acid for two weeks in Hoagland solution for adaptation (reaching up to 30-40 cm). For Hoagland medium: macronutrient, micronutrient, $ZnSO_4$ and Fe-EDTA stock solutions were prepared separately. Macronutrient solution contained 6 mM KNO_3, 1 mM $NH_4H_2PO_4$, 2.0 mM $MgSO_4$, 4.0 mM $Ca(NO3)_2$. Micronutrient solution contained 30 μM (control) or 500 μM and 1000 μM H_3BO_3 (excess boron), 9 μM $MnCl_2$, 0.3 μM $CuSO_4$. Fe-EDTA concentration was 25 μM. Zinc was supplied as $ZnSO_4$ at concentration of 0.8 μM for zinc sufficient growth conditions. The final concentrations of these compounds in the hydroponics medium are as follow; 4mM calcium, 15 mM nitrogen, 2 mM magnesium, 5.8 mM potassium, 1 mM phosphorus, 30 μM boron, 2 μM manganese, 0.2 μM copper, 25 μM iron, 10 μM ammonium. Plants were let grow at 20 °C 18 h in light and 6 h in dark, at 60% humidity. Afterwards, they were split into 4 sets, two set of samples were grown in 500 μM, one set in 1000 μM, and the control set was in

30 μM boron concentrations, thus the age of samples and all the subsequent growth conditions were kept identical. Excess boron treated leaf samples and leaves from control plants were collected as a mixture of old and new at 3, 12, and 24 hrs upon boron additions. All of the leaf materials were stored at –80 °C until RNA isolations; four sets of RNA isolations were performed as three time point samples at different boron treatments. First set of RNA isolates of all the time points from the control and 500 μM boron treated samples were used to perform DDRT-PCR. For qRT-PCR analysis, only the RNA isolates of the 24^{th} hr time point from the two replicates of 500 μM (one is the same as DDRT-PCR analysis) and one from 1000 μM boron treated samples together with control samples were used.

Figure 2.5 *Gypsophila perfoliata* L. pictures from **a)** natural habitat (METU campus*, the picture was kindly made available by the permission of Z. Kaya, Biology Department and Doğa Topluluğu of METU); **b)** thawed sample which was frozen after adopted in Hoagland medium from Kirka, and herbarium samples; collected from **c)** Aksaray, Sultanhani at 940 m altitude in 1996 by M. Vural, N. Adiguzel, and M. Oztekin **d)** and **e)** Ankara, Bala at 1000 m altitude by Z. Aytac and N. Adiguzel, in Plant Herbarium of Gazi University, Biology Department. *Printed in 'Field Guide to Wild Flowers of METU Campus' by Dönmez Ofset-Ankara, 1999.

2.10.2 RNA isolation and purification

Total RNA was extracted from the collected leaf samples using Trizol® (Invitrogen, CA, USA) according to suggested procedure by manufacturers. RNA pellets in ethanol were stored at -80 ^{o}C until use. The total RNA of 10 µg was treated with 10 Us of RNase free Turbo DNaseI (Ambion, Inc., TX, USA) in 20 µL reaction volume at 37 ^{o}C for 30 min followed by 65 ^{o}C incubation for 10 min. DNAse treated RNA samples were precipitated in 2.5 M LiCl by incubating at -20 ^{o}C and collected by centrifugation at 4 ^{o}C, 12.000 g for 15 min. The RNA pellets were washed in 70% Ethanol and suspended in 10 µL DEPC-treated ddH_2O just before use.

2.10.3 cDNA synthesis

First strand cDNA was synthesized using SuperScript^{TM} III- RNase H^{-} Reverse Transcriptase (Invitrogen CA, USA). cDNA constructions were performed according to manufacturers instructions as follow: Total RNA (5 µg) was incubated in the presence of 0.1 mM dNTPs, 30 pmol oligo$(dT)_{20}$ and sterile ddH_2O in 13 µL total volume at 65 ^{o}C for 5 min and set on ice. The synthesis was performed in 1X First Strand Buffer, 5 µM DTT, 200 U SuperScript^{TM} III- RNase H^{-} Reverse Transcriptase and 40 U RNase inhibitor (Invitrogen CA, USA) in 20 µL total volume, incubated for 90 min at 50 ^{o}C and stopped at 70 ^{o}C by 15 min incubation.

2.10.4 Differential display RT-PCR (DDRT-PCR)

DDRT-PCR (Liang and Pardee, 1992), was performed on single stranded-cDNAs (ss-cDNA) using different primer combinations; P1-P10/T1-T9 (primer sequences are available in Clontech (Delta Differential Display Kit, Ref. 637405). cDNAs from different time points (3, 12, 24 hrs after the addition of excess boron) were combined appropriately in equal amounts to be used in DDRT-PCR experiments and amplified with 1 U 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 at 42 °C and 5 min at 72 °C; followed by 30 cycles of three steps of 94 °C for 1 min; 60 °C for 1 min; 72 °C for 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). PCR products were separated on a standard DNA denaturing sequencing gel (6% denaturating gel 5.7% acrylamide, 0.3% N, N'methylene-bis-acrylamide, 8 M urea, 1X TBE). Prior to loading, PCR products were denaturized at 94 ^{o}C for 5 min. DNA sequencing gels were dried and radiolabeled PCR products were visualized on X-ray films after overnight exposure.

Table 2.2 List of sequences of the primers used in differential display analysis

Primers	Primer sequence (5'-3')	T_m 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
T9	CAT TAT GCT GAG TGA TAT CT $_{(9)}$ GG	68,5
P1	ATTAACCCTCACTAAATGCTGGGGA	66.1
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

2.10.5 Reamplification and subcloning of cDNA probes

The fragments determined as differentially expressed bands were excised cut off from the gels with aligning to the autoradiograph and suspended in 10 µL ddH_2O. DDRT-PCR fragments were re-amplified in the absence of radiolabeled nucleotides as above with the same primer combinations. Products of reamplified bands were visualized on 1% agarose gels. DNA was eluted from the cut off gel pieces in 5 µL of sterile ddH_2O by multiple freeze thaw steps and used directly for ligation into 5 ng pGEM®-T Easy TA cloning vector (Promega, Madison, WI) in 10 µL reaction volume in the presence of 6 Us of T4 DNA ligase and 1X Ligase Buffer at 4 ^{o}C for overnight. The half of the ligation products were transformed into *E. coli* Dh5-α.

Several white colonies were randomly picked and the presence of the inserts was confirmed prior sequencing.

2.10.6 Sequencing and homology analysis

Plasmids were isolated from the randomly selected clones using QIAprep Spin Miniprep kit (Qiagen, USA). Plasmids were sequenced at The Ohio State University Plant-Microbe Genomics Facility. Sequences were manipulated using Editseq 4.0 (DNA Star Inc.), sequence analysis software. NCBI DNA sequence database were searched for homologies using tblastn, tblastx, and tblastp for homology identification (Altschul et al., 1990)

2.10.7 qRT-PCR for confirmation of DDRT-PCR excised and cloned fragments

To confirm the detected genes, the comparative qRT-PCR was used. Expression level differences of the identified genes were confirmed and quantified by Stratagene Mx3005p qPCR System. Using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548) the expression level differences were calculated. Total RNAs of the 24th boron treated and control plant leaf samples have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *Arabidopsis thaliana* 18S rRNA (GenBank accession: X16077) due to of the lack of *Gypsophila perfoliata* sequence information. (forward 5' TTTGACTCAACACGGGGAAA 3' Reverse 5'CAGACAAATCGCTCCACCAA 3'). We have also tried Arabidopsis Rubisco (GenBank accession number is: AB042066) primers on *Gypsophila perfoliata* (forward: 5' GTGAGCACAACGCATCTCCT 3'; reverse: 5' TTGACCTCCTCCACCTCGTT 3'). Primer pairs (Table 2.3) for real-time PCR analyses were designed using Primer3 software (Rozen and Skaletsky, 2000). These primers were able to amplify the target genes in *Gypsophila perfoliata*. The 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 according to Pfaffl's model

(Pfaffl, 2001), in comparison to constitutively expressed *18S rRNA* and *Rubisco* genes for normalizations, using three technical repeats of each sample.

Table 2.3 qRT-PCR primers designed to confirm the differential expression levels based on the gene fragments cloned in DDRT-PCR. The accession numbers of the detected homologies are presented.

Primer Name	Clones	Accession Numbers	Sequences 5'-3' Directions
DD1 Bor2 F	DD1 Bor 2	NP196044.2	TTCTTGGACGTCAGAAAGTTGTTC
DD1 Bor2 R			TAATTCTTCCTCGGTGGCAGACTC
DD1 Bor4 F	DD1 Bor 4	NP190169.1	CAGCCTACAAGGTTACATCCTAGG
DD1 Bor4 R			TAAATTCAATCTCTTAATCAAATG
DD1 Bor6 F	DD1 Bor 6	AAM66946.1	CTAAGACTGCTTCCTGCTGTAGTT
DD1 Bor6 R			TGTAGCCGACAGACACCCATCCC
DD2 Bor10 F	DD2 Bor 10	ABE94292.1	ATCATTGGTATCCGCTCATCATCT
DD2 Bor10 R			GAGAAGCTAGATCAAGGATTGAG
DD2 Bor14 F	DD2 Bor 14	AAL77110.1	CATCTTCAAATGAATCATCCTTGT
DD2 Bor14 R			ACTCTGAGCCAAGGTGACTGCATC
DD3 Bor2 F	DD3 Bor2	NP566212.2	AGCTTGTGAGGCTCAGGCAGTTGT
DD3 Bor2 R			CCTCACTAAAGATCTGACTGCAAC
DD3 Bor5 F	DD3 Bor 5	ABK42076.1	GATAGAGTGTCGCTCAAACAAAAG
DD3 Bor5 R			GACCGTGATTGGAAAAATTCCGTA
DD3 Bor7 F	DD3 Bor 7	NP114244.1	CAAGGATTTCATAATTGGACGTTG
DD3 Bor7 R			AAGAGCGTTTCCACGTGGTAGAAC
DD3 Bor13 F	DD3 Bor 13	ABE77689.1	GATCAAAGTGCTGCTTTAGCAGGC
DD3 Bor13 R			ACTTTGTCATACCTGTTAATGGAA
DD3 Bor26 F	DD3 Bor 26	CAB41122.1	GTGCTGAGCTTCCTCCACGCTCTG
DD3 Bor26 R			CAATACTTTGATAACTTGGGCTGG
DD3 Bor34 F	DD3 Bor 34	BAD22997.1	ACATTGTGGGTTGGAGGAAA
DD3 Bor34 R			ATCAACATGTGATGGTGTGAAAA
DD3 Bor36 F	DD3 Bor 36	AAM62497.1	TCGCTGCATCTAACCAACA
DD3 Bor36 R			TCGCTGCATCTAACCAACAA
DD3 Bor39 F	DD3 Bor 39	No hit	GCCATTGGTCTCAACCTCAC
DD3 Bor39 R			TTCGTGGCACAAAATTACCC
DD3 Bor40 F	DD3 Bor 40	BAD15474.1	GTGAGCACAACGCATCTCCT
DD3 Bor40 R			TTGACCTCCTCCACCTCGTT
DD3 Bor41 F	DD3 Bor 41	AAD04292.1	ACATTGTGGGTTGGAGGAAA
DD3 Bor41 R			ATCAACATGTGATGGTGTGAAAA
DD3 Bor42 F	DD3 Bor 42	AAF03437.1	CGGGGAGTTCAGTATTTGGAG
DD3 Bor42 R			TCGGCCAAACAGTACAATAGG
DD3 Bor72 F	DD3 Bor 72	T07697	GATACCATCAGGGAGCTGGA
DD3 Bor72 R			AGTACTTGACGGGAGCGAGA
18SRRNA F	18SRRNA	AT2G01010	TTTGACTCAACACGGGGAAA
18SRRNA R			CAGACAAATCGCTCCACCAA

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Infections and *Rad6* gene expression level changes during inoculations

Rad6 gene was previously identified and has been shown to be potentiallyinvolved in yellow rust disease resistance in wheat (Bozkurt O, PhD thesis supervised by Akkaya MS, 2007). Among them DD Yr10-1 (GenBank accession number: FE900153) is one of the most important clone to be functionally analyzed. It has high homology with *OsRad6* gene at amino acid sequence (96%) and nucleotide level (83%) with possible role in plant disease resistance (Bozkurt et al., 2007). This gene is down-regulated upon incompatible interaction and it has E2 ubiquitin conjugating enzyme homology. Therefore it may be taking role in plant disease resistance by its involvement in ubiquitination.

3.1.1 *Puccinia striiformis* f. sp. *tritici* (yellow rust) interaction with Avo-Yr1 wheat plants

Rad6 gene expression level changes were determined during incompatible (resistance) and compatible (disease) pathogen infections of wheat plants. *Puccinia striiformis* f. sp. *tritici* (Pst), Pst-232E137 is avirulent agent of Avo-Yr1 wheat and Pst-169E136 is virulent agent of Avo-Yr1 (causes yellow rust disease). Pst-232E137 and Pst-169E136 inoculations were performed on Avo-Yr1 plants and leave samples were collected at 6, 12, 24, and 72 hours after inoculation (hai).

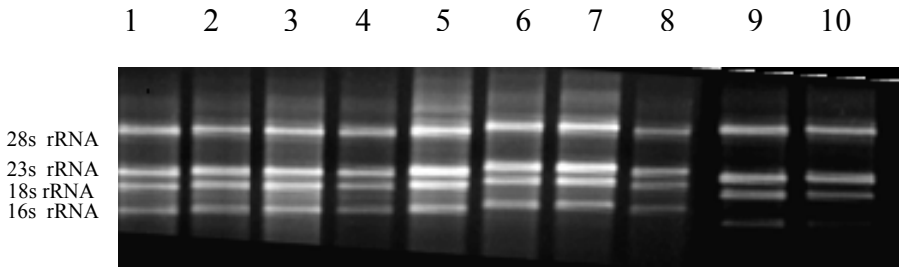


Figure 3.1 Total RNA samples from avirulant, virulent and mock inoculated Avo-Yr1 wheat plants. **1-4**: Avo-Yr1 inoculated with Pst-169E136 (6, 12, 24, and 72th hai samples, respectively), **5-9**: Avo-Yr1 inoculated with Pst-232E137 (same time points above), **9-10**: Avo-Yr1 and Avo-Yr10 mock inoculated namely no pathogen interacted control samples (2% agarose gel).

Total RNA isolated from the pathogen inoculated and mock inoculated samples of wheat plant were normalized with constituvely expressed *18s rRNA* gene and used for determination of gene expression level changes. All of the RNAs were treated with DNAse then they were precipitated with 2.5 M lithium chloride. Purified RNAs were reverse transcribed for cDNA synthesis which was used for quantitative Real-time qRT-PCR (as detailed in Section 2.6.3.6-8).

Table 3.1 Gene expression fold changes of *Rad6* upon Pst inoculations on Avo-Yr1

Gene	Pathogen **Pst**	Inoculation time points			
		6 hai	12 hai	24 hai	72 hai
Rad6 **FE900153**	Pst-232E137	1.90↑ 1.83↑ 1.79↑	2.02↓ 1.90↓ 1.96↓	3.85 ↓ 3.42↓ 3.65↓	1.16↓ 1.16↓ 1.29↓
	Pst-169E136	1.78↑ 1.83↑ 1.69↑	NC NC NC	NC NC NC	NC NC NC

NC: Not changed, data presented as compared with mock inoculated same time point samples.

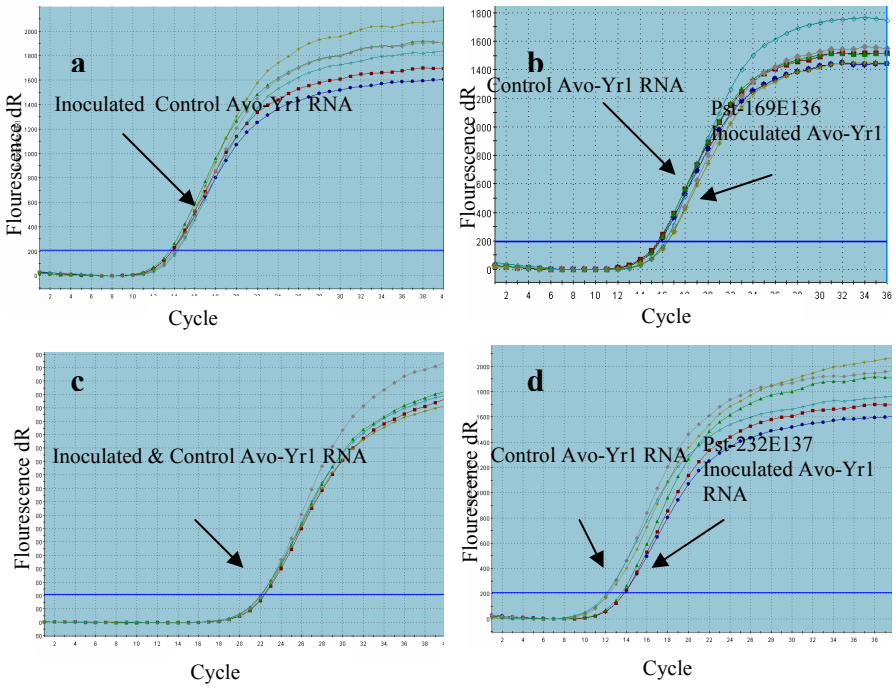


Figure 3.2 qRT-PCR determination of *Rad6* gene transcript level of 24 hai after virulent Pst-169E136 on (disease causing) and avirulent Pst-232E137 on Avo-Yr1 wheat plants (defense response) inoculations. Firstly, all the RNA levels in both cases were normalized using *18s rRNA* gene specific primers. **a** and **c** show the normalization of three cases. **b** is the *Rad6* gene expression level after virulent inoculation and **d** is the level of decrease in *Rad6* gene after avirulent inoculation.

Rad6 gene expression was detected as decreasing upon incompatible infection compared to mock inoculation in wheat (Figure 3.2). Upon virulent pathogen attack, the level of gene expression in susceptible plant was found to be unchanged. The same results were also obtained in another plant-pathogen system which is barley-powdery mildew host-pathogen interaction (Figure 3.5).

3.1.2 *Blumeria graminis* f. sp. *hordei* (Bgh, powdery mildew) interaction with Pallas-01/Mla1 barley lines

Rad6 gene expression level changes were also measured in incompatible and compatible interactions of powdery mildew pathogen on barley. *Blumeria graminis* f. sp. *hordei* (Bgh) Bgh95(53/01) is a virulent agent of Pallas-01/Mla1 barley plant (causes powdery mildew disease on barley) and Bgh103(64/01) is an avirulent agent of Pallas-01/Mla1 barley plant. Both Bgh95(53/01) and Bgh103 (64/01) inoculations were performed on Pallas-01/Mla1. The leaf samples were collected at 24 hour after inoculation (hai). The barley cultivar Bülbül which is known as universal susceptible has been inoculated with Bgh103(64/01) to follow up the success of infection. So the pathogen viability can be assessed by expected mildew formation on the leaves.

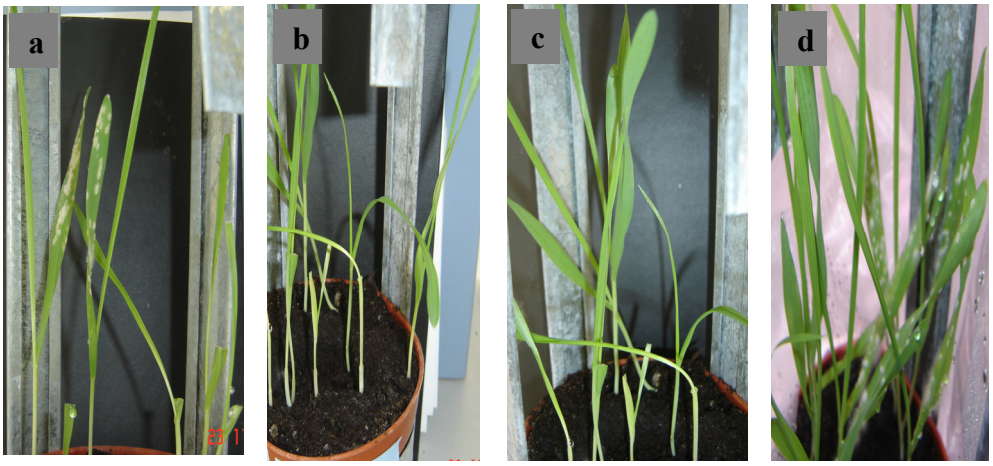


Figure 3.3 Pallas-01/Mla1 (*Mla1* resistance gene containing barley variety) inoculated with virulent and avirulent powdery mildew pathogens. **a**: inoculated with Bgh95(53/01) 9 day post inoculation (dpi) (susceptible, compatible interaction), **b**: 9 dpi mock inoculated **c**: 9 dpi Bgh103(64/01) (resistant, incompatible interaction) **d**: Bülbül, inoculated with Bgh103(64/01) (control).

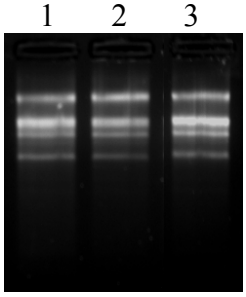


Figure 3.4 Pallas-01/Mla1 (*Mla1* resistance gene containing barley variety) inoculated with virulent and avirulent powdery mildew pathogens and mock inoculated Pallas-01/Mla1 barley plant RNAs collected 24th hai were isolated. **1**: Pallas-01/Mla1 inoculated with Bgh95(53/01) 24th hour **2**: Pallas-01/Mla1 inoculated with Bgh 103(64/01) 24th hour. **3**: Pallas-01/Mla1 mock inoculated 24th hour (the concentration of RNAs loaded 1: 1.5 µg/µL, 2: 1.5 µg/µL, 3: 1.5 µg/µL).

Inoculated barley leaf RNA samples were used for determination of gene expression level changes. Prior to qRT-PCR analysis all the RNAs was treated with DNAse and precipitated with 2.5M Lithium chloride. Cleaned RNAs were reverse transcribed and finally cDNAs were synthesized to use for quantitative Real-time RT PCR. These RNAs were normalized with *18s rRNA* gene (detailed in Section 2.6.3.9).

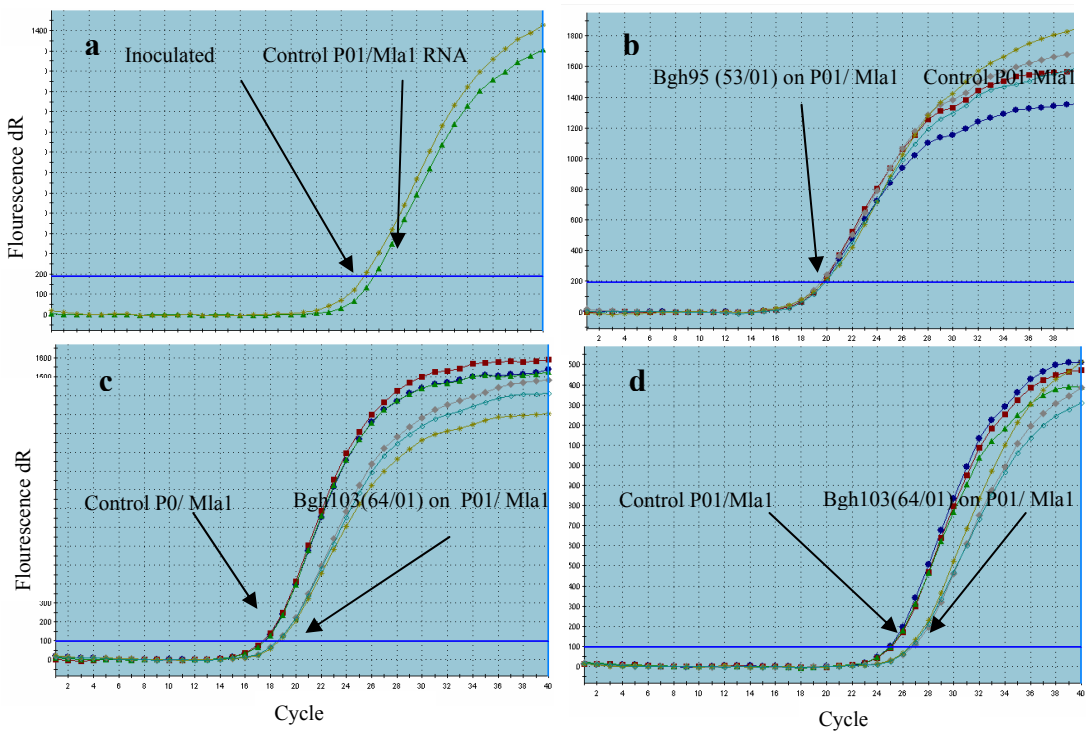


Figure 3.5 qRT-PCR determination of *Rad6* gene transcript level in virulent Bgh95 (53/01) inoculated Pallas-01/Mla1 barley (disease causing) and avirulent Bgh103 (64/01) inoculated Pallas-01/Mla1 barley (defense response) plants. Firstly, all the RNA levels were normalized using *18s rRNA* gene specific primers. **a** and **c** show the normalization of three cases. **b** is the *Rad6* gene expression level suppression after avirulent inoculation and **d** is the level of decrease in *Rad6* gene after virulent inoculation.

Rad6 gene expression level has been detected upon virulent and avirulent Pst attack on wheat and Bgh attack on barley lines. It has been presented that *Rad6* gene expression level 3.65 fold decreased in incompatible wheat-Pst interaction (Figure 3.2 **d**) and 3.28 fold decreased in incompatible barley-Bgh interaction (Figure 3.5 **d**) at 24 hai. Moreover there is no *Rad6* gene expression level difference in both compatible interactions (wheat-Pst and barley-Bgh) (Figure 3.2 **b** and 3.5 **b**). *Rad6* expression levels were found to be suppressed upon incompatible interaction compared to mock inoculation. As a result *Rad6* gene expression is

down-regulated upon avirulent race specific pathogen attack; the situation was proved in two different plant-pathogen interaction systems.

3.1.3 Barley stripe mosaic virus (BSMV) inoculation on wheat and barley plants

BSMV mediated gene silencing on plants especially on wheat and barley were successfully and effectively performed. Our laboratory is one of the very few applicants of this technology being pioneer in the world (Akkaya MS and Unver T, 2007; Unver et al., 2007). BSMV infects both barley and wheat. Firstly, the examination is required if the viral infection alone is effecting the expression of *Rad6* gene. Therefore during the inoculation of BSMV:00 (having no plant target gene for a particular gene silencing, virus alone), the expression level change of *Rad6* was also detected. Figure 3.6 shows the transcript level decrease of *Rad6* gene by BSMV:00 inoculation.

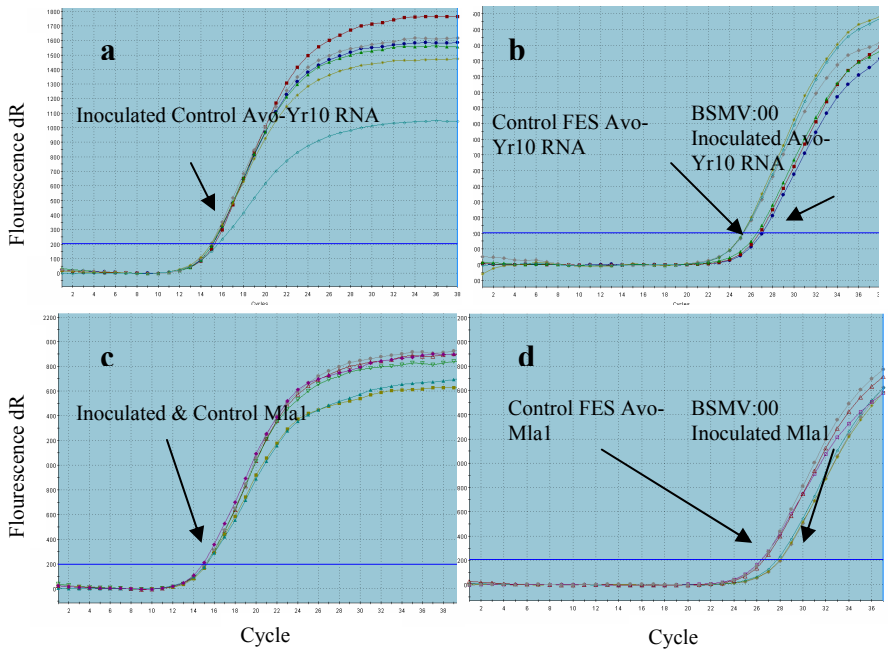


Figure 3.6 qRT-PCR determination of *Rad6* gene transcript level after virus inoculation on Avo-Yr10 wheat plants. FES is the solution which causes wounding for viral infection on plants. **a** is the normalization of FES and BSMV:00 treated wheat samples. **b** is the *Rad6* gene expression level suppression after viral inoculation. **c** is the normalization of FES and BSMV:00 treated barley samples. **d** is the *Rad6* gene expression level suppression after viral inoculation.

Rad6 expression level was detected to be decreasing in BSMV:00 inoculated wheat and lines (~3.81 fold) compared to FES no virus inoculated, negative control, (Figure 3.6 **b**) and similar results had been obtained upon BSMV:00 inoculation on barley lines ~3.65 fold decrease in *Rad6* gene expression was measured in BSMV:00 inoculated samples compared to FES treated barley lines (Figure 3.6 **d**). Normalizations were performed according to *18s rRNA* gene expression levels (Figure 3.6 **a** and **c**). Therefore BSMV:00 inoculation as a positive viral inoculated control sample for silencing confirmation experiments was decided.

3.2 Virus induced gene silencing (VIGS) studies

3.2.1 VIGS of PDS and GFP expression *via* BSMV

Prior to BSMV mediated gene silencing of targeted genes for functional analysis *Phytoene desaturase* (*Pds*) gene was silenced to determine the effectiveness of the silencing by BSMV in wheat. Using *Pds* gene silencing, the experimental conditions were optimized. Virus spreading and propagation was also visualized by Green Flourescent Protein (GFP) fluorescence detection with microscopy. BSMV vectors carrying the (*Hordeum vulgare*) *Pds* gene fragment either in sense or antisense orientations (constructed by Holzberg et al., 2002) were used to silence the endogenous wheat *Pds*. The viral inoculation requires wounding, thus the transcripts of the viral genome was robbed on the plant leaves in FES solution having celite for prior wounding. So the FES alone treated samples used as negative controls. BSMV:00 is the combination of three plasmid generated viral RNA; α, βΔβa and γ (without engineered construct) transcripts, inoculated at equal amount on plants with FES solution. BSMV:00 is a positive viral inoculated control (Figure 3.7-3.8).

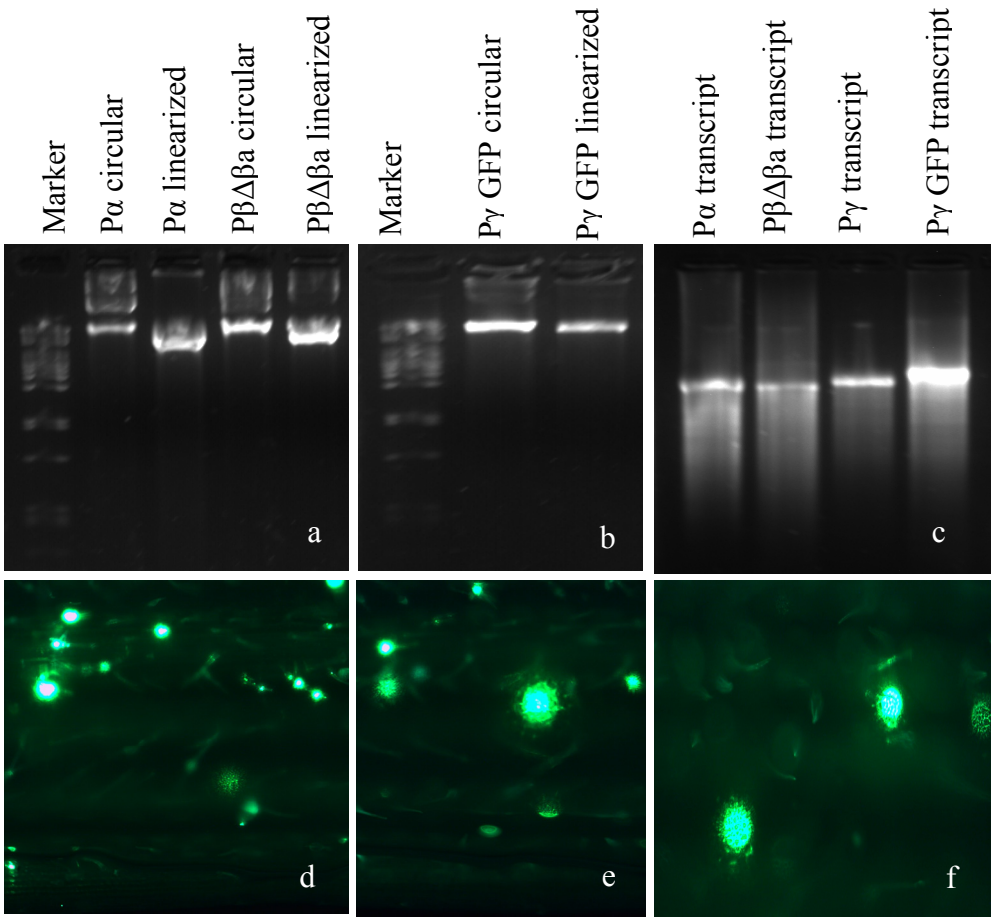


Figure 3.7 GFP expressions in wheat plants *via* BSMV infection. **a** and **b** circular plasmids and linearized three genomes required for effective silencing and virus spreading in plants. **c**: Transcripts of viral genomes separated on 2% agarose gel. **d**: GFP visualization the expression of GFP was performed by using BSMV:GFP construct (2 dpi) on Avo-Yr1 **e** and **f:** pictures were taken from 3rd day of viral inoculation on Avo-Yr10 and Avo-Yr1 respectively (**d-e** were visualized at 40X magnification).

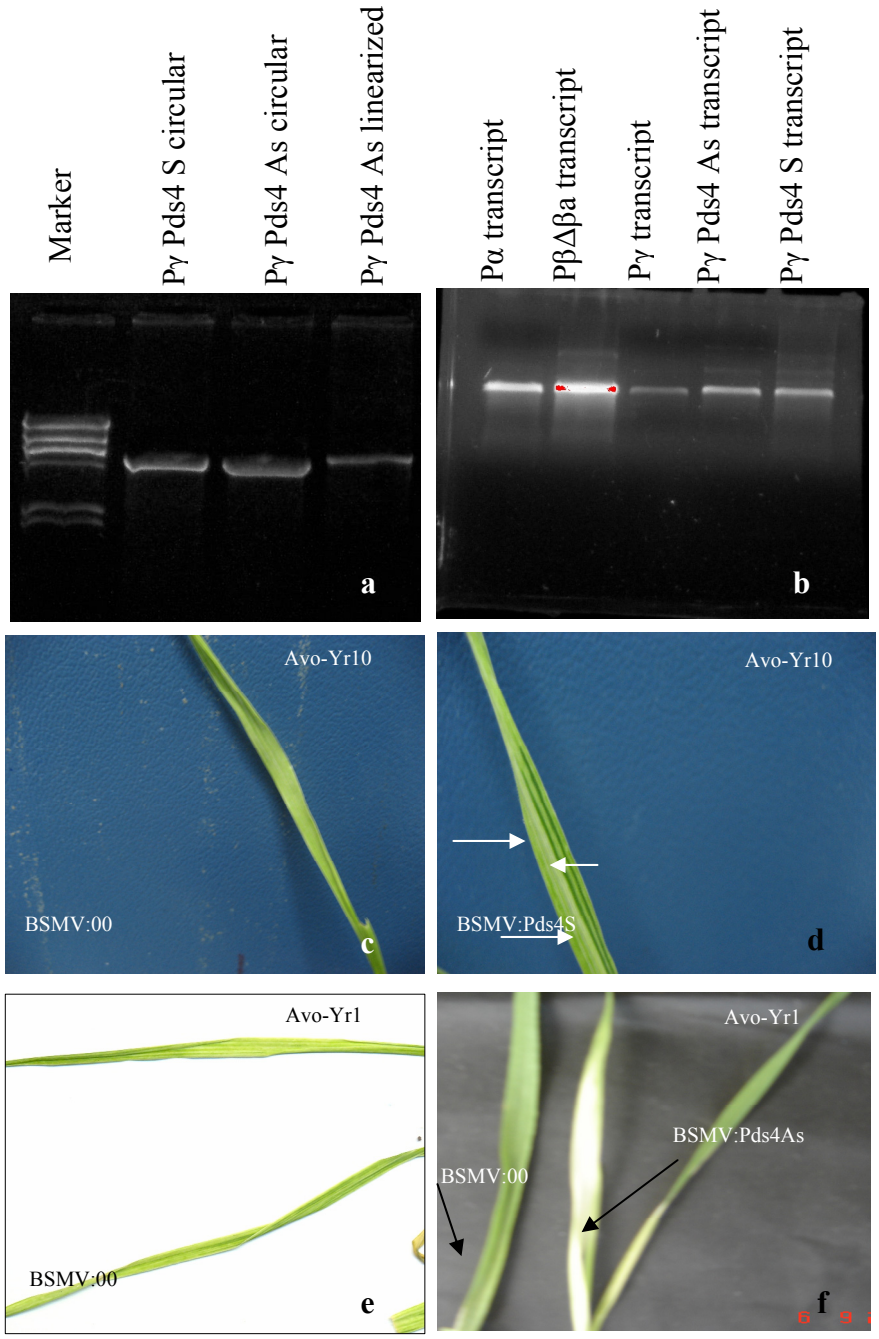


Figure 3.8 *Pds* gene silencing of wheat. **a**: BSMV:PDS4 Sense and Anti-sense constructs circular and linearized versions. **b**: transcripts of viral genomes **c**: FES control means no viral inoculations on Avo-Yr1 wheat plants after 15th dpi **d**: BSMV:PDS4As silencing of *Pds* gene for Avo-Yr1 wheat plants the picture was taken from 15 dpi. **e**: BSMV:00 of Avo-Yr10 wheat plants 15 dpi **f**: BSMV:PDS4As silencing of *Pds* gene for Avo-Yr10 wheat plants and FES control for Avo-Yr10 wheat plants the picture was taken from 15 dpi pictures are seen.

Linearized BSMV constructs were transcribed and purified for silencing prior to inoculation of all the transcripts which were analyzed in terms of concentration, quality and purity. The effect of BSMV:PDS4As silencing inoculation on Avo-Yr1, Avo-Yr10 wheat plants together with BSMV:00 viral inoculation and FES, solution treatment negative controls were pictured at 15 dpi (Figure 3.8 **f**).

3.2.1.1 qRT-PCR analyses for the determination of *Pds* gene expression

Silencing determinations or level of gene expression decreases were measured by quantitative real-time RT-PCR. Prior to the virus alone treated and negative control (only FES treated) plant RNAs were isolated (Figure 3.9). Leaf samples were collected at different time points (7, 15, and 21 dpi). All RNAs were purified and then converted to cDNAs. For qRT-PCR efficiency control standard curve analysis were performed at two different times (Figure 3.10 **a-b**).

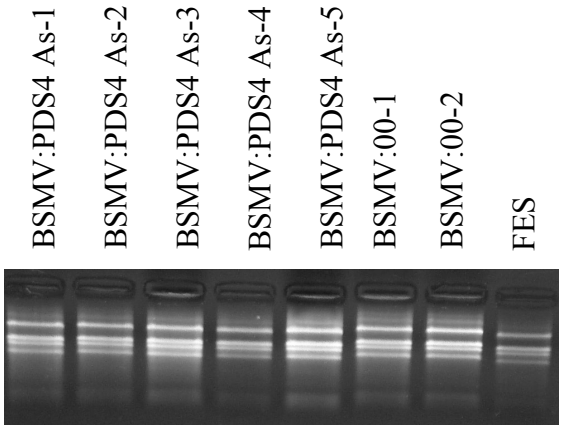


Figure 3.9 RNA samples of *Pds* gene silenced Avo-Yr10 plants with BSMV:00 and FES control samples collected 15 dpi. RNA samples were purified for further analysis. The numbers are referring to the biological replicates. RNAs were loaded on 2% agarose gel with varying 1-2 μg/μL concentrations.

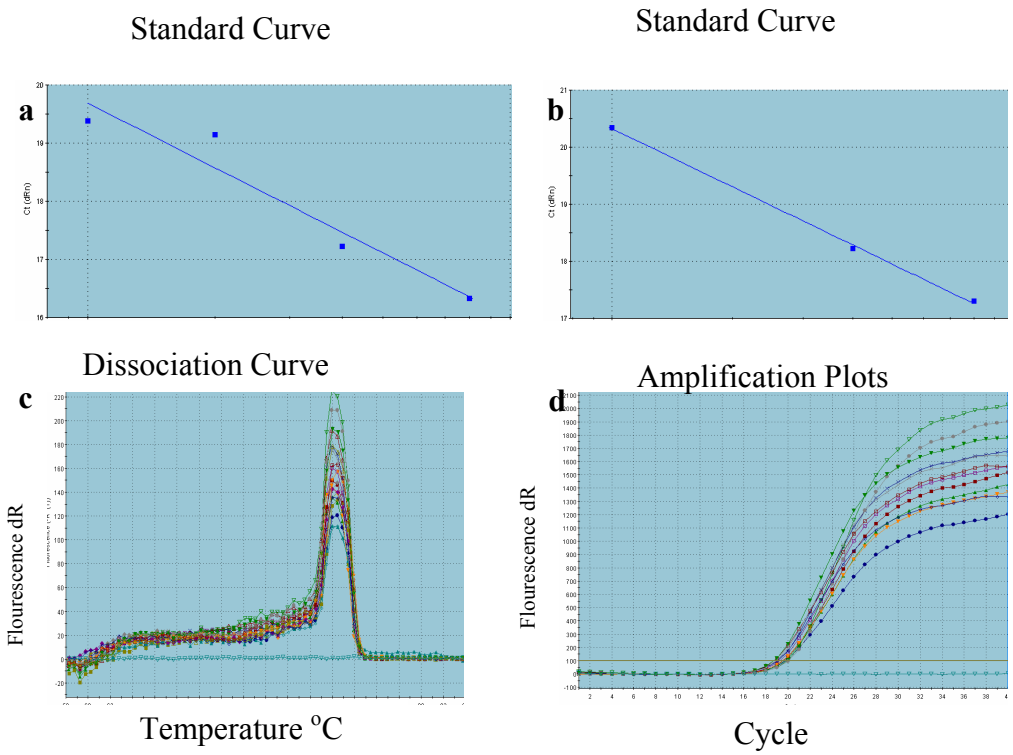


Figure 3.10 qRT-PCR Normalizations and standard curves of the (**a**, **b)** two different experiments for standard curve analysis are seen. **c**: Disassociation curve of different cDNA samples using (4 different dilutions with 3technical repeats) *18s rRNA* gene specific primers. **d**: Amplification plots of *Pds* gene silenced and non-silenced plant samples for normalization.

Silenced Avo-Yr10 wheat plants were pictured in Figure 3.8 **e-f** and the silencing levels are presented in the table 3.2. Three different experiments for analysis of *Pds* gene silencing were performed with three biological and technical replicates. The appearances (photo-bleaching) are presented in Figure 3.8 of the *Pds* silenced Avo-Yr10 plants. The first experiment represents the efficiency of PCR as 87.1% and the second produces 97.0% efficiency. Therefore using 90% efficiency was decided in the qRT-PCR calculations for fold change measurements.

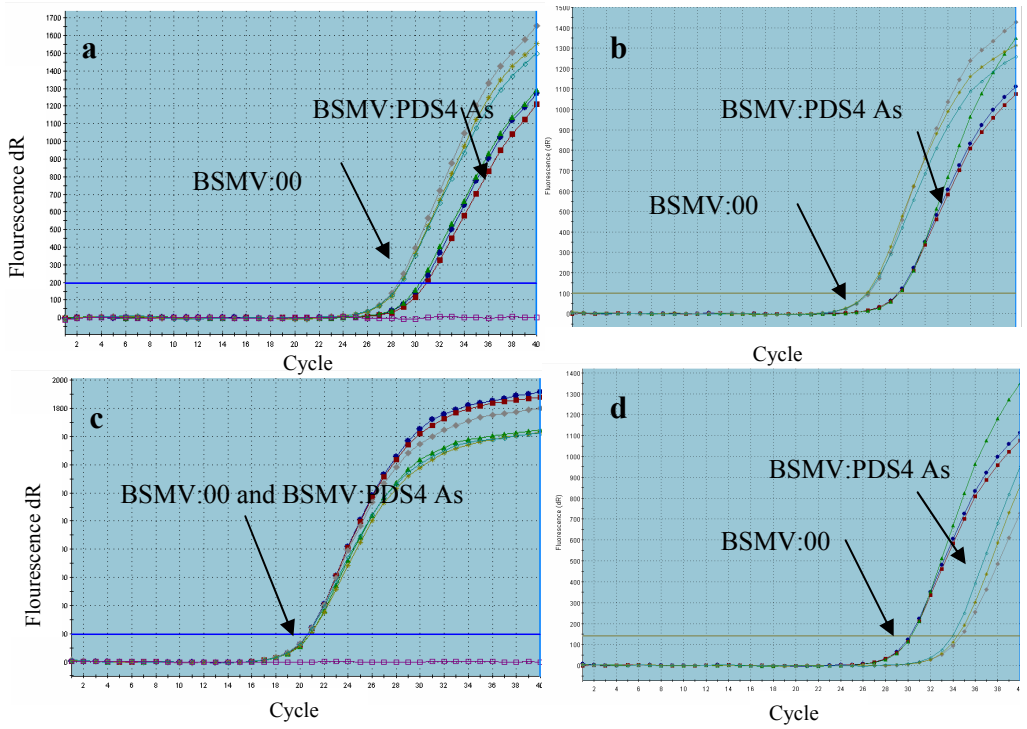


Figure 3.11 qRT-PCR confirmation experiments of *Pds* gene expression level decrease by BSMV mediated gene silencing in wheat. **a**: *Pds* gene silencing of Avo-Yr10 using BSMV:PDS4As (3.61 fold decrease). **b**: *Pds* gene silencing of Avo-Yr10 using BSMV:PDS4As (5.58 fold decrease). **d**: *Pds* gene silencing of Avo-Yr10 using BSMV:PDS4As (13.03 fold decrease). **c**: Normalization of silenced and non-silenced plant RNAs using *18 s rRNA* gene primers.

Table 3.2 qRT-PCR results of *Pds* silenced Avo-Yr10 wheat plants with three biological and three technical replicates. qRT-PCR real-time analysis of silencing of *Pds* expression by BSMV mediated VIGS in the third leaves of Avocet-Yr10 wheat plants

	Relative *PDS* expression*			
	Exp.1	Exp.2	Exp.3	
dpi				qPCR replicates
7	0.335	0.425	0.358	3
15	0.277	0.179	0.115	3
21	0.076	0.041	0.076	3

* Relative expression of *Pds* gene was calculated by dividing the level of *Pds* RNA from BSMV:PDS4As silenced plants by the level of *Pds* RNA from BSMV:00 non *Pds* silenced plants. Exp: experiment

Virus induced gene silencing (VIGS) method to silence a specific gene target of wheat and barley was performed. *Pds* gene was silenced using an anti-sense orientation construct; pγPDS4as (see Appendix for the sequence). Barley *Pds* gene (GenBank accession number: AY062039) has 96% homology to wheat *Pds* (GenBank accession number: DQ270237) (see Appendix for alignment figure done by CLC Bio.). 1:1:1 mixture of the *in vitro* transcripts synthesized from the plasmids pα, pβΔβa and pγ in FES were inoculated the plant samples. The derivative of β genome transcript, βΔβa, which does not encode viral coat protein, was used for faster viral spreading. We have observed white color due to photolysis on wheat leaves silenced with BSMV:PDS4as at 10^{th} day of silencing inoculation. Transcripts were applied on second leaves of plants then viral and photo-bleaching symptoms were detectable on second, third and fourth leaves. qRT-PCR reactions were also confirmed the silencing of *Pds* as visual phenotypic marker for VIGS of wheat. BSMV:00 silencing was also applied on wheat and barley plants. BSMV virus inoculated plant RNAs were used as positive control to detect the silencing level changes and FES mock inoculated plants used as negative control too.

3.2.2 Virus induced gene silencing of *Rad6* gene *via* BSMV

Pds gene as a phenotypic marker was silenced successfully and it has been proven that the method can be effectively used to silence the other genes of interests in wheat. Therefore, *Rad6* gene has been silenced to predict the specific biological function in plant disease resistance. The gene was cloned from wheat cDNA in the previous study in our lab (Bozkurt et al., 2007). The gene has a high (96%) homology with E2 enzyme of *Oryza sativa* at amino acid level. E2 is ubiquitin conjugating enzyme required for ubiquitination. Ubiquitination is an important mechanism for all living organisms moreover its importance in disease resistance mechanisms has been indicated in many publications in the literature (Devoto et al., 2003; Azevedo et al., 2002; Peart et al., Liu et al., 2002; Xu et al, 2002; Gonzalez-Lamothe et al., 2006). Due to the suppression of *Rad6* gene during race specific pathogen infections (see Figure 3.2, 3.5 and 3.6) the gene has been subjected to be silenced and functionally analyze the effect on pathogenesis in *planta*.

3.2.2.1 *Rad6* gene silencing construct preparation

Using the sequence information of the gene (see Appendix) and appropriate restriction enzyme cleavage sites adding to 5' ends, PCR primers have been designed. *Pac*I and *Not*I restriction enzyme cleavage sites added 185 bp lengths *Rad6* gene specific amplification was performed.

ATGTGATATGATACAACATTACAAACTCACACAAGACGTTACAAAATGTTTGCGTTCCAG
ATACAGACAGTGTAGACACAGGCCATGACAGAGCAATCTCCAACCGTCCAGCCAAACGAT
GCAACAAAGCAAGTTGCTGTTTCAGGTGGTAACGACAGAGTTCAGTCATTTTAGTTACAA
ACATTTTGGTACAGAAACCGCATGATAACACATGGTGAGCTCAACTTATTAGTCTGCCGT
CCAGCTCTGCTCAACAATCTCCCGCACTTTGCGGTTGTACTCTCGCTTGTTCTCACTGAA
CATGCGGGCAGCTTCTGAGTTAGCAGGCGAATTTGGGTTAGGATCACACAGCAGCGACTG
GATAGATGTAAGTATAGCAGCTACATCATATATTGGGCTCCACTGATTCTGTAGAATATC
TAAGCATATGCTTCCATCAGCATAAATGTTAGGGTGAAACATCCGAGAAATAAACCGAAC
TGTTGGTGGCTTATTAGGATATTCTTCATTAAACTGGAGAGTCAGCTTAA

Figure 3.12 *Rad6* gene nucleotide sequence and the amplified region (colored) for silencing construct.

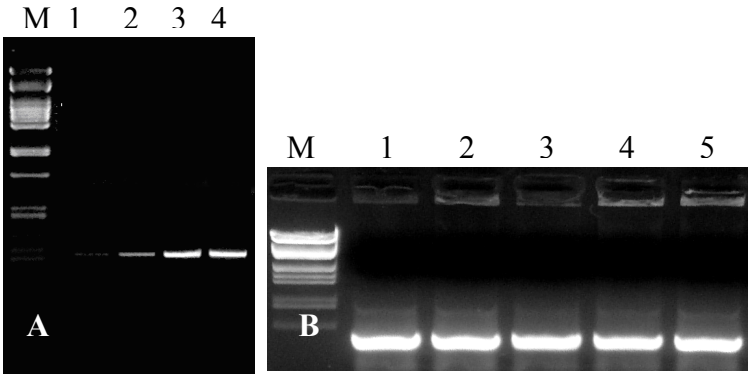


Figure 3.13 Amplification of *Rad6* gene part for silencing construct preparation. **A**: Avo-Yr10 plant cDNA was used as template and the *PacI* and *NotI* restriction enzyme sites added expected amplification was done (1-4 replicates of amplification with 185 bp). **B**: Ligated Rad6.2 clone with silencing vector is transformed into *E. coli* competent cells and colony PCR (1-5 replicates of colony amplification with 185 bp).

If *Rad6* gene in wheat has another copy is currently unknown. Therefore, Open Reading Frame (ORF) of *Rad6* gene for silencing has been performed. Amplified fragment was ligated into was targetted pγPDS4As by substituting PDS vector then *E. coli* competent cells was transformed with this ligation product. Using colony PCR with specific primers colonies with inserts were selected. As a second confirmation, colonies were subjected to sequencing. The insert sequence was validated as expected fragment. pγRad6.2As construct was linearized with appropriate restriction enzyme (*BssH* II) for *in vitro* transcription (Figure 3.14 **a-b**).

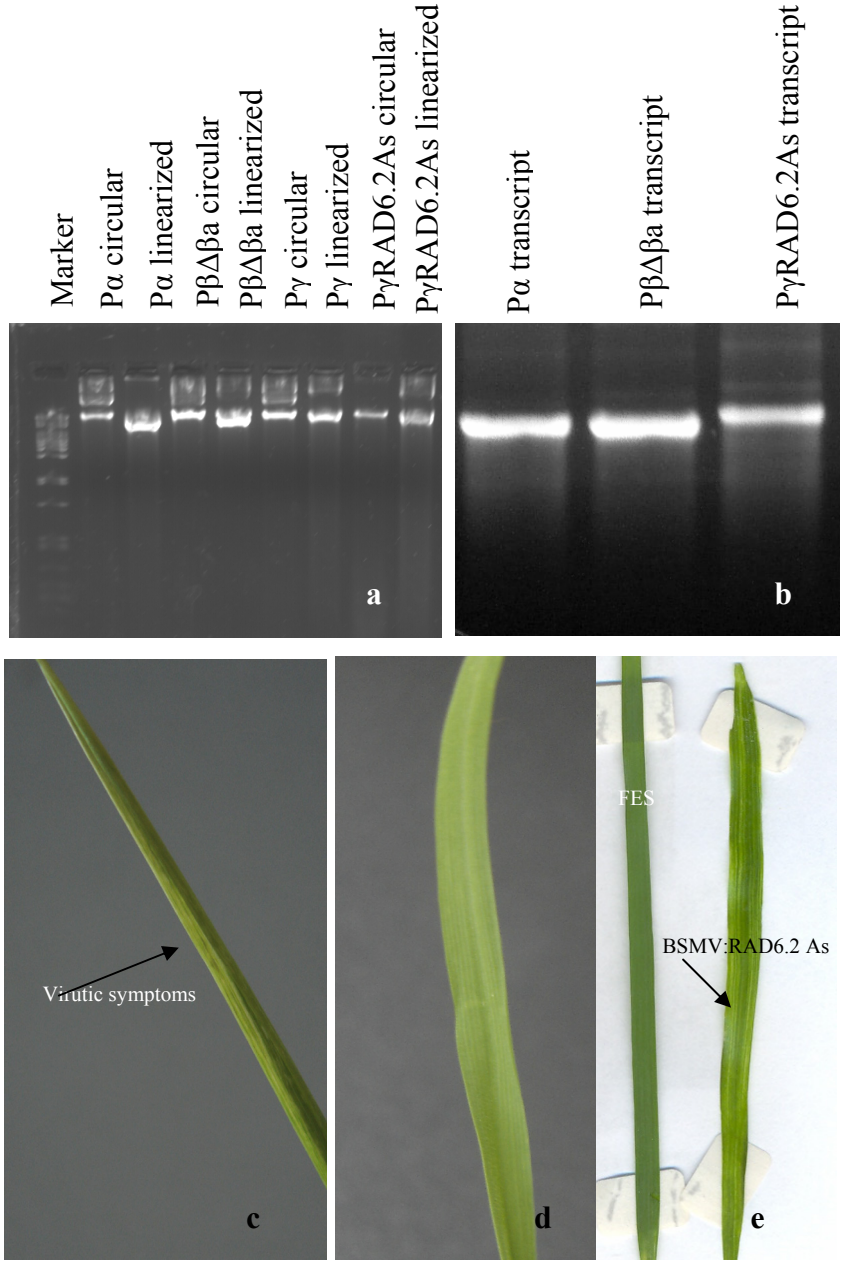


Figure 3.14 *Rad6* gene silencing of wheat. **a**: BSMV:RAD6.2 Anti-sense constructs circular and linearized versions with circular and linear pα, pβΔβa, pγ plasmids. **b**: Those linearized BSMV constructs were transcribed and purified for silencing. Prior to inoculation all the transcripts were analyzed in terms of concentration, quality and purity. **c**: BSMV:RAD6.2As silencing of *Rad6* gene for Avo-Yr10 wheat plants the picture was taken from 15 dpi. **d**: FES control means no viral inoculations on Avo-Yr10 wheat plants after 15th dpi **e**: BSMV:RAD6.2As

silencing of *Rad6* gene for Avo-Yr10 wheat plants and FES control for Avo-Yr10 wheat plants the picture was taken from 30 dpi.

3.2.2.2 *Rad6* is a negative regulator of cell death

BSMV mediated *Rad6* gene silencing was performed on (Avo-Yr1, Avo-Yr10) wheat and (Golden promise Myc-Mla1, Golden promise Myc-Mla6 and Bülbül) barley plants. 10 days old seedlings were used for gene silencing *via* BSMV. The second leaves of seedlings were BSMV inoculated with FES solution and then silencing symptoms were observed on third and forth leaves. Firstly the Avo-Yr10 wheat plants have been analyzed which are BSMV:RAD6.2As inoculated on microscopy. DAB staining, trypan blue staining and auto-flourescence phenotypic appearances has shown that programmed cell death (PCD) like symptoms are produced on *Rad6* silenced plants. However, these symptoms were not observed on BSMV:00 non-silenced, virus inoculated plant leaves (Figure 3.15).

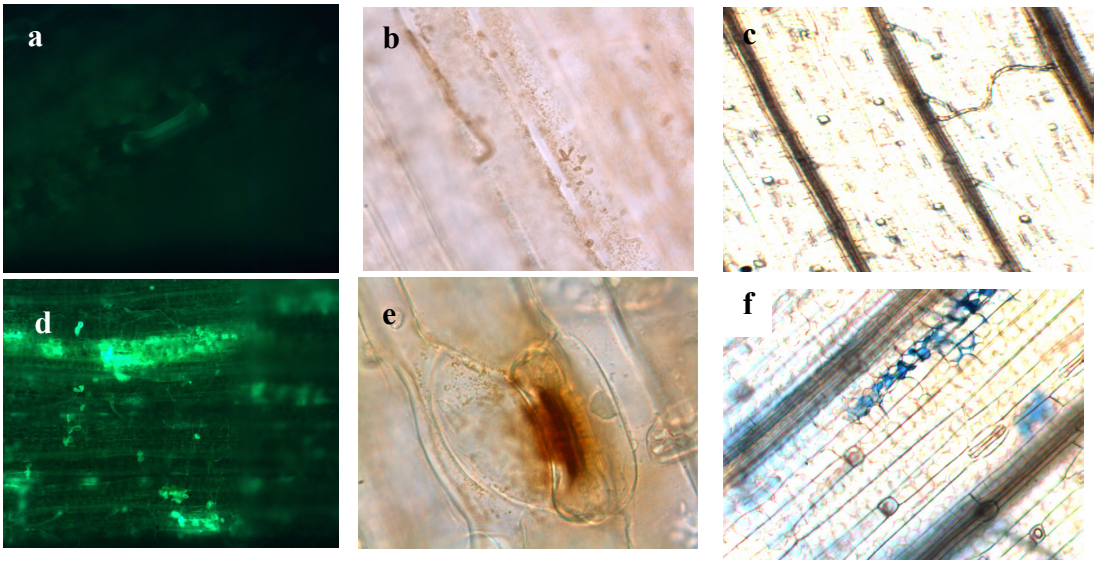


Figure 3.15 BSMV:RAD6.2As silenced and viral control BSMV:00 Avo-Yr10 wheat plants pictures. Panel **a** to **c** the non-silenced but virus inoculated plant leave samples are seen. Panel **d** to **f** silenced plant leave profiles are pictured. **a** and

d: auto-flourescence, **b** and **e**: DAB staining, **c** and **f**: trypan blue staining microscopy pictures are shown.

Hypersensitive cell death response in the *Rad6* gene silenced suppressed Avo-YR10 wheat plants was observed. I wanted to confirm the success and reproducibility of *Rad6* gene silencing in another wheat cultivar and barley plants as well. Therefore the *Rad6* gene has been silenced in another wheat cultivar which is Avo-Yr1. I observed the similar results as in the Avo-Yr10. Figure 3.16 shows the BSMV:RAD6.2As silenced Avo-Yr1 plant microscopy pictures. Symptoms were similar to hypersensitive response in avirulent pathogen infected resistant plant leaves. Moreover, knock-down of this gene generates HR is obvious. Barley *Rad6* gene silencing experiments prove the HR production idea (Section 3.2.2.5). It was also well stated in "Introduction" HR is a strong resistance response against avirulent pathogen attack, always observed in incompatible biotrophic pathogen and plant interactions.

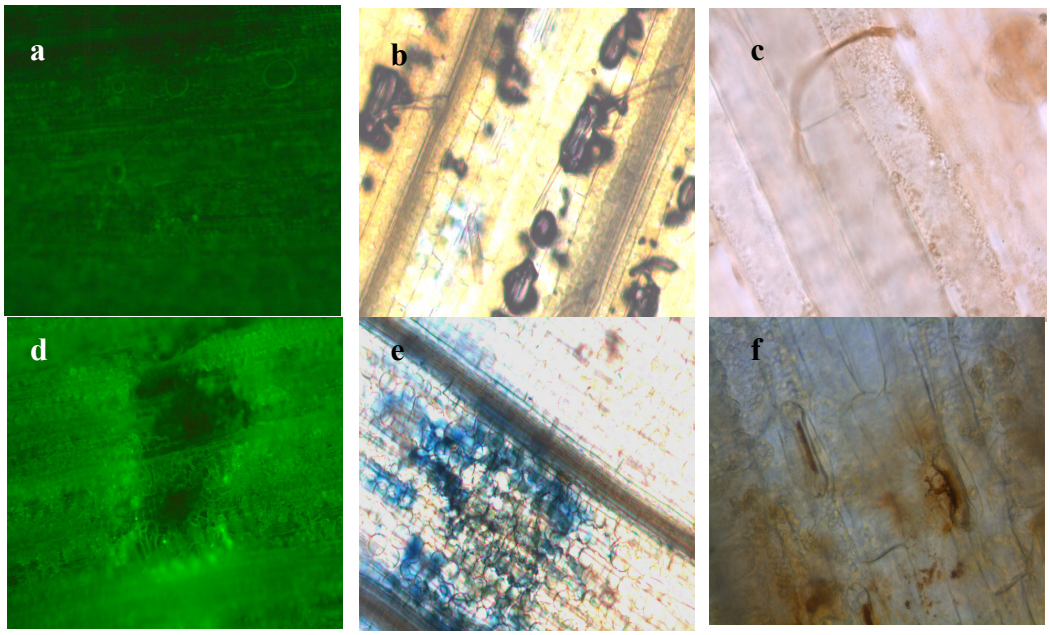


Figure 3.16 BSMV:RAD6.2As silenced and viral control BSMV:00 Avo-Yr1 wheat plants pictures. Panel **a** to **c** the non-silenced but virus inoculated plant leave samples. From **d** to **f** silenced plant leave profiles are pictured. **a** and **d**: auto-

flourescence, **b** and **e**: DAB staining, **c** and **f**: trypan blue staining microscopy pictures are shown.

3.2.2.3 qRT-PCR analyses for *Rad6* gene expression level determination in silenced wheat lines

Rad6 gene has been efficiently silenced using the VIGS system in wheat lines. The silencing of the gene caused 60-85% suppression of transcription level in the transcriptome of analyzed plants. BSMV:RAD6.2As silencing on Avo-Yr10 and Avo-Yr1 wheat plants contains pα, pβΔβa and pγRad6.2As transcript mixture of 1:1:1 quantity. 1.5 µg of each *in vitro* synthesized and purified transcripts were used for one silencing reaction. As a positive control BSMV:00 silencing was performed. BSMV:00 contains pα, pβΔβa and pγ transcript mixture of 1:1:1 quantity with only virus inoculation. It does not cause any endogenous specific plant gene silencing. Silenced wheat plants, positive and negative control plant samples were collected for RNA isolations and cDNA synthesis. qRT-PCR analyzes showed the decrease of *Rad6* transcript levels in plants as shown in Table 3.5. Figure 3.15 and 3.16 indicate that the silencing of *Rad6* gene causes programmed cell death in wheat plants. The reason for that may be the up regulation of some disease resistance related genes listed in Table 3.4 and 3.5.

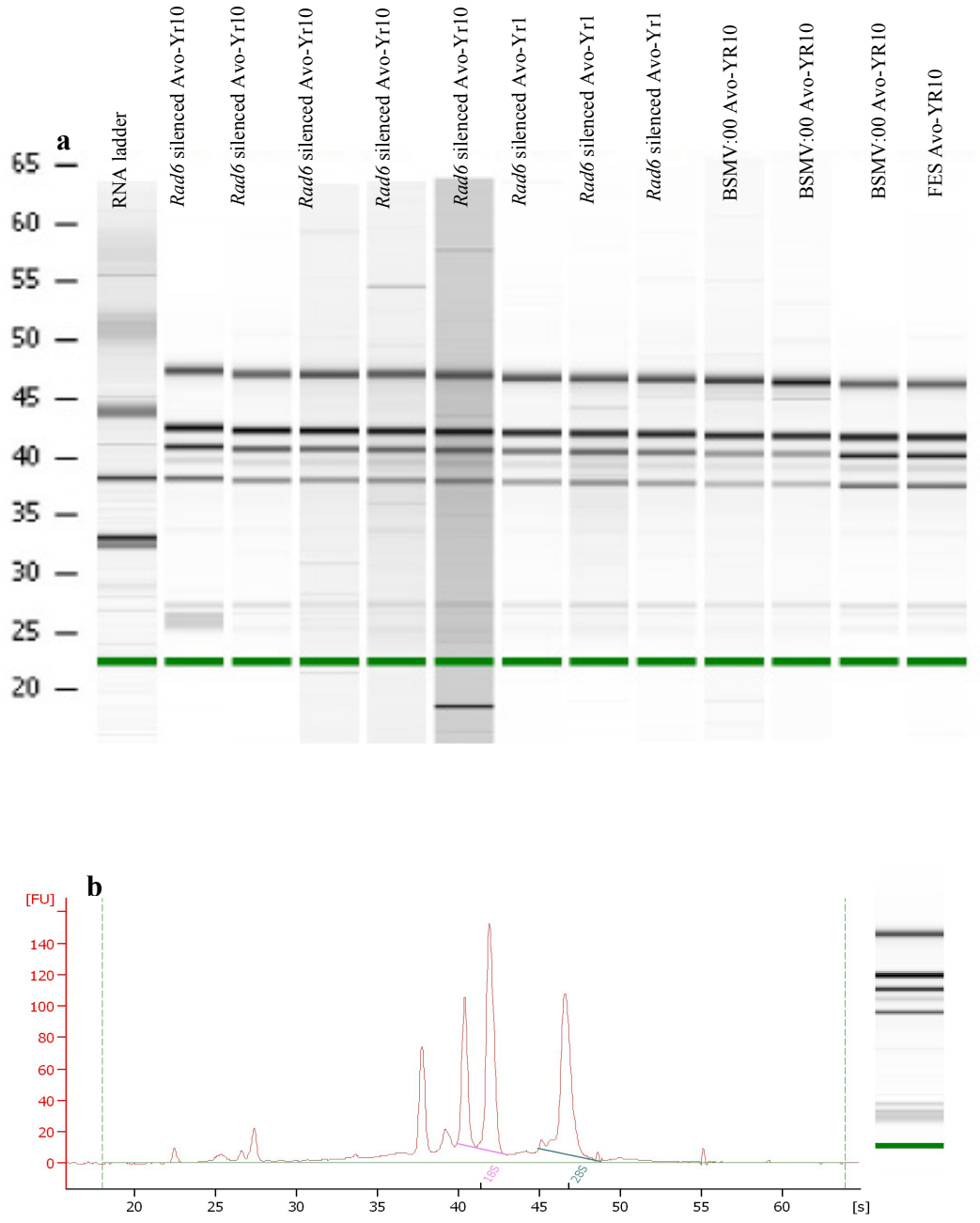


Figure 3.17 RNA analysis of *Rad6* silenced and non-silenced Avo-yr10 and Avo-Yr1 plants on RNA Nano-chips. **a:** All the RNAs that were run on RNA Nano-chip analyzed on Agilent 2100 Bioanalyzer. **b**: Specific illustration of sample 1, Rad6 silenced Avo-Yr10 plant leaf RNA analysisis shown as an example for RNA analysis.

Table 3.3 Overall results of sample 1* for RNA analysis for sample 1.

Overall RAN analysis for sample 1 : *Rad6* silenced Avo-Yr10				
RNA Area: 960.3				
RNA Concentration: 2,118 ng/µl				
rRNA Ratio [28s / 18s]: 0.0				
RNA Integrity Number (RIN): 7.8 (B.02.03)				
Fragment table for sample 1: *Rad6* silenced Avo-Yr10				
Name	Start Time [s]	End Time [s]	Area	% of area
18S	41.69	43.68	184.7	19.2
rRNA 2	46.35	48.36	133.8	13.9

*: Same as Figure 3.17. The RAN analysis reports of the other samples are presented in Appendix-E.

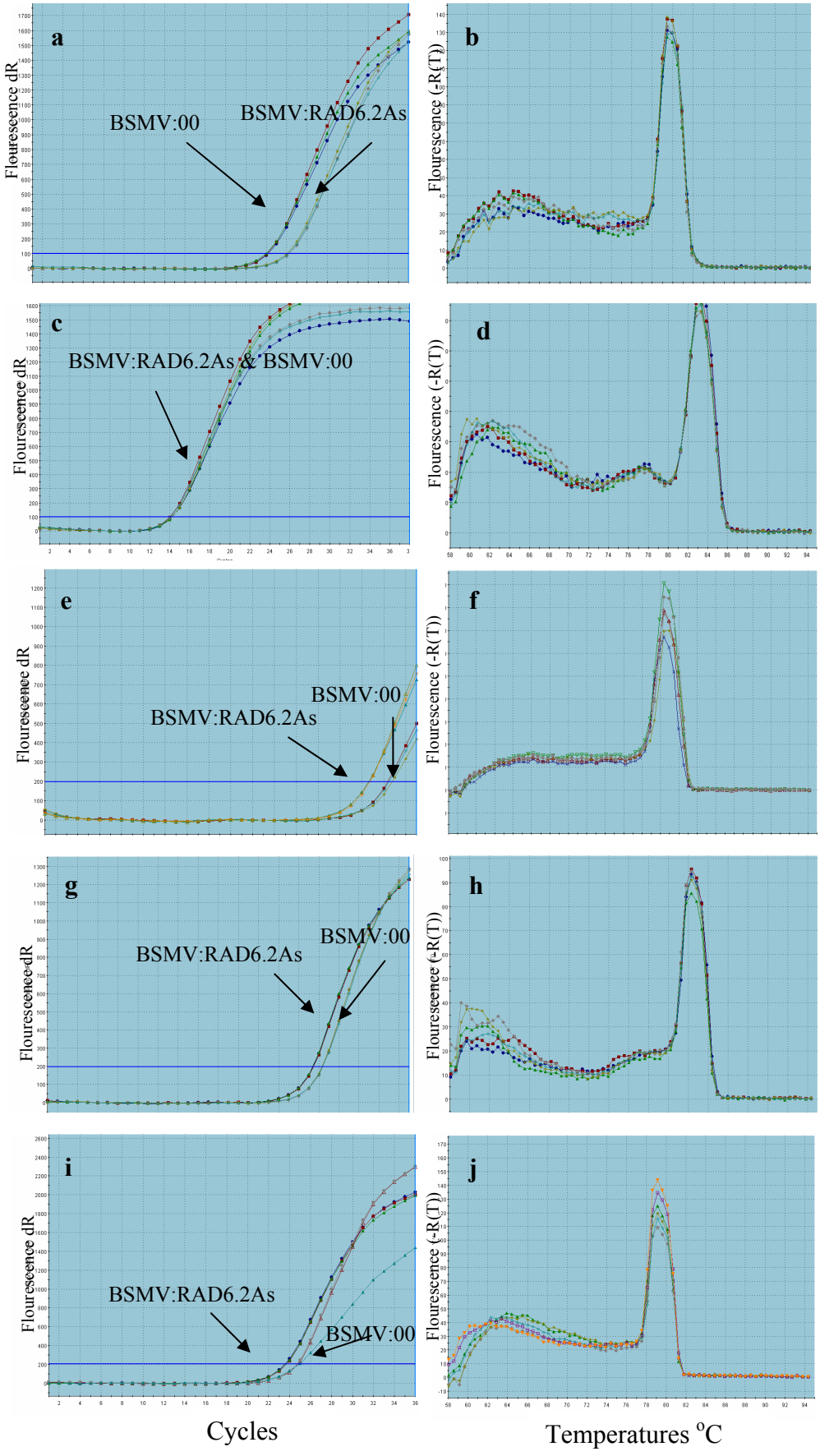

a
BSMV:00
BSMV:RAD6.2As
Flouresecence dR
b
Flouresecence (-R(T))
c
BSMV:RAD6.2As & BSMV:00
Flouresecence dR
d
Flouresecence (-R(T))
e
BSMV:00
BSMV:RAD6.2As
Flouresecence dR
f
Flouresecence (-R(T))
g
BSMV:RAD6.2As
BSMV:00
Flouresecence dR
h
Flouresecence (-R(T))
i
BSMV:RAD6.2As
BSMV:00
Flouresecence dR
j
Flouresecence (-R(T))
Cycles
Temperatures °C

Figure 3.18 (in previous page) *Rad6* gene silencing and the effect of *Rad6* level decrease on other disease resistance related gene expression levels. **a**: BSMV:RAD6.2As silenced plant *Rad6* gene expression level was decreased by approximately 4.11 fold relative to BSMV:00 inoculated Avo-Yr10 wheat plants. **b**: Disassociation curves showing the *Rad6* gene specific real-time PCR primers work correctly. **c**: BSMV:RAD6.2As inoculated and BSMV:00 inoculated Avo-Yr10 wheat plants RNAs normalized by *18s rRNA* gene level equalization. **d**: Disassociation curves show that the *18s rRNA* gene specific real-time PCR primers work correctly. **e**: *Rar1* gene transcript level in the transcriptome of BSMV:RAD6.2As inoculated wheat plant is increased approximately 4.03 fold according to BSMV:00 inoculated Avo-Yr10 wheat plants. **f**: Disassociation curves show that the *Rar1* gene specific real-time PCR primers work correctly. **g**: *Hsp90* gene expression is also increased by 2.16 fold in BSMV:RAD6.2 As silenced avo-Yr10 wheat plant relative to BSMV:00 inoculated Avo-Yr10 wheat plants. **h**: Disassociation curve shows that the *Hsp90* gene specific real-time PCR primers work correctly. **i**: *Sgt1* gene expression is also increased by 2.30 fold in BSMV:RAD6.2 As silenced avo-Yr10 wheat plant relative to BSMV:00 inoculated Avo-Yr10 wheat plants. **j**: Disassociation curves show that the *Sgt1* gene specific real-time PCR primers work correctly.

Table 3.4 qRT-PCR real-time analysis of silencing of *Rad6* by BSMV mediated VIGS in the third leaves of Avocet-Yr10 wheat plants and the level of *Rad6, Rar1, Sgt1,* and *Hsp90* expression. Silencing of *Rad6* by BSMV mediated VIGS affect the expression levels of *Rar1, Sgt1,* and *Hsp90* genes.

Experiment	Relative gene expression*			
	Rad6	*Rar1*	*Sgt1*	*Hsp90*
1	↓ 0.396	↑ 2.10	↑ 1.90	↑ 1.67
2	↓ 0.317	↑ 2.17	↑ 1.94	↑ 1.31
3	↓ 0.407	↑ 2.16	↑ 2.45	↑ 2.95
4	↓ 0.145	↑ 2.02	↑ 2.01	↑ 1.191
5	NC	NC	NC	NC
6	↓ 0.243	↑ 4.03	↑ 2.30	↑ 2.16
7	↓ 0.414	↑ 1.90	↑ 1.90	↑ 1.67
8	↓ 0.295	↑ 3.11	↑ 2.09	↑ 1.87

* Relative expression of *Rad6, Rar1, Sgt1, Hsp90* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:RAD6.2As silenced plants by the level of *the genes* transcripts from BSMV:00 virus alone but non silenced plants. ↓: decrease ↑: increase NC: Not Changed.

Rad6 gene expression level decrease was observed in BSMV:RAD6.2As inoculated Avo-Yr10 wheat plants compared to BSMV:00 virus alone inoculated Avo-Yr10. Due to the *Rad6* gene silencing *Rar1*, *Sgt1,* and *Hsp90* gene expression levels were increased. The *Rad6* gene silencing was also performed in Avo-Yr1 wheat plants and followed to detect similar phenotype and same expression level differentiation of the genes observed before.

Table 3.5 qRT-PCR real-time analysis of silencing of *Rad6* by BSMV mediated VIGS in the third leaves of Avocet-Yr1 wheat plants and the level of *Rad6, Rar1, Sgt1,* and *Hsp90* expression. Silencing of *Rad6* by BSMV mediated VIGS affects the expression levels of *Rar1, Sgt1, Hsp90* genes.

Experiment	Relative gene expression*			
	Rad6	*Rar1*	*Sgt1*	*Hsp90*
1	↓ 0.383	ND	ND	ND
2	↓ 0.341	ND	ND	ND
3	↓ 0.526	↑ 1.88	↑ 1.81	↑ 1.56
4	↓ 0.327	↑ 2.45	↑ 2.28	↑ 2.70
5	↓ 0.276	↑ 2.61	↑ 2.45	↑ 2.79

* Relative expression of *Rad6, Rar1, Sgt1, Hsp90* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:RAD6.2As silenced plants by the level of *the gene* transcripts from BSMV:00 virus alone but non silenced plants. ↓: decrease ↑: increase ND: Not determined

Silencing of *Rad6* gene in Avo-Yr1 wheat plants leads to same physiologic and molecular results with BSMV:RAD6.2As inoculated Avo-Yr10 wheat plants (Table 3.4, Figure 3.16).

3.2.2.4 qRT-PCR analyses present *Rar1*, *Sgt1*, *Hsp90* genes expression level increase *via* silencing of *Rad6* gene in wheat lines

The silencing success of BSMV:RAD6.2As has been proven by qRT-PCR. The phenotype which was observed as HR symptoms is detected and analyzed in silenced wheat leaves (Figure 3.15-16). Determination of the expression levels of some important genes might provide the explanation of HR-like symptoms caused by *Rad6* gene suppression. The genes, *Rar1, Sgt1,* and *Hsp90* which are related with disease resistance and their roles in the mechanisms have been already proven (see discussion part for detailed information). Our analysis aimed to search out the expression level of those genes in silenced and non-silenced plants. Relative expression levels of that genes were found to be induced upon BSMV:RAD6.2As silencing (Figure 3.18 **e, g, i** and Table 3.4-5). The results confirm the HR phenotype of BSMV:RAD6.2As silenced wheat plants.

3.2.2.5 *Rad6* gene silencing in barley (*Hordeum vulgare*) lines

BSMV mediated gene silencing in wheat has been performed efficiently. Previously, *Rad6* gene silencing was optimized for wheat plants then the *Rad6* gene silencing for barley plants was attempted to apply. Primary goals for silencing of *Rad6* gene in barley were; 1: Could we see the similar phenotype and molecular results in different plants? 2: What would be observed after inoculation of avirulent and/or virulent race specific pathogens on *Rad6* gene silenced barley plants?

First plant to apply BSMV:RAD6.2As silencing was the *Mla6* resistance gene containing Golden promise barley plant, the second silencing host was *Mla1* resistance gene containing Golden promise barley and the last was the universal susceptible Bülbül barley cultivar. The BSMV:RAD6.2As and BSMV:00 inoculations have been applied on barley plants as following optimized VIGS method which is previously used. The gene specific suppression was analyzed in terms of expression level gene expression differentiation, phenotypic changes and pathogenesis (virulent, avirulent) tests.

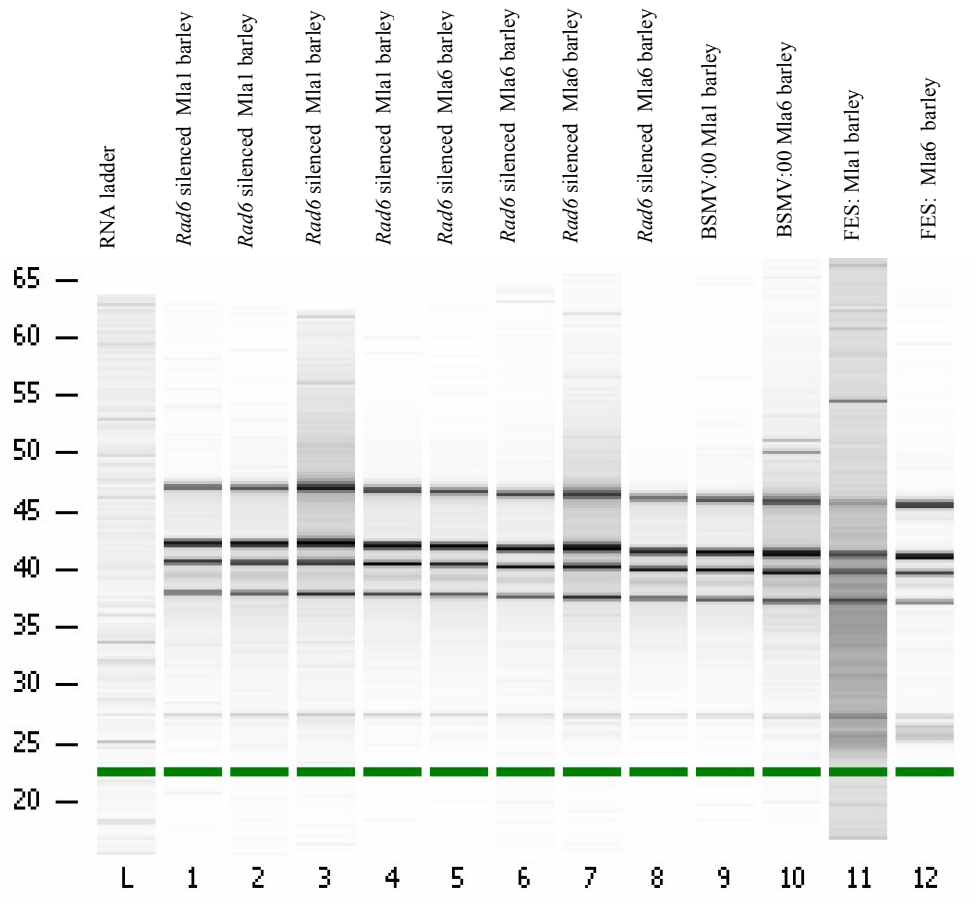


Figure 3.19 RNA analysis of *Rad6* silenced and non-silenced Myc-Mla1 containing Golden promise barley and Myc-Mla6 Golden promise barley plants. All of the RNAs was run on RNA Nano-chip and analyzed on Agilent 2100 bioanalyzer (RAN analysis reports for this run is presented in Appendix-F).

3.2.2.6 qRT-PCR analyses for *Rad6* gene expression level determination in silenced barley lines

Rad6 gene was efficiently silenced using the VIGS system in barley plants. The VIGS mediated silencing reaction caused 55-80% suppression of the gene expression of analyzed plant samples. BSMV:RAD6.2As silencing on Myc-Mla6 and Myc-Mla1/Golden promise barley plants contains pα, pβΔβa and pγRad6.2As transcript mixture of 1:1:1 quantity. 1.5 µg of each purified *in vitro* synthesized

transcripts was used for one silencing reaction. As a positive control BSMV:00 silencing has been applied. BSMV:00 contains pα, pβΔβa and pγ transcript mixture of 1:1:1 quantity with only virus inoculation. Silenced, positive and negative control plant samples were collected for RNA isolations and cDNA synthesis. qRT-PCR analyzes showed the decrease of *Rad6* expression levels in plants as shown in Table 3.5 and 3.6. Figure 3.20 to 3.32 indicate that the silencing of *Rad6* gene causes programmed cell death in barley plants, too. The reason for that might be the up regulation of some disease resistance related genes listed in the table 3.4- 7.

Myc-Mla6/Golden promise barley plants were silenced with BSMV:RAD6.2As then evaluated in terms of the level of *Rar1*, *Sgt1,* and *Hsp90* expression. Table 5 shows the effective silencing results as Avo-Yr10 wheat plants *Rad6* level decrease causes the expression of some disease resistance related genes such as increase in transcript level of *Rar1*, *Sgt1,* and *Hsp90*.

Table 3.6 qRT-PCR real-time analysis of silencing of *Rad6* by BSMV mediated VIGS in the third leaves of golden promise Mla6 barley plants and the level of *Rad6, Rar1, Sgt1,* and *Hsp90* expression. Silencing of *Rad6* by BSMV mediated VIGS affects the expression levels of *Rar1, Sgt1,* and *Hsp90* genes

Experiment	Relative gene expression*			
	Rad6	*Rar1*	*Sgt1*	*Hsp90*
1	↓ 0.495	↑ 1.81	↑ 1.90	↑ 1.41
2	↓ 0.393	↑ 1.90	↑ 2.20	↑ 1.67
3	↓ 0.471	↑ 1.88	↑ 1.82	↑ 1.56
4	↓ 0.227	↑ 2.40	↑ 2.01	↑ 1.91
5	↓ 0.413	↑ 1.89	↑ 1.81	↑ 1.64

*Relative expression of *Rad6, Rar1, Sgt1,* and *Hsp90* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:RAD6.2As silenced plants by the level of the gene transcripts from BSMV:00 virus alone but non silenced plants. ↓: decrease ↑: increase

The other *R* gene containing barley plant, *Mla1* gene, has been used for efficient *Rad6* gene silencing. Thus, the effect of the gene silencing can be search for *R* gene specifity. As a result, same outcome has been obtained with the silencing

of *Rad6* in Myc-Mla1/Golden promise barley by two aspects; first; disease resistance related genes *Rar1* and *Sgt1* gene expressions were induced (Table 3.6), second; silenced plants has produced HR, rapid cell death.

Table 3.7 qRT-PCR real-time analysis of silencing of *Rad6* by BSMV mediated VIGS in the third leaves of golden promise Mla1 barley plants and the level of *Rad6, Rar1, Sgt1, Hsp90* expression. Silencing of *Rad6* by BSMV mediated VIGS affects the expression levels of *Rar1, Sgt1,* and *Hsp90* genes.

Experiment	Relative gene expression*			
	Rad6	*Rar1*	*Sgt1*	*Hsp90*
1	↓ 0.277	↑ 1.90	↑ 1.90	↑ 1.64
2	↓ 0.165	↑ 2.30	↑ 2.40	↑ 1.91
3	↓ 0.314	↑ 2.01	↑ 1.90	↑ 1.56
4	↓ 0.227	ND	ND	ND
5	↓ 0.413	ND	ND	ND

* Relative expression of *Rad6, Rar1, Sgt1, Hsp90* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:RAD6.2As silenced plants by the level of *the gene* transcripts from BSMV:00 virus alone but non-silenced plants. ↓: decrease ↑: increase, ND: Not determined.

3.2.2.7 Physiological results of *Rad6* gene silenced barley lines

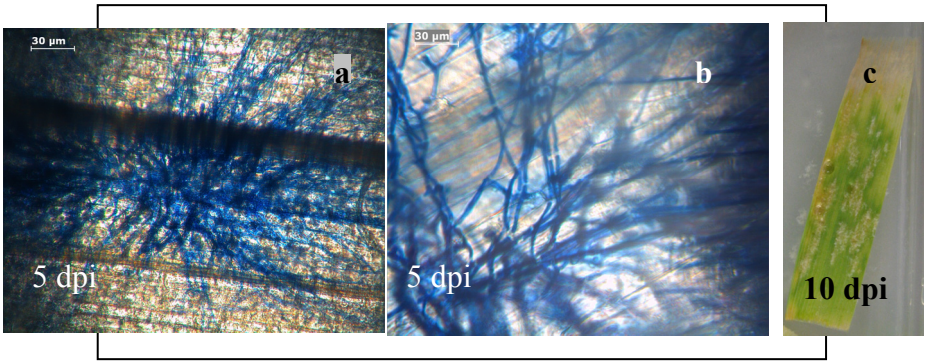
BSMV:RAD6.2As silencing has been applied on different types of barley plants with the positive and negative controls which are BSMV:00 and FES control inoculations respectively. Following the seventh day of silencing virulent and avirulent fungal pathogen inoculations have been performed. The schema of silencing was:

1-BSMV:RAD6.2As, BSMV:00 and FES inoculations have been applied on *Mla1* gene having barley plants and then virulent Bgh95(53/01) and avirulent Bgh 103(64/01) pathogens were inoculated.

2-BSMV:RAD6.2As, BSMV:00 and FES inoculations have been applied on *Mla6* gene having barley plants and then virulent Bgh103(64/01)and avirulent Bgh95(53/01) fungal inoculations were done.

3-BSMV:RAD6.2As, BSMV:00 and FES inoculations have been applied on universal susceptible barley Bülbül plants and then virulent Bgh95(53/01) fungal inoculation has been performed.

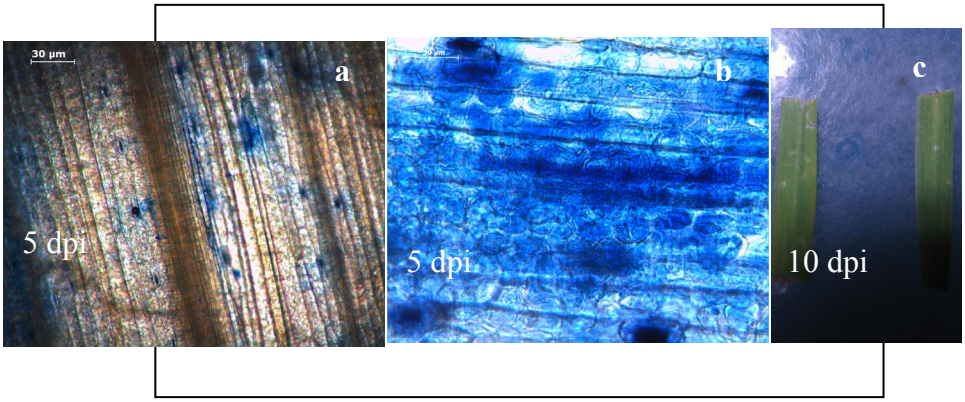
BSMV:00 virus alone



Bgh95(53/01)

Figure 3.20 Myc-Mla1/Golden promise barley plants treated with BSMV:00 **a** and **b** (trypan blue staining) was pictured 5 dpi and **c** was pictured 10 dpi the powdery mildews are easily seen on plant leave, interaction is still compatible.

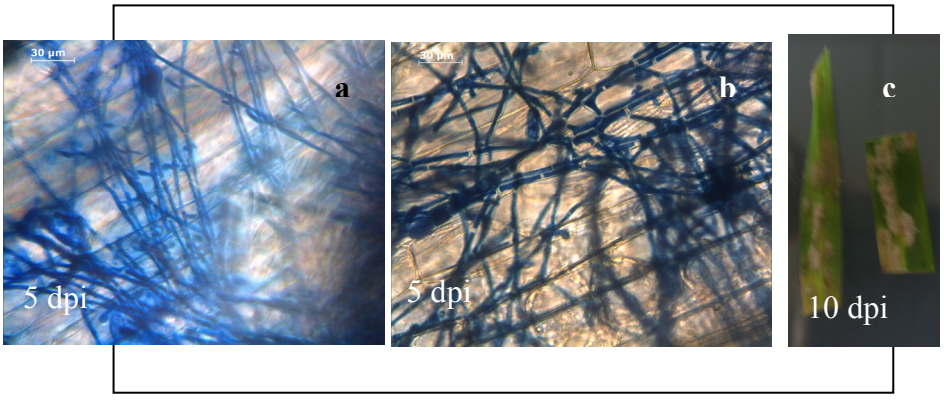
BSMV:RAD6.2 As



Bgh95(53/01)

Figure 3.21 Myc-Mla1/Golden promise barley plants treated with BSMV:RAD6.2As **a** and **b**: (trypan blue staining) were pictured 5 dpi **c**: no powdery mildew can produce spor formation on silenced plant leaves as pictured 10 dpi, interaction is turned into incompatible.

BSMV:00 virus alone



Bgh103(64/01)

Figure 3.22 Myc-Mla6/Golden promise barley plants treated with BSMV:00 **a** and **b** (trypan blue staining) were pictured 5 dpi and **c** was pictured 10 dpi the powdery mildews are easily seen on plant leaves, interaction is still compatible.

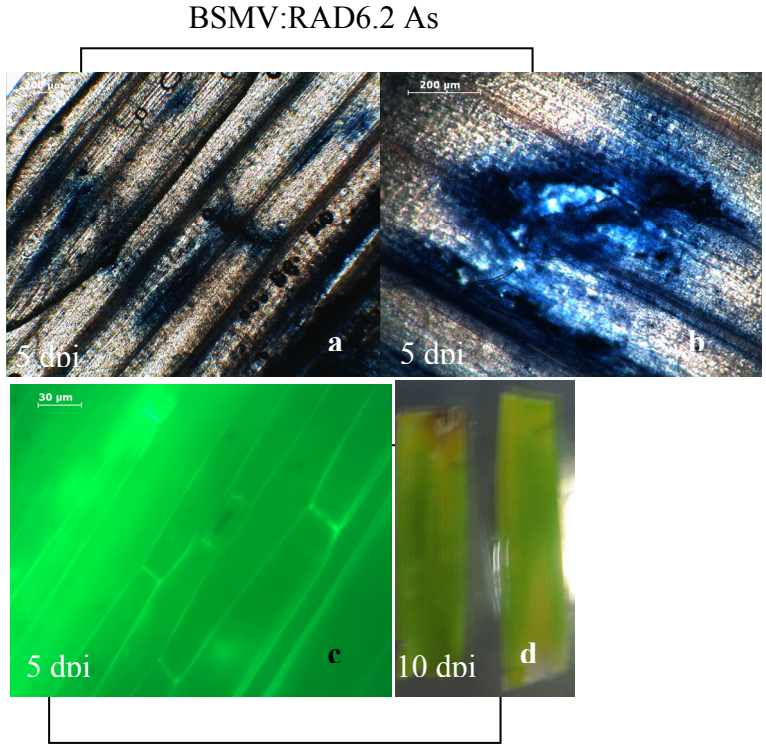
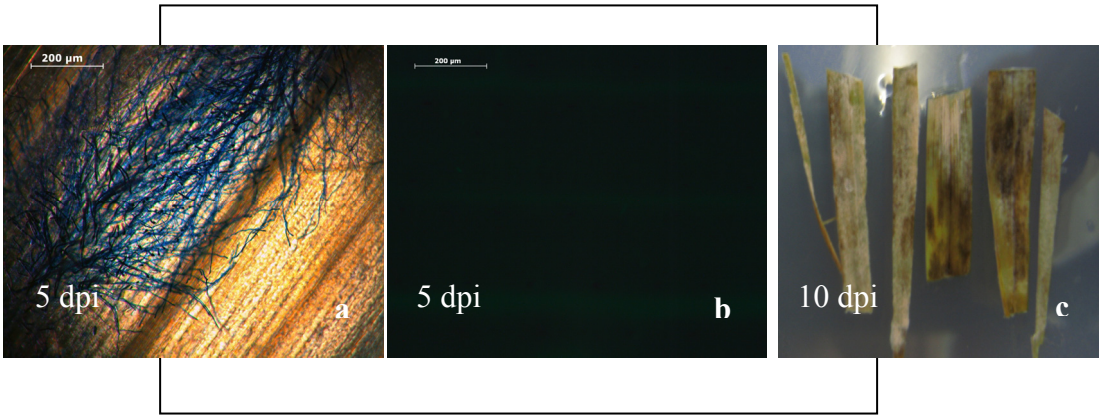


Figure 3.23 Myc-Mla6/Golden promise barley plants treated with BSMV:RAD6.2As **a** and **b:** (trypan blue staining) were pictured 5 dpi **c:** Auto-flouresence in the silenced plant leave cells was detected and **d** no powdery mildew can produce spor formation on silenced plant leaves as pictured 10 dpi, interaction is turned into incompatible.

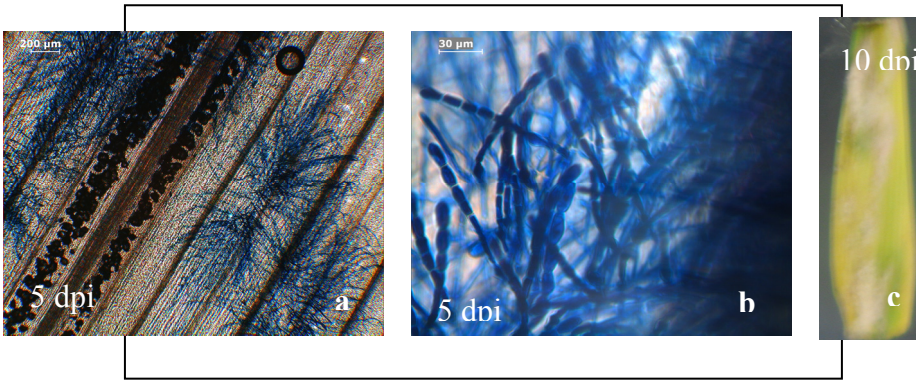
BSMV:00 virus alone



Bgh95(53/01)

Figure 3.24 Bülbül barley plants treated with BSMV:00 **a** (trypan blue staining) were pictured 5 dpi **b**: No auto-flouresence was detected in the BSMV:00 inoculated plant leave cells and **c** was pictured 10 dpi the powdery mildews are easily seen on plant leaves, interaction is still compatible.

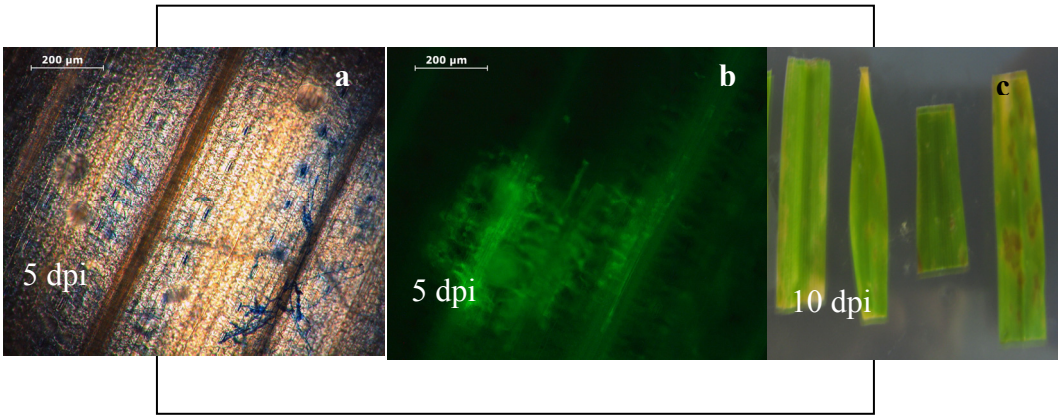
FES negative control



Bgh95(53/01)

Figure 3.25 Bülbül barley plants treated with FES **a** and **b** (trypan blue staining) were pictured 5 dpi and **c** was pictured 10 dpi the powdery mildews are easily seen on plant leaves, interaction is stil compatible.

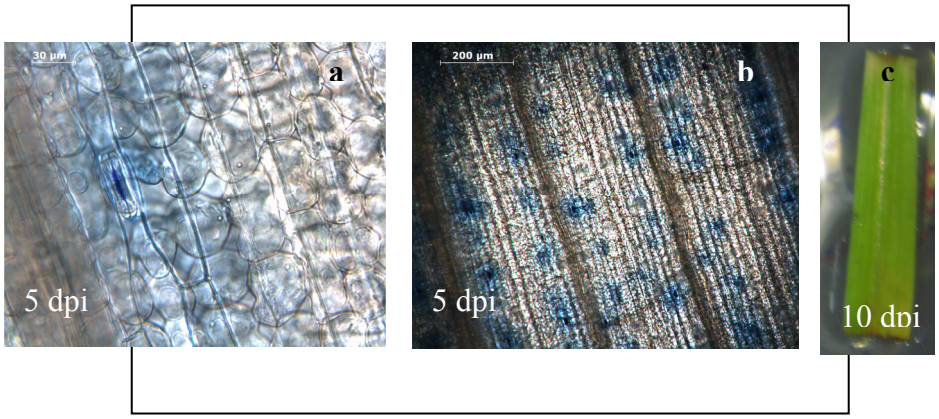
BSMV:RAD6.2 As



Bgh95(53/01)

Figure 3.26 Bülbül barley plants treated with BSMV:RAD6.2As **a**: (trypan blue staining) were pictured 5 dpi **b**: Auto-flouresence in the silenced plant leave was detected **c**: no powdery mildew can produce spor formation on silenced plant leaves as pictured 10 dpi, interaction is turned into incompatible.

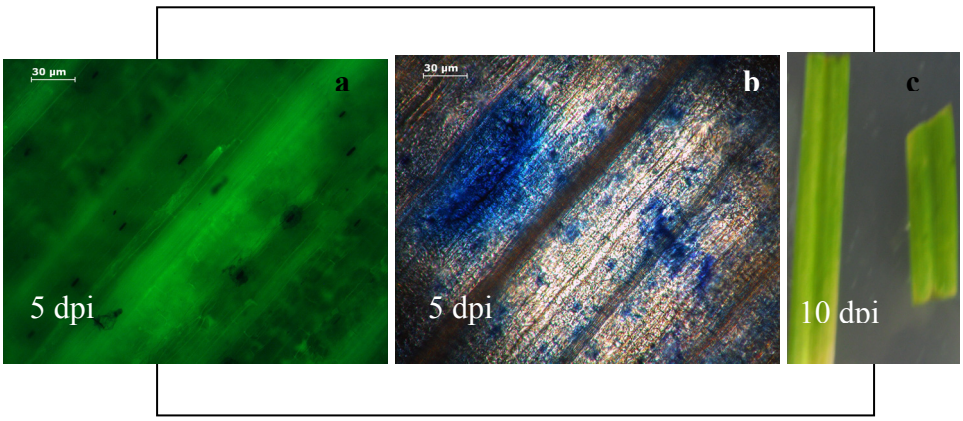
FES negative control



Bgh103(64/01)

Figure 3.27 Myc-Mla1/Golden promise barley plants treated with FES **a** and **b:** (trypan blue stainings) were pictured 5 dpi **c**: no powdery mildew can produce spor formation on silenced plant leaves as pictured 10 dpi, no silencing and the interaction is incompatible.

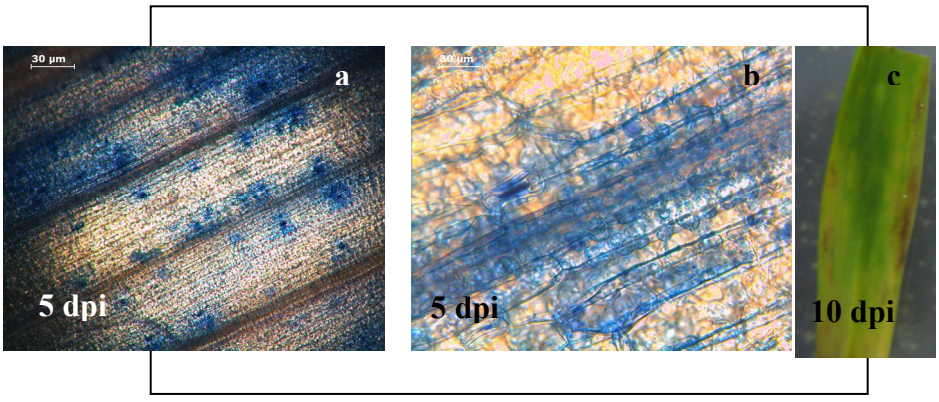
BSMV:00 virus alone



Bgh103(64/01)

Figure 3.28 Myc-Mla1/Golden promise barley treated with BSMV:00 **a** Auto-flouresence in the BSMV:00 inoculated plant leave was detected and **b:** (trypan blue staining) was pictured 5 dpi **c**: no powdery mildew can produce spor formation on BSMV:00 inoculated plant leaves as pictured 10 dpi, interaction is still incompatible.

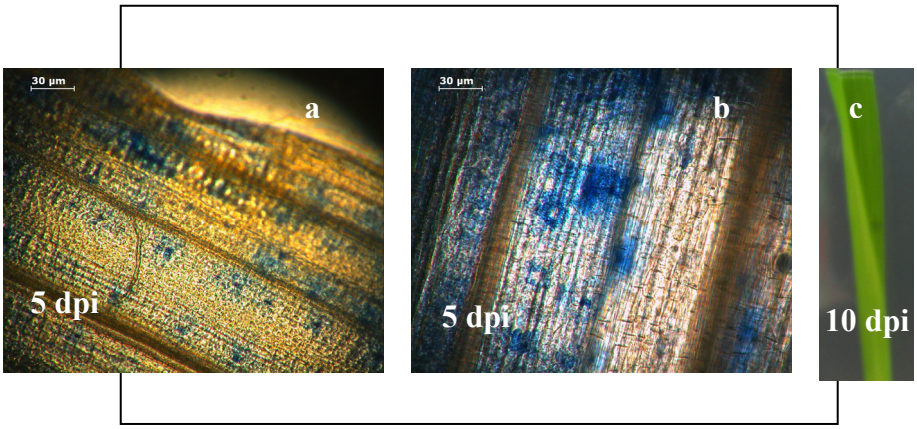
BSMV:RAD6.2As



Bgh103(64/01)

Figure 3.29 Myc-Mla1/Golden promise barley plants treated with BSMV:RAD6.2As **a** and **b**: (trypan blue staining) was pictured 5 dpi **c**: no powdery mildew can produce spor formation on BSMV:00 inoculated plant leaves as pictured 10 dpi, interaction is still incompatible.

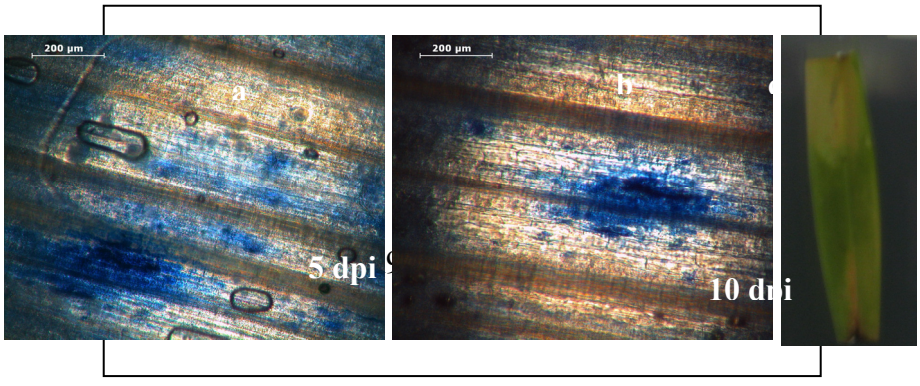
FES negative control



Bgh95(53/01)

Figure 3.30 Myc-Mla6/Golden promise barley plants treated with FES **a** and **b** (trypan blue staining) were pictured 5 dpi **c**: no powdery mildew can make spor formation on silenced plant leaves as expected pictured 10 dpi, no gene silencing interaction is incompatible.

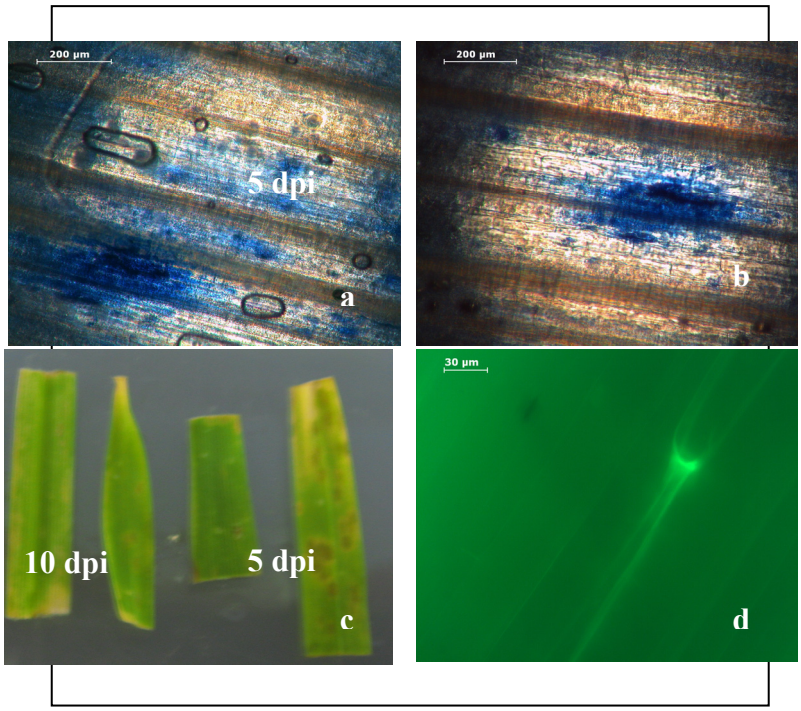
BSMV:00 virus alone



Bgh95(53/01)

Figure 3.31 Myc-Mla6/Golden promise barley plants treated with BSMV:00 **a** and **b:** (trypan blue staining) were pictured 5 dpi **c**: no powdery mildew can produce spor formation on silenced plant leaves as expected pictured 10 dpi, interaction is still incompatible.

BSMV:RAD6.2As



Bgh95(53/01)

Figure 3.32 Myc-Mla6/Golden promise barley plants treated with BSMV:RAD6.2As **a** and **b:** (trypan blue stainings) were pictured 5 dpi **c**: no powdery mildew can produce spor formation on silenced plant leaves as pictured 10 dpi **d**: Auto-flouresence in the BSMV:RAD6.2 As inoculated plant leave cells was detected 5 dpi, interaction is still incompatible.

BSMV mediated *Rad6* gene silencing reactions have been successfully applied on different types of barley plants. That provides us some interesting results about the disease resistance mechanisms in plants. Susceptible plants were silenced by VIGS system and these were subjected to virulent fungal inoculations. The level

of susceptibility was dropped out because of the formation of programmed cell death in the silenced plants. The susceptible plants were identified to behave as resistant. In three different conditions my hypothesis has been proven and confirmed. Figure 3.21 shows the changes from susceptibility to resistance, susceptible Myc-Mla1/Golden promise barley plant inoculation after *Rad6* gene silencing with its virulent pathogen Bgh95(53/01). Level of fungal inoculation success and susceptibility were pictured in Figure 3.20. Figure 3.23 shows the resistance plant HR symptoms of susceptible Myc-Mla6/Golden promise plant inoculation after *Rad6* gene silencing with its virulent pathogen Bgh103(64/01). Level of fungal inoculation success and susceptibility was pictured in Figure 3.22. Figure 3.25 shows infection success of virulent pathogen Bgh95(53/01) and disease level of universal susceptible Bülbül plants the inoculation was done with FES solution. On the other hand BSMV:00 silencing applied Bülbül plants was also inoculated with Bgh 95(53/01) and the pathogen was still virulent so the viral inoculation does not affect on disease resistance Figure 3.24. However BSMV:RAD6.2As silenced Bülbül plants turned into resistance case Figure 3.26. HR is generated in the silenced plants so the *Rad6* silencing in all barley plants causes programmed cell death in plants. The effect of *Rad6* silencing on resistance level was also checked. Figure 3.32 shows the *Rad6* silenced Myc-Mla6/Golden promise plants with the HR responses so the resistance is conserved with the inoculation of avirulent Bgh95(53/01). Same results is seen in Figure 3.29 there was no change means no return from resistance case to susceptible case in the *Rad6* silenced Myc-Mla1/Golden promise with the inoculation of avirulent Bgh103(64/01). Control experiments for those silencing results were performed Figure 3.27, 3.28, 3.30, 3.31 show resistance conditions in plants.

3.2.2.8 Proteomics aspects of *Rad6* gene silenced wheat plants

Rad6 gene was successfully silenced and the suppression of this gene was confirmed by qRT-PCR. Another outcome of *Rad6* gene silencing was the increased level of the resistance related genes. Physiological experiments gave idea about the function of that gene. The silencing of *Rad6* gene causes HR in wheat and plants therefore leads to enhanced resistance. To gather all kinds of data obtained from

Rad6 gene silencing experiments and understand the role of that gene in resistance mechanism, the silenced plant and non-silenced plant were also searched at proteome level. Therefore the *Rad6* gene was silenced in Avo-Yr10 wheat plants and selected one of them which have 85.5% suppression of *Rad6* gene expression. BSMV:RAD6.2As inoculated (silenced) Avo-Yr10 wheat plant proteome has been compared with BSMV:00 (without any endogenous plant gene) virus alone inoculated Avo-Yr10 wheat plants. To subtract the virus alone induced or silenced plant proteins BSMV:00 positive control plant proteome was used for comparison. I aimed to investigate that how plant proteome differs in the RAD6 protein suppressed wheat to investigate. For this purpose two-dimensional poly-acrylamide gel electrophoresis (2-D PAGE) experiment was performed. Totally 47 protein spots were analyzed which are differentially synthesized in silenced plant leaves. 9 of the protein spots give no hit with the amino acid database. 30 protein spots were detected as down-regulated or suppressed in the silenced Avo-Yr10 wheat plant according to non-silenced but viral inoculated Avo-Yr10 wheat leaves and 17 protein spots were detected as up-regulated in the silenced plant proteome (Table 3.8-9). A total of 38 unique proteins from these 47 spots were identified by Liquid chromatography/Mass /Mass spectrometry (LC-MS/MS).

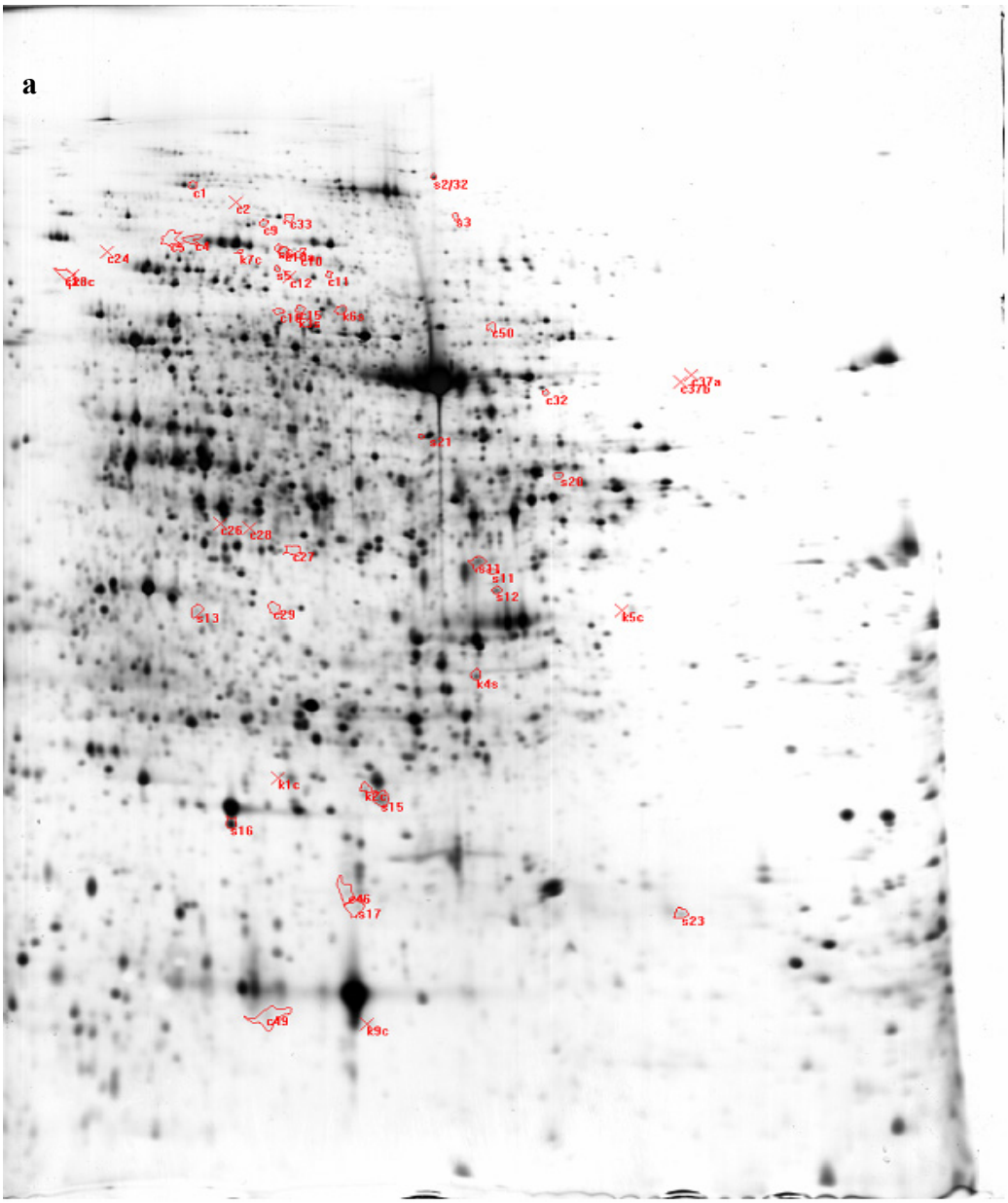


Figure 3.33 2-D PAGE results of *Rad6* gene silenced Avo-Yr10 wheat plant versus BSMV:00 inoculated Avo-Yr10 wheat plant. **a**: BSMV:00 inoculated Avo-Yr10 wheat plants 30 dpi were used for 2-D PAGE. Silver stained gels were pictured with detected spots.

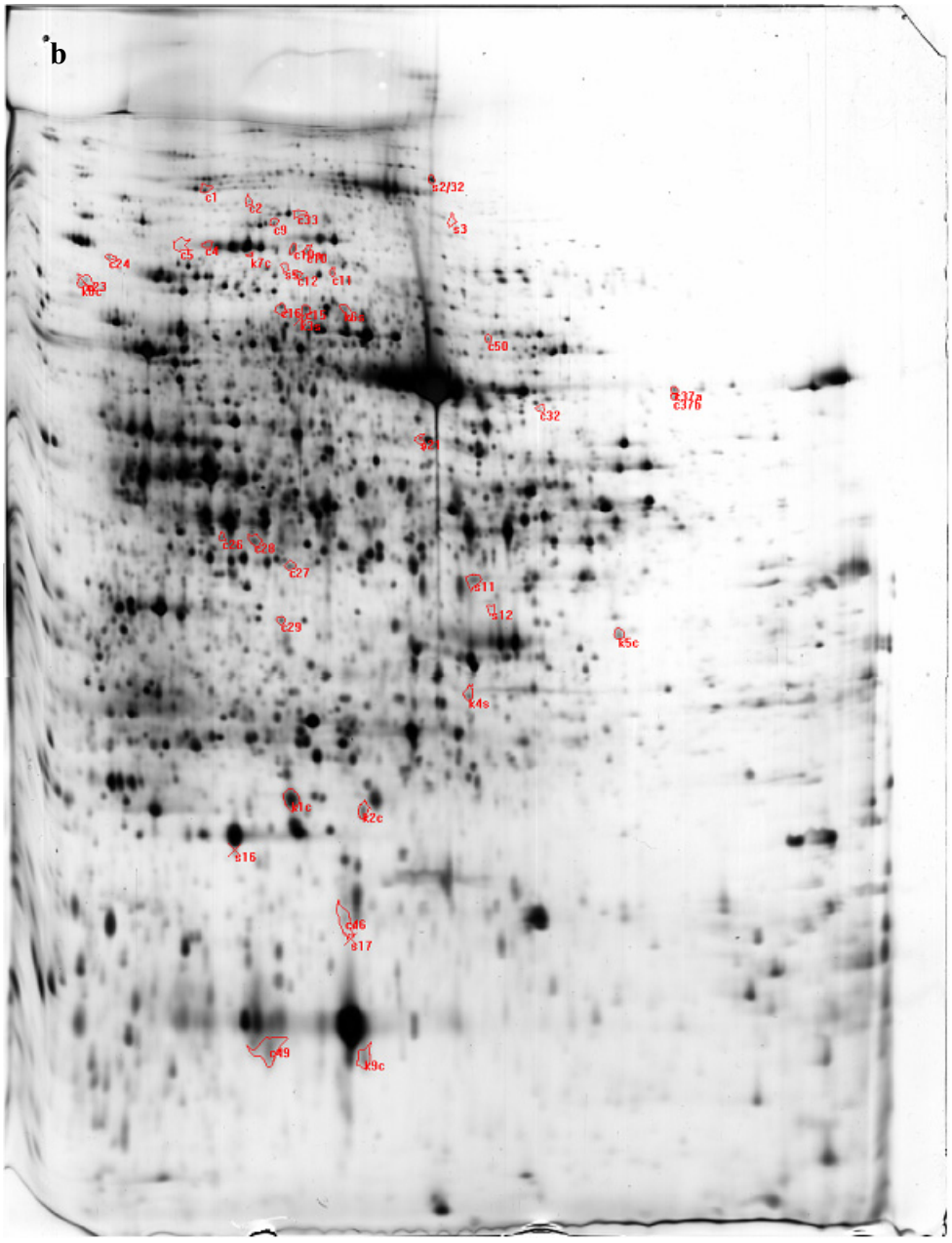


Figure 3.33 (continued) **b**: BSMV:RAD6.2As inoculated (*Rad6* gene silenced) Avo-Yr10 wheat plant leave samples 6.89 fold (85.5% of the gene expression was suppressed) silenced according to BSMV:00 virus alone inoculated Avo-Yr10 wheat plant 30 dpi were used for 2-D PAGE. Silver stained gels were pictured with detected spots.

Table 3.8 List of protein spots that are down-regulated after *Rad6* gene silencing (BSMV:RAD6.2As)

No	Name	ID
1	c01	Cell Division Cycle Protein 48
2	c02	-
3	c04	Transketolase, Chloroplast
4	c05	Transketolase, Chloroplast
5	c09	Osjnba0039c07.4 ATP-Dependent Clp Protease ATP-Binding Subunit Clpa
6	c10	Putative Transketolase
7	c10a	Transketolase 1
8	c11	-
9	c12	Phosphoglucomutase
10	c15	ATP Synthase CF1 Alpha Chain
11	c16	ATP Synthase CF1 Alpha Chain
12	c23	-
13	c24	-
14	c26	Unknown Protein (Blast: Ribosomal L4)
15	c28	Unknown Protein Os04g0459500
16	c29	-
17	c32	ATP Synthase CF1 Alpha Chain
18	c33	Putative Transketolase
19	c37a	Beta-D-Glucan Exohydrolase Beta-D-Glucosidase
20	c37b	Signal Recognition Particle 54 Kda Protein, Chloroplast Precursor
21	c46	Cytochrome B6-F Complex İron-Sulfur Subunit
22	c49	Ribulose Bisphosphate Carboxylase Small Chain
23	c50	2-Oxoglutarate Dehydrogenase, E3 Subunit
24	K01c	Germin-Like Protein 1
25	K02c	Os02g0259600 RNA Binding / Structural Constituent Of Ribosome
26	K05c	Peroxidase 6
27	K07c	-
28	K08c	-
29	K09c	-
30	S02/32	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Large Subunit

Table 3.9 List of protein spots that are up-regulated after *Rad6* gene silencing (BSMV:RAD6.2As)

No	Name	ID
1	K03s	Methionine Synthase
2	K04s	Glutathione-S-Transferase 19E50
3	K06s	ATP Synthase CF1 Alpha Chain
4	S02/32	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Large Subunit
5	s03	-
6	s05	Methionine Synthase
7	s06	Putative Transketolase
8	s10	UCW116, Putative Lipase
9	s11	Beta-1,3-Glucanase
10	s12	Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase
11	s13	ATP Synthase Subunit Beta, Mitochondrial Precursor
12	s15	Ribulose Bisphosphate Carboxylase Large Chain Precursor
13	s16	Ribulose 1,5-Bisphosphate Carboxilase/Oxigenase Large Subunit
14	s17	Cytochrome B6-F Complex İron-Sulfur Subunit, Chloroplast Precursor
15	s20	UDP-D-Glucose Epimerase 3
16	s21	Putative Heterogeneous Nuclear ribonucleoprotein A2
17	s23	ATPase Alpha Subunit

Silver stained PAGE gels were used for detection of differentially expressed protein spots. Some of the spots were excised from the commassie stained 2-D PAGE gel and then amino acid sequences of protein spots were analyzed by Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC-MS/MS). Sequence results were analyzed and detected amino acids were searched for protein hits in the protein databank.

Proteins that were down-regulated in the BSMV:RAD6.2 As silenced wheat plants were listed in Table 3.8. Some of the proteins related indirectly with disease resistance which are suppressed during infection in resistant plants. The up-regulated or induced proteins in *Rad6* gene silenced plant were listed in Table 3.9. Some of the proteins which are known as Pathogenesis Related (PR) protein are also induced in

Rad6 silenced plant. Especially, peroxide mechanism related proteins for peroxide synthesis are up regulated as well.

3.2.2.9 *Rar1* and *Sgt1* gene expression levels are increase upon avirulent infection

In the literature many genes were detected as up regulated during infection of certain pathogen attacks. Especially in race-specific disease resistance some well known genes are induced upon avirulent inoculations. *Rar1* and *Sgt1* genes are both related with disease resistance and they are induced upon incompatible plant-pathogen interaction. qRT-PCR analyses proved the expression of those genes are also up-regulated in our plant- pathogen system which is Avo-Yr1 wheat – yellow rust Pst-232E137 incompatible interaction. Figure 3.34 shows the expression level induction of *Rar1* and *Sgt1* genes upon yellow rust inoculation compared to mock inoculated Avo-Yr1 control wheat plants.

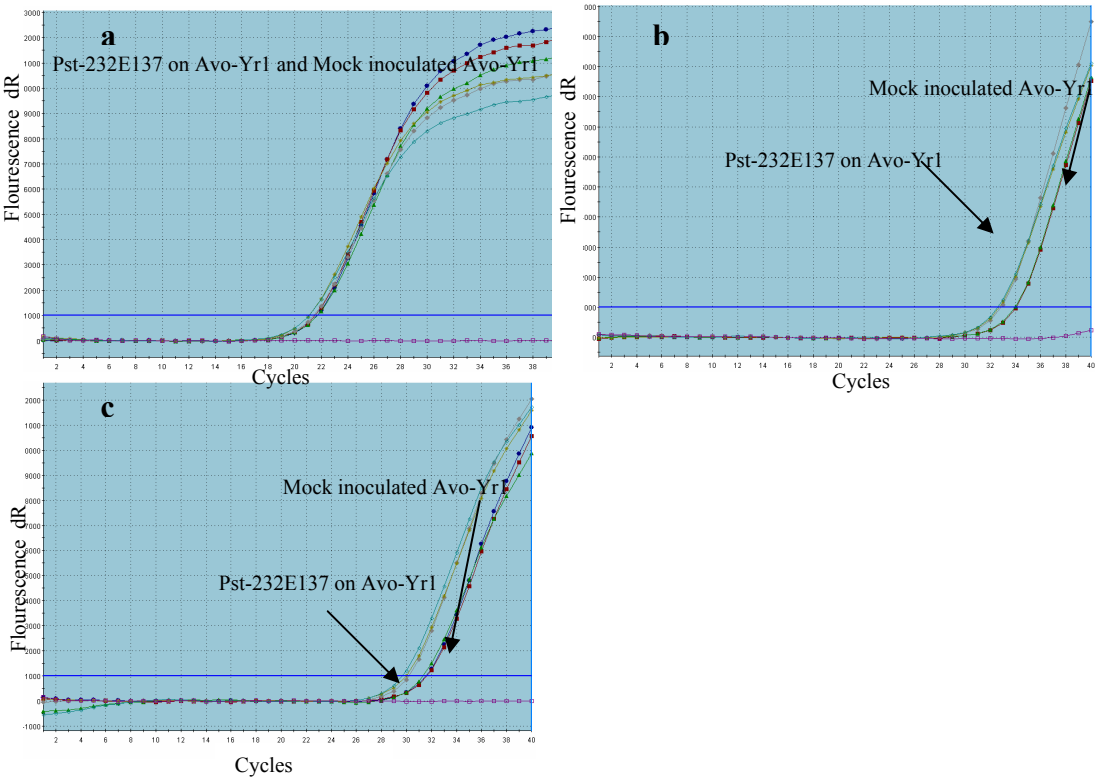


Figure 3.34 qRT-PCR determination of *Rar1* and *Sgt1* gene transcript levels after avirulent Pst-232E137 on Avo-Yr1 wheat plants (defense response) inoculations. **a**: Firstly all the RNA levels in both cases were normalized using *18s rRNA* gene specific primers. **b** is the *Rar1* gene expression level increase after avirulent inoculation nearly 2.61 fold up-regulation is seen and Figure 3.34. **c** shows the level of induction in *Sgt1* gene after avirulent inoculation nearly 2.45 fold increase is presented.

3.2.2.10 BSMV virus inoculation decreases the expression level of *Rar1*, *Sgt1* and *Hsp90* genes

BSMV infects wheat and barley and the time course of inoculation, it tries to suppress some of the host gene expressions for efficient infection. The expression levels of three important disease resistance related genes which are *Rar1 Sgt1* and *Hsp90* have been measured upon viral inoculation. In Figure 3.35 the decrease in expression of those genes were pictured. BSMV:00 inoculated Avo-Yr10 wheat plant cDNA was compared with (mock) FES inoculated Avo-Yr10 cDNA. It has been

shown here the expression of those genes was suppressed by BSMV virus for efficient infection and break down of resistance.

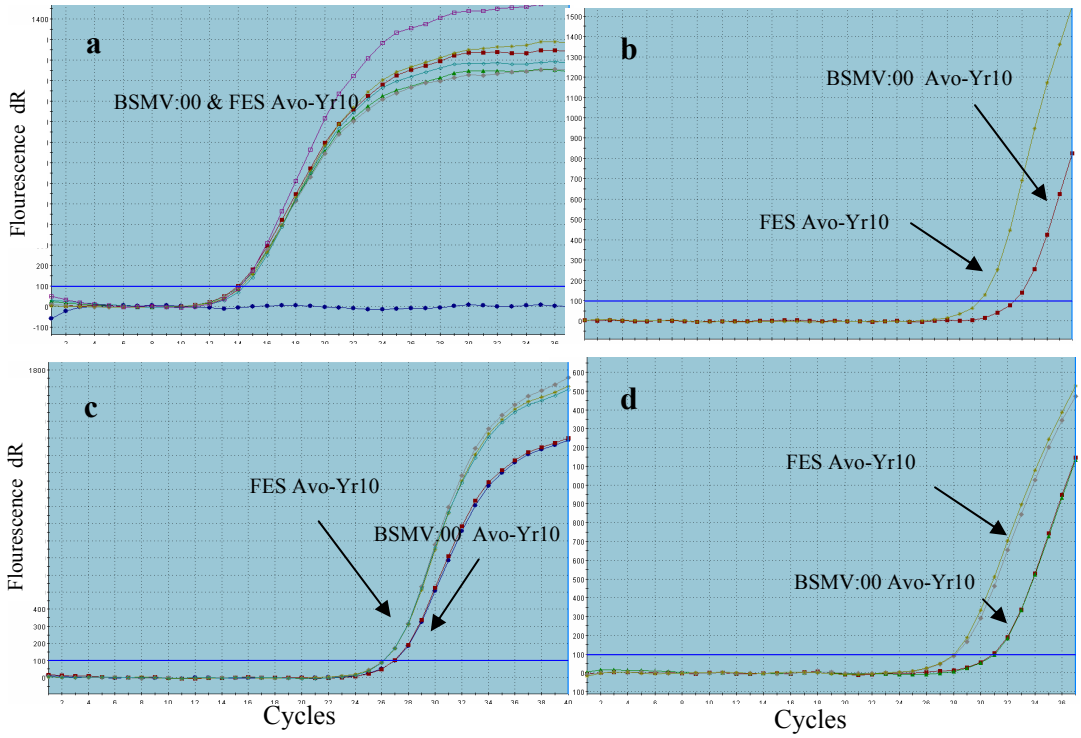


Figure 3.35 qRT-PCR determinations of *Rar1*, *Sgt1,* and *Hsp90* gene transcript levels after virus inoculation on Avo-Yr10 wheat plants. Firstly the RNA levels in both cases BSMV:00 inoculated and FES negative control inoculated samples RNAs were normalized using *18s rRNA* gene specific primers. FES is the solution causing wounding for viral infection on plants. **a** is the normalization of FES and BSMV treated wheat RNAs. **b** is the *Rar1* gene expression level suppression after viral inoculation approximately 5.65 fold down-regulation is seen. **c** shows the *Sgt1* gene expression level suppression after viral inoculation approximately 6.23 fold down-regulation is seen. **d** is the *Hsp90* gene expression level suppression after viral inoculation approximately 1.72 fold down-regulation is seen.

3.2.3 *Rar1* gene silencing *via* BSMV in wheat

To demonstrate the relationship of *Rad6* gene expression with *Rar1* expression the *Rad6* gene was silenced and the induction of *Rar1* gene was detected. Systemic RNA silencing of *Rar1* gene was also performed to determine the effect of *Rar1* gene silencing on *Rad6* gene expression.

3.2.3.1 *Rar1* gene silencing construct preparation

Prior to silencing experiment I have cloned the full length of *Rar1* gene from wheat transcriptome. Then obtained sequence information and clone have been used for VIGS construct preparation. Using gene-specific primers harboring appropriate (*Pac*I and *Not*I restriction enzyme) cleavage restriction enzyme sites at their 5' ends VIGS construct has been engineered. 148 bp lengths *Rar1* gene specific amplification was performed.

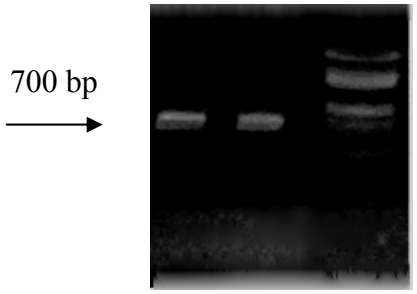


Figure 3.36 Full length *Rar1* gene was cloned from Avo-Yr10 wheat plant and sequenced. Sequence analysis confirmed the cloning

ATGTCGGCGGAGACGGAGACGAGCGCCGCCGCGCCCGCGCCCGCGCCCAT
GCGGTGCCAGCGAATAGGCTGCGACGCCATGTTCACCGACGACGACAACC
CCGACGGCTCCTGCCACTACCACCCCTCCGGACCTATGTTTCATGATGGC
ATGAAAGAGTGGAGCTGTTGCAAGCAAAGAAGCCATGATTTTAGCTTATT
TTTAGCTATTCCTGGATGTGCCACAGGGAAGCATACAACTGAGAAACCAG
TCACAAAAGCTGTTTCTCTTAACTCAAAGGCAACCCCACCAAAGTTAGCT
CCAATCCAGTCTTCTAAGCAGGGTGTGGAAACCGAGGCCTGCTCCAGGTG
CCGTCAGGGTTTCTTTTGCTCCGACCATGGATCACAGCCCAAGGCACAAA
AACCAGTTGCTGTAAATGGTACAAATACGGAACCTGTCGAAAAATGCTCA
GTTCCACAGCCCAGGAAAAAAGTTGTTAAT

Figure 3.37 *Rar1* gene nucleotide sequence and the amplified region (colored) for silencing construct.

Amplified fragment was ligated with silencing pγ As vector then this ligation product was transformed into *E. coli* competent cells. Using colony PCR with specific primers right colonies were selected. Being a second confirmation, sequencing results were analyzed then the expected pγRAR1.1As construct was linearized with appropriate (*BssH* II) restriction enzyme for *in vitro* transcription (Figure 3.38 **a-b**).

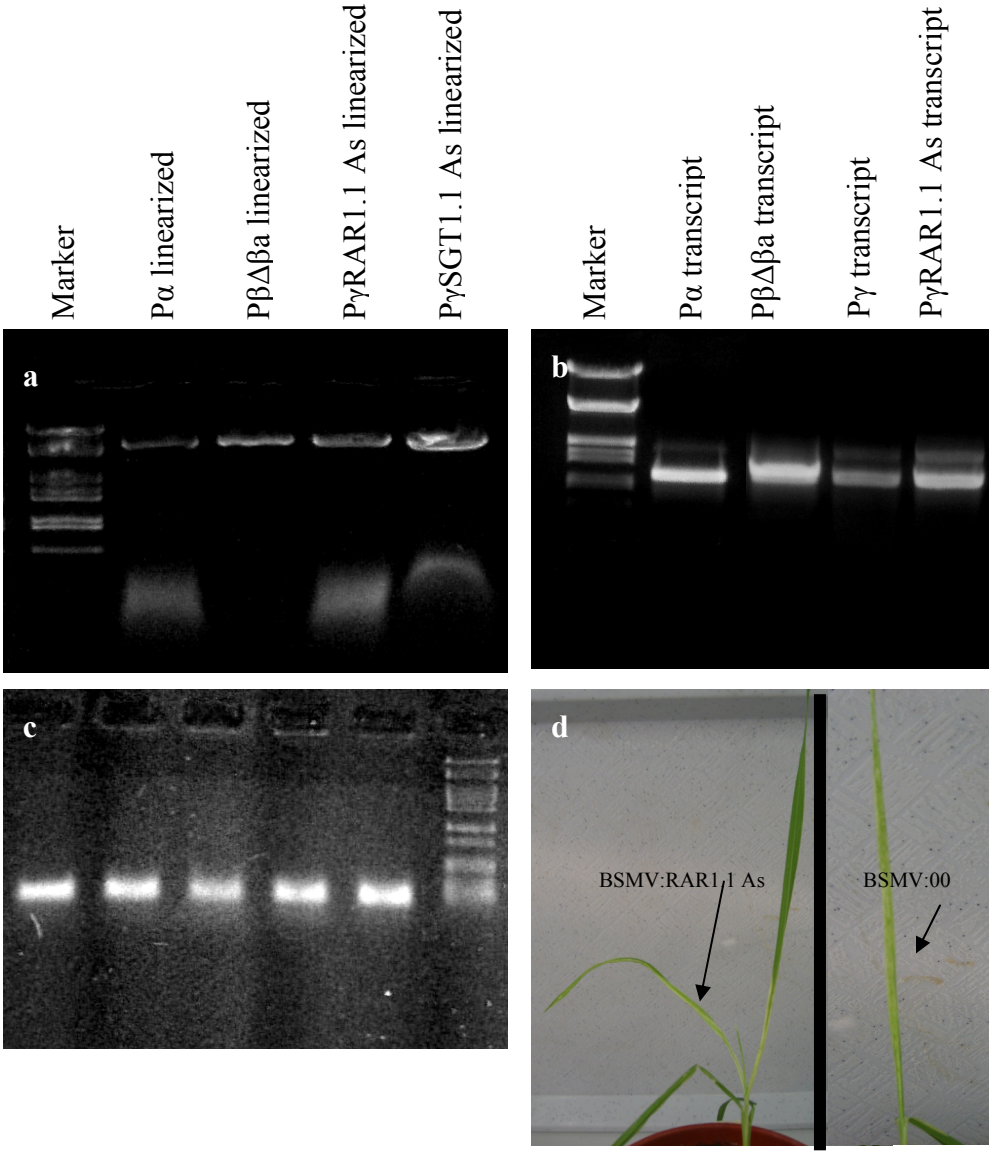


Figure 3.38 *Rar1* gene silencing of wheat. **a**: BSMV:RAR1.1 Anti-sense construct linearized plasmid version with linear pα, pβΔβa, pγ plasmids. **b**: Those linearized BSMV constructs were transcribed and purified for silencing prior to inoculation all the transcripts were analyzed in terms of concentration, quality and purity. **c**: Rar1.1 145 bp *NotI* and *PacI* enginered fragment was amplified from Avo-Yr10 wheat plant cDNA **d**: BSMV:RAR1.1 As silencing of *Rar1* gene for Avo-Yr10 wheat plants and BSMV:00 inoculated Avo-Yr10 plants the pictures were taken from 15 dpi.

3.2.3.2 qRT-PCR analyses for *Rar1* gene expression level determination in silenced wheat lines

Gene expression of *Rar1* in BSMV:RAR1.1As silenced Avo-Yr10 and BSMV:00 inoculated Avo-Yr10 were compared by using qRT-PCR. It has been shown that *Rar1* gene silencing was efficiently performed (Figure 3.39 and Table 3.10). Detection the level of *Rad6* gene expression in BSMV:RAR1.1As silenced wheat plants was performed. Figure 3.39 **d** and Table 3.10 show the *Rad6* gene level of expression was not changed in *Rar1* silenced wheat plants.

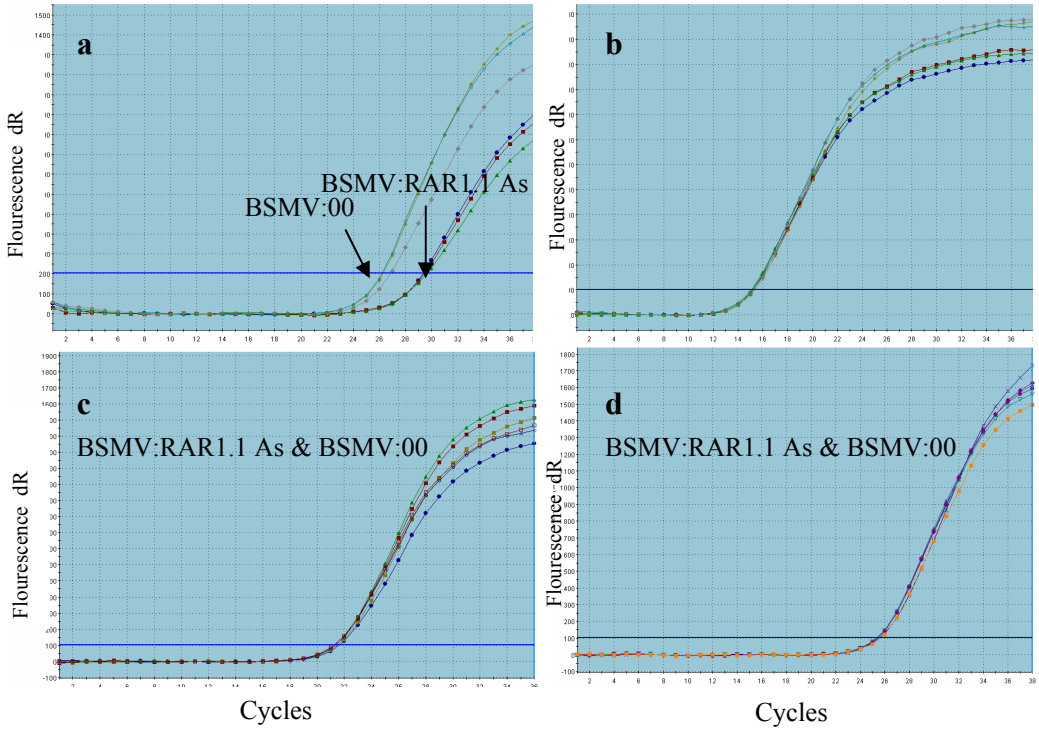


Figure 3.39 *Rar1* gene silencing and the effect of *Rar1* level decrease on *Rad6* gene expression level. **a**: BSMV:RAR1.1As silenced Avo-Yr10 wheat plant *Rar1* gene expression level was decreased by approximately 6.03 fold relatively to BSMV:00 inoculated Avo-Yr10 wheat plants. **b** and **c**: BSMV:RAR1.1As inoculated and BSMV:00 inoculated Avo-Yr10 wheat plants RNAs normalized by *18s rRNA* gene level equalization. **d**: BSMV:RAR1.1As inoculated, *Rar1* gene silenced, and BSMV:00 inoculated Avo-Yr10 wheat plants RNAs *Rad6* gene expressions relative levels normalized by *18s rRNA* gene.

Table 3.10 qRT-PCR real-time analysis of silencing of *Rar1* gene expression by BSMV mediated VIGS in the third leaves of Avocet-Yr10 wheat plants

Experiment	Relative gene expression*	
	Rar1	*Rad6*
1	↓ 0.260	NC
2	↓ 0.165	NC
3	↓ 0.462	NC
4	↓ 0.434	NC
5	↓ 0.295	NC

* Relative expression of *Rar1* and *Rad6* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:RAR1.1 As silenced plants by the level of *the gene* transcripts from BSMV:00 virus alone but non silenced plants. ↓: decrease NC: No change.

3.2.4 *Sgt1* gene silencing *via* BSMV in wheat

To demonstrate the relationship of *Rad6* gene expression with *Sgt1* expression the *Rad6* gene was silenced and the induction of *Sgt1* gene was detected. Systemic RNA silencing of *Sgt1* gene was also performed to determine the effect of *Sgt1* silencing on *Rad6* gene expression. Using qRT-PCR *Rar1,* and *Hsp90* gene expression differentiation have also been detected in *Sgt1* silencedAvo-Yr10 wheat plants.

3.2.4.1 *Sgt1* gene silencing construct preparation

Prior to VIGS experiments for *Sgt1* silencing the full length *Sgt1* gene have been cloned from wheat cDNA. Then this sequence information and clone have been used for VIGS construct preparation. Using gene specific primers harboring appropriate (*Pac*I and *Not*I restriction enzyme) cleavage restriction enzyme sites at their ends VIGS construct was engineered. 83 bp lengths of *Sgt1* gene specific amplification was performed.

ATGGCCGCCGCCGCCGCGTCGGATCTGGAGAGCAAGGCCAAGGAGGCCTTCGTCGACGACGA
CTTCGAGCTGGCCGCCGAGCTCTACACCCAGGCCATCGAGGCGGGGCCGGCTACCGCGGAAC
TCTACGCCGACCGAGCCCAGGCGCACATCAAGCTGGGCAGTTACACTGAGGCTGTAGCTGAT
GCCAACAAAGCAATTGAACTTGATCCTTCGATGCATAAAGCATACCTTCGGAAGGGCTCTGC
TTGCATCAAGCTGGAGGAATACCAAACTGCAAAGGCTGCTCTTGAAGTGGGTTCTTCTTATG
CATCTGGTGACTCGAGGTTTACTCGTCTGATGAAGGAGTGTGATGATCGTATTGCTGAGGAG
GCTAGCCAGGTGCCAGTAAAGAATGCCGCTGCGGCTGTTGCTTCAGCTACATCTTCGGGGGC
ATCTTCCGGGGCTACAACTGTGGCTACTGAAGCTGAGGACCAGGATGGCGCAAATATGGAGA
ATGCACAGCCAACGATAGAAGTGCCAAGCAAGCCCAAATACAGGCATGACTACTACAATACT
CCTACAGAAGTGGTACTGACTATATTTGCTAAGGGTGTTCCAGCTGACAGCGTGGTTGTTGA
CTTTGGTGAACAGATGCTGAGTGTCTCAATTGAACTTCCCGGTGAGGAACCATACCATTTTC
AGCCTCGTCTGTTTTCAAAGATCGTCCCAGATAAGTGCAAGTATACTGTGTTGTCTACAAAG
GTTGAAATGCGCCTTGCAAAAGCTGAGCCAGTAACTTGGACATCATTGGATTATACTGGTAA
ACCAAAGGCTCCTCAGAAGATAAATGTACCAGCTGAATCAGCCCAGAGGCCATCTTATCCAT
CATCAAAATCCAAAAAGGACTGGGATAAGCTTGAAGCTGAAGTGAAAAAACAGGAGAAGGAT
GAAAAACTTGATGGCGATGCTGCATTGAACAAATTTTTCCGTGAAATTTACAGTGATGCTGA
TGAAGATATGCGTAGAGCAATGATGAAGTCTTTTGTGGAGTCTAATGGAACCGTTCTCTCAA
CCAACTGGAAGGATGTCGGGAAAAAGACGGTTGAAGGAAGCCCTCCTGATGGAATGGAGCTC
AAGAAGTGGGAGTATTAATACTCCCACTTGAGCTC

Figure 3.40 *Sgt1* gene nucleotide sequence and the amplified region (colored) for silencing construct.

Amplified fragment was ligated with silencing pγ As vector then this ligation product was transformed into E. coli competent cells. Using colony PCR with specific primers right colonies were selected. Being a second confirmation sequencing results were analyzed then the expected pγSGT1.1As construct was linearized with appropriate (*BssH* II) restriction enzyme for *in vitro* transcription (Figure 3.41 **b** and **d**).

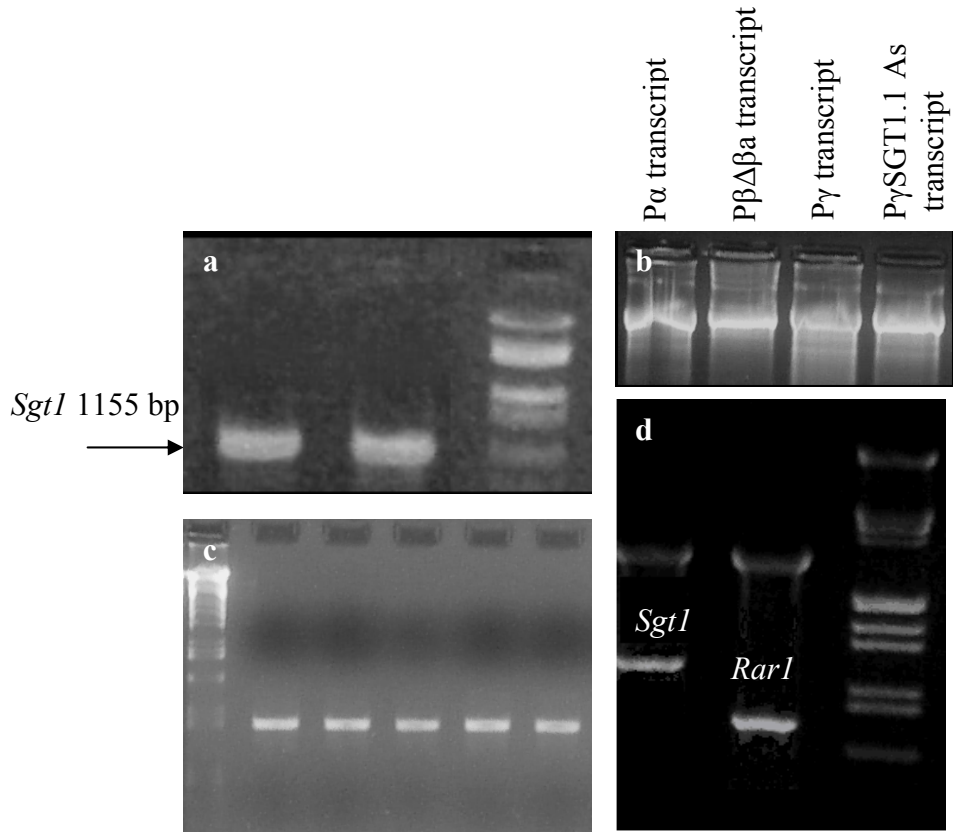


Figure 3.41 *Sgt1* gene silencing of wheat. **a**: *Sgt1* gene as full length was cloned from Avo-Yr10 wheat plant and sequenced. Sequence analysis confirmed the cloning **b**: Linearized BSMV constructs were transcribed and purified for silencing prior to inoculation all the transcripts were analyzed in terms of concentration, quality and purity. **c**: Sgt1.1 *NotI* and *PacI* enginered fragment was amplified from Avo-Yr10 wheat plant cDNA **d**: Double digestion were performed on isolated plasmids containing *Sgt1* gene and *Rar1* gene of Avo-Yr10 wheat (*Triticum aestivum*) after cloning of the full length *Rar1* and *Sgt1* genes.

3.2.4.2 qRT-PCR analyses for *Sgt1* gene expression level determination in silenced wheat plants

Gene expression of *Sgt1* in BSMV:SGT1.1As silenced Avo-Yr10 wheat plants and BSMV:00 inoculated Avo-Yr10 wheat plants were compared by using qRT-PCR. It has been shown that *Sgt1* gene silencing was efficiently performed

(Figure 3.42 and Table 3.11). Detection of the level of *Rad6* gene expression in BSMV:SGT1.1As silenced wheat plants was performed. Figure 3.42 **c** and Table 3.11 show the *Rad6* gene level of expression was not affected in *Sgt1* silenced wheat. *Rar1* and *Hsp90* expression levels have been detected as decreased by the suppression of *Sgt1* gene in wheat (Figure 3.42 **d-e**).

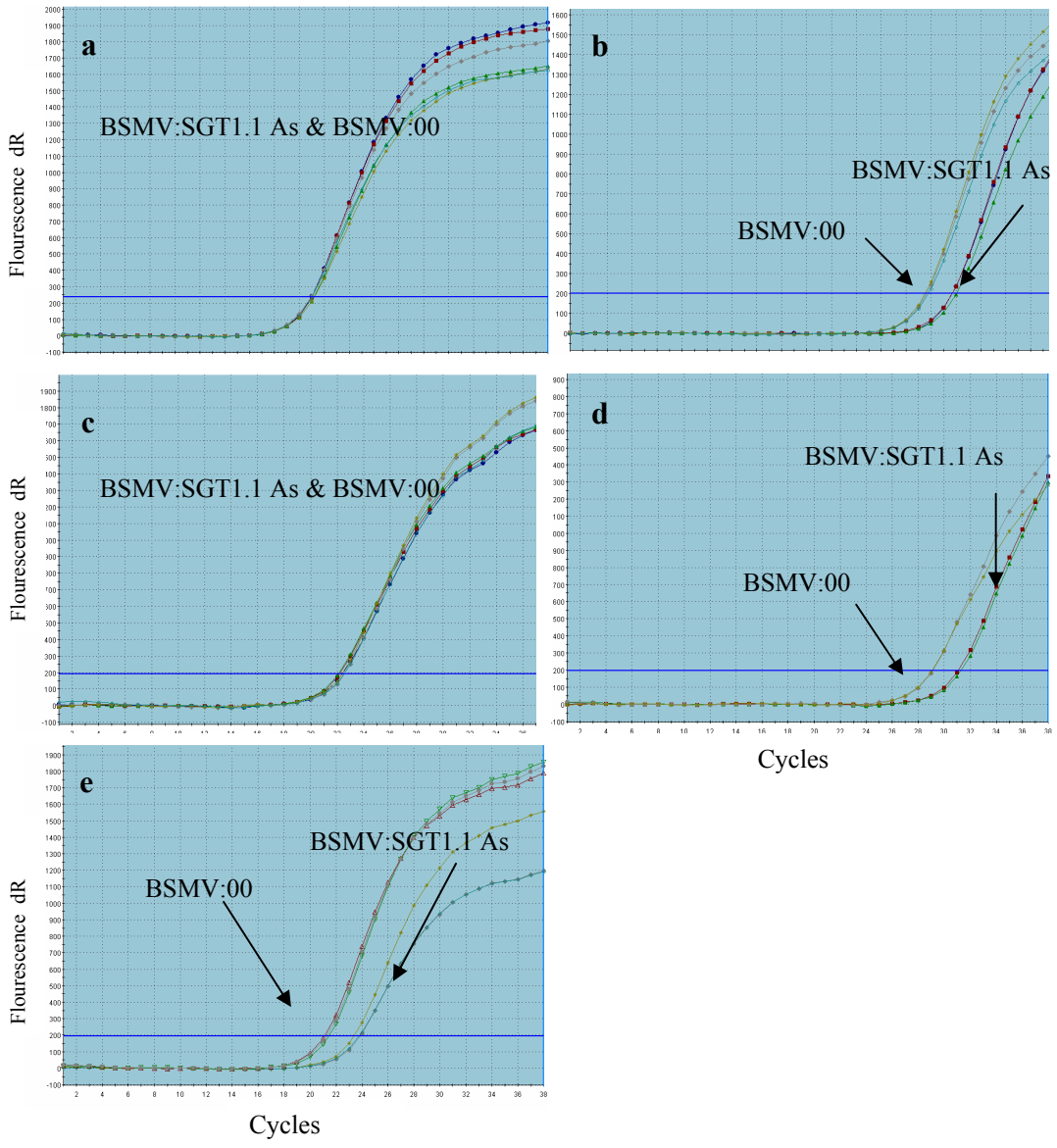


Figure 3.42 *Sgt1* gene silencing and the effect of *Sgt1* level decrease on *Rad6* gene and other disease resistance related gene expression levels. **a**: BSMV:RAR1.1As inoculated and BSMV:00 inoculated Avo-Yr10 wheat plants RNAs normalized by *18s rRNA* gene level equalization **b**: BSMV:SGT1.1As silenced Avo-Yr10 wheat plant *Sgt1* gene expression level was decreased by

approximately 3.85 fold relatively to BSMV:00 inoculated Avo-Yr10 wheat plants. **c**: *Rad6* gene expression level is not changed in BSMV:SGT1.1As silenced Avo-Yr10 wheat according to BSMV:00 inoculated Avo-Yr10 wheat plants RNAs normalized by *18s rRNA* gene. **d**: *Rar1* gene expression level in BSMV:SGT1.1As silenced Avo-Yr10 wheat is decreased by 3.61 fold relative to BSMV:00 inoculated Avo-Yr10 wheat plants RNA. **e**: *Hsp90* gene expression level in BSMV:SGT1.1As silenced Avo-Yr10 wheat is decreased by 4.97 fold relative to BSMV:00 inoculated Avo-Yr10 wheat plants RNA

Table 3.11 qRT-PCR real-time analysis of silencing of *Sgt1* by BSMV mediated VIGS in the third leaves of Avocet-Yr10 wheat plants and the level of *Rad6, Rar1, Sgt1, Hsp90* expression. Silencing of *Sgt1* by BSMV mediated VIGS affects the expression levels of *Rar1, Rad6,* and *Hsp90* genes.

	Relative gene expression*			
Experiment	*Sgt1*	*Rad6*	*Rar1*	*Hsp90*
1	↓ 0.259	NC	↓ 0.277	↓ 0.203
2	↓ 0.214			
3	↓ 0.407	NC	↓ 0.462	↓ 0.361
4	↓ 0.277			
5	↓ 0.128	NC	↓ 0.282	↓ 0.680

* Relative expression of *Rad6, Rar1, Sgt1, Hsp90* genes were calculated by dividing the level of the *silenced* gene transcripts from BSMV:SGT1.1As silenced plants by the level of the gene transcripts from BSMV:00 virus alone but non silenced plants. ↓: decrease ↑: increase NC: Not changed.

3.3 Yeast-Two-Hybrid (Y2H) studies

To this point, *via* VIGS experiments it was shown that silencing of *Rad6* gene causes *Rar1*, *Sgt1*, *Hsp90* expression level change. On the other hand silencing of *Rar1* and *Sgt1* do not affect *Rad6* expression level. It is well characterized that RAR1, SGT1, and HSP90 proteins interact with each other (see Chapter I). Their required interactions are important for disease resistance mechanisms in plants. Since their gene expressions related with each other, there might be a possible link between RAD6 protein with RAR1 and SGT1 proteins. In order to see the probable connection one to one protein-protein interaction experiments were performed.

Gateway based cloning strategies were used for yeast two hybrid analysis. Interaction studies would provide clues to explain the reason of HR or PCD in *Rad6* silenced wheat and barley plants.

3.3.1 Cloning of genes for Y2H

Cloning of *Rar1*, *Rad6,* and *Sgt1* genes from Avo-Yr10 wheat cDNA was started with the gene-specific primer design. Since having all the sequence information for those genes allowed designing the AttB1 and AttB2 flanking gene specific primers according to their sequence information. Full length gene with AttB1 and AttB2 sequences obtained by PCR with appropriate primers (Table 2.1) and shown in Figure 3.43. Purified products were used for BP reaction then entry clones were generated. Sequencing results confirmed the cloning (Appendix-F). The selected colonies were used for LR reaction. Destination vectors for Y2H studies were chosen as pDEST32 (bait vector) and pDEST22 (prey vector). So *Rad6* was cloned into pDEST32, *Rar1* and *Sgt1* were cloned into pDEST22 (Figure 3.44). Clonings were re-confirmed after sequencing. The N-terminal and C-terminal domains of Yr10 protein interactions with RAR1 and SGT1 were also analyzed. Therefore, transformation into Mav203 yeast cells and further analysis of the pDEST32-YR10-1506 and pDEST32-YR10-1562 constructs were performed, too (Figure 3.45).

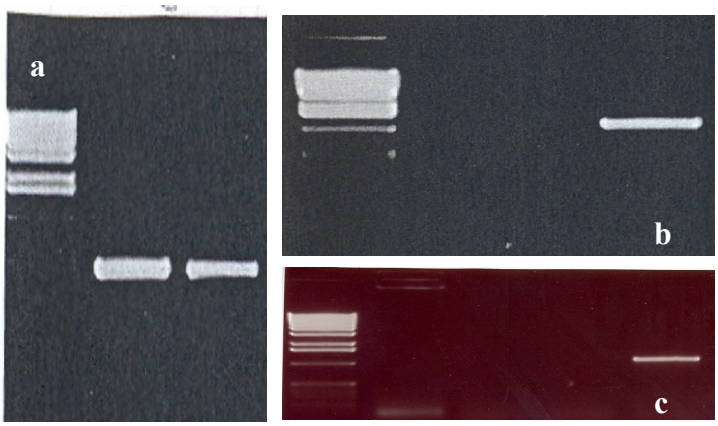


Figure 3.43 *Rad6*, *Rar1* and *Sgt1* gene amplifications from wheat cDNA **a**: *Rad6,* **b**: *Sgt1,* **c**: *Rar1* amplifications with AttB1 and AttB2 additions.

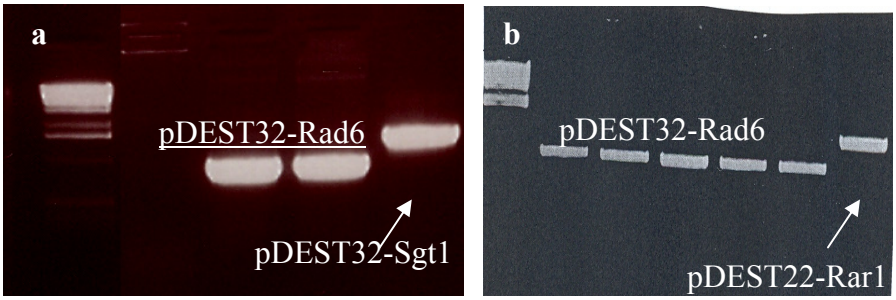


Figure 3.44 *Rad6*, *Rar1* and *Sgt1* gene amplifications from destination plasmid clones **a**: *Rad6* from PDEST32-Rad6 *Sgt1* and *Rar1* amplifications from **b**: pDEST22-Sgt1 and pDEST22-Rar1.

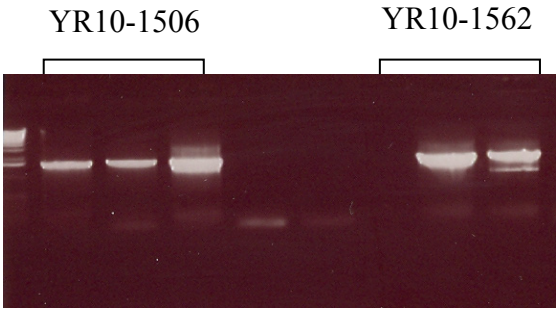


Figure 3.45 Yr10-1506 and Yr10-1562 gene fragment amplifications from destination plasmid clones pDEST32-Yr10 1506 and pDEST32-Yr10 1562.

3.3.2 Yeast selection media and interaction controls

Mav203 is the host yeast cell. Invitrogen Y2H screening and one-to-one protein-protein interaction system which is called ProQuest Two Hybrid system provides many controls in a single host yeast cell. Two vectors (pDEST22 and pDEST32) are transformed into single yeast competent cell and first selection is performed by using selective medium (-leu and –trp yeast droup out medium). So the interaction chart has been planned and the prepared vectors were transformed as listed below.

1: pDEST32-Rad6 with pDEST22-Sgt1
2: pDEST32-Rad6 with pDEST22-Rar1
3: pDEST32-Yr10 1506 with pDEST22-Sgt1
4: pDEST32-Yr10 1562 with pDEST22-Sgt1
5: pDEST32-Yr10 1562 with pDEST22-Rar1
A: none interaction control
B: weak interaction control
C: moderately strong interaction control
D: strong interaction control
E: very strong interaction control
I. self control transformation of pDEST32-Rad6 with pEXP-AD502
II: self control transformation of pDEST22-Sgt1 with pDBLeu
III: self control transformation of pDEST22-Rar1with pDBLeu
IV: self control transformation of pDEST32-Yr10 1506 with pEXP-AD502
V: self control transformation of pDEST32-Yr10 1562 with pEXP-AD502

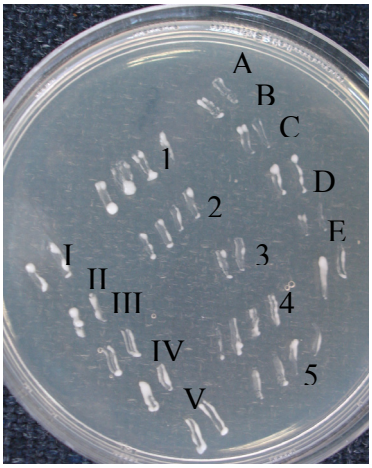


Figure 3.46 Master plate, only pDEST32 and pDEST22 transformed yeast cells were efficiently grown in -Leu and -Trp medium.

Nitrocellulose membrane placed on the surface of YPAD medium for use in the X-gal assay and master plate was replicated on it. Following subsequently required incubation, membrane was picked up and used for X-gal assay. Blue color shows the interaction between two proteins cloned into yeast cells.

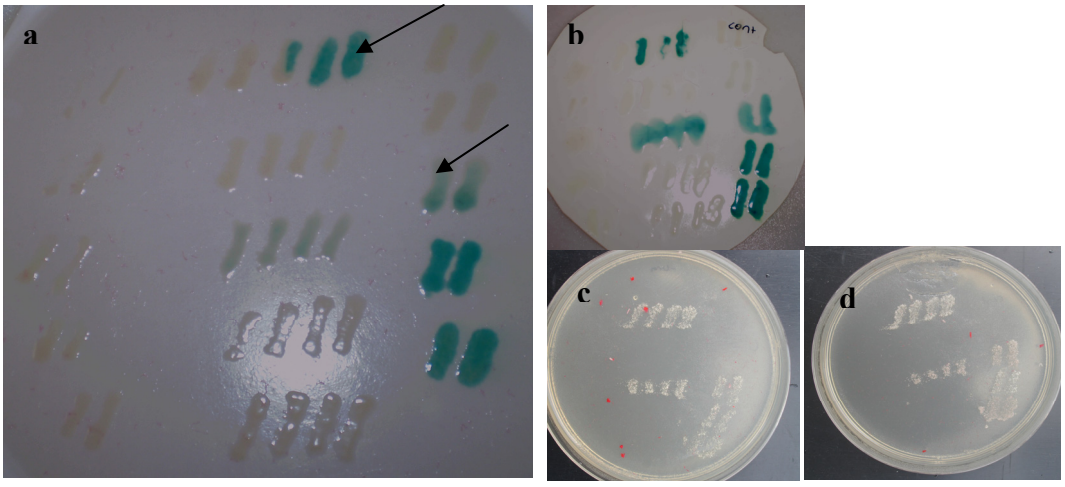


Figure 3.47 Y2H assays, **a** and **b** are X-Gal confirmation assays, **c** is -leu –trp –ura medium yeast complementation assay, and **d** is -leu –trp –his medium yeast complementation assay.

Protein interaction studies showed that there is a possible interaction between RAD6 and SGT1 proteins. Previous experiment in the literature reported the same result; the case is discussed in the discussion part. On the other hand Y2H presents that RAD6 protein does not interact with RAR1. Control transformations (from A to E) show the blue color contrast with respect to their interaction strength. There is no self activity for proteins (in Figure 3.47 **a**, left panel) RAD6, RAR1 and SGT1 proteins does not lead to self-binding or self-activity. The interaction (between RAD6-SGT1) was obvious as shown in the Figure 3.47 on top line of the colonies.

3.4 *Arabidopsis thaliana Rad6* gene mutant based experiments

To test biological function of *Rad6* gene in a model organism, Arabidopsis *Rad6* gene mutants have been searched. These mutants would be used for pathogenesis infection tests. *Arabidopsis thaliana* has two copies of *Rad6* gene in its genome named as *Rad6.1* and *Rad6.2* were identified in mutant seeds. Then these mutants have been tested with race specific *Arabidopsis* pathogens (*Hyaloperonospora parasitica*).

3.4.1 *Rad6* mutant *Arabidopsis* plants

Arabidopsis, in its genome, has two copies of *Rad6* gene which are called as homozygote mutant lines of *Arabidopsis* (rad6-1 and rad6-2) were subjected to analysis for mutation confirmation. They were also looked for disease resistance phenotype. Characterization of the T-DNA Insertion Lines was performed and confirmed using the protocols described by Siebert et al. (1995), Rad6-1 mutant (SALK Accession no: 060994) and Rad6-2 mutant (SAIL Accession no: N622055) (http://www.arabidopsis.org/servlets/Search?type=general&search_action=detail&method=1&show_obsolete=F&name=N622055&sub_type=seed) (Figure 3.48 and 3. 49). The location of the T-DNA insertions within each line was originally identified at the Salk Institute Genomic Analysis (La Jolla, CA) and Syngenta Arabidopsis Insertion Library Line Information (SAIL). All the lines were grown and their DNAs were isolated. PCR reactions were performed with specific primers (Section

2.8) flanking the T-DNA region and gene specific regions. Homozygote mutant plants were selected and seeded for further analysis.

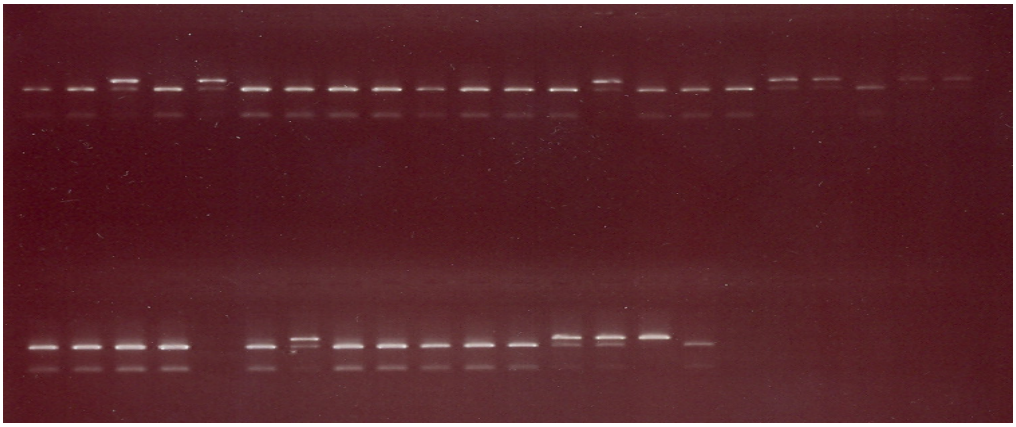


Figure 3.48 Homozygote selections PCR for mutant plant screenings. The upper and single band containing plant DNAs were selected as Rad6-1 SALK mutant (arrow shows the homozygote mutant PCR product).

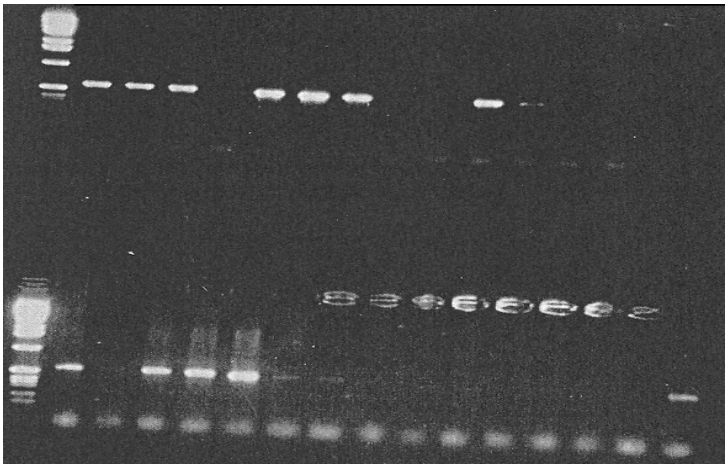


Figure 3.49 Homozygote selections PCR for mutant plant screenings.

Rad6-2 SAIL mutant plants were selected *via* tissue PCR. Specific primers were designed (given in Section 2.8) and the DNA fragments including T-DNA insertion were produced with PCR amplification. Agarose gel analysis provides the identification of homozygote Rad6-1 and Rad6-2 mutant selection (Figure 3.48 and 3.49).

Col5

SALK 060994
Rad6-1M17/Col8

Col5

SALK 060994
Rad6-1M19/Col8

Figure 3.50 Rad6-1 mutant *Arabidopsis* plants show dwarfism. The picture was taken from 30 day old control Col-5 (left) and Rad6-1 SALK mutants (right)

3.4.1.1 Physiological experiments on *Arabidopsis* Rad6 mutants

The detailed experiments of this thesis showed that the suppression of *Rad6* gene in susceptible barley results HR and ceases the growth of virulent race specific pathogen. So, a model organism *Arabidopsis thailana* which has mutated *Rad6* gene was subjected to test the possible HR and pathogenesis phenotype. For which Rad6 Arabidopsis mutants (which are Rad6-1 and Rad6-2 gene copy mutants) were identified and biologically tested. Incompatible and compatible race specific pathogens of *Hyaloperonospora parasitica* (which is a distinct fungi division; Oomycete) were used to infect *Arabidopsis thaliana* mutants. As a result, both of the mutant plants showed resistance against virulent (means disease causing) *H. parasitica* pathogen. Therefore, susceptible Arabidopsis plants show resistant phenotype. These results confirm the barley *Rad6* gene silencing and pathologic function test results. On the other hand, when these *Arabidopsis* mutants which are resistant to their race specific avirulent pathogens. There was no change at resistance level as it was observed in barley *Rad6* gene silencing experiment results.

Namely, once the *Rad6* gene is mutated in resistant plant, the plant still is resistant against its pathogen.

3.4.1.2 Two different types of *Arabidopsis* Rad6 mutants present enhanced disease resistance against virulent *H. parasitica* (Oomycete)

Virulent (*H. parasitica* Noks1) and avirulent (*H. parasitica* Emoy2) inoculations were applied on rad6-1 mutant *Arabidopsis* Col8, rad6-2 mutant *Arabidopsis* Col8 and *Arabidopsis* Col8 (control) individual. Virulent inoculations on Col8 were resulted expected disease phenotype and pathogen can grow on susceptible *Arabidopsis* Col8. Unexpectedly, yet similarly in barley studies the mutants showed resistance and restriction of fungal growth (Figure 3.51). These results confirm our previous wheat and barley *Rad6* gene silencing experiment results. Additionally, on resistant plant, *H. parasitica* inoculation did not cause disease in all cases (means mutants and resistant wild type plants are still resistant against avirulent *H. parasitica* inoculation) (Figure 3.52) which is also supporting the data observed in barley experiments.

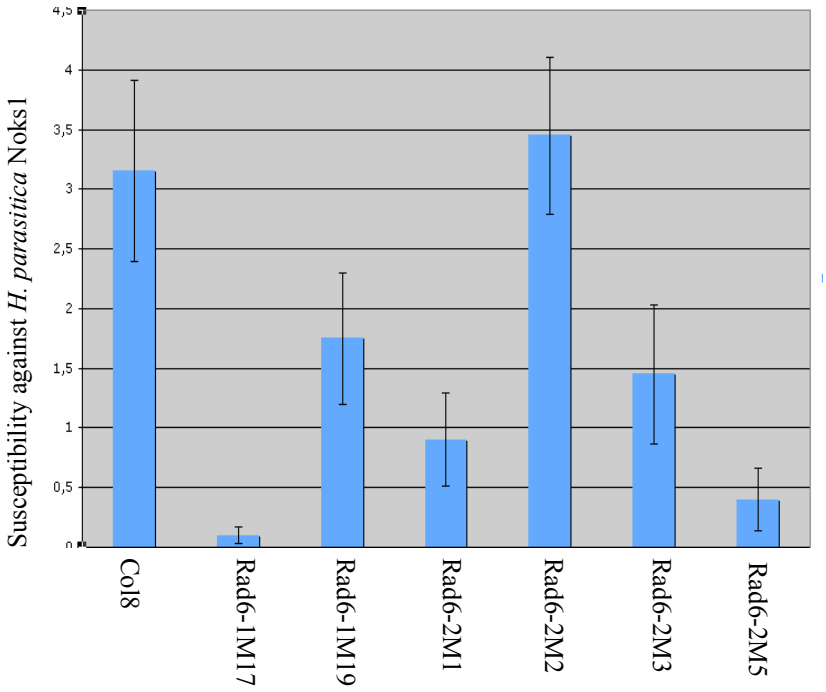


Figure 3.51 *H. parasitica* Noks1 (virulent) inoculation results on wild type *A. thaliana* Col8, Δrad6-1 *A. thaliana*, Δrad6-2 *A. thaliana*. (Data was kindly provided by Dr. Mahmut Tör and obtained by his postdoc Dr. Kate Bailey).

Table 3.12 Score table of *H. parasitica* on Δrad6-1 *A. thaliana*

Noks1 3days	Infection scores																			
Col-8	5	1	0	0	0	3	3	2	12	6	5	7	0	0	0	3	0	3	9	4
Rad6-1 M17	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Rad6-1 M19	6	0	0	1	0	1	3	0	5	0	0	0	2	3	3	1	0	9	0	1
Rad6-2 M1	1	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	3	7	1	2
Rad6- M2	9	5	4	1	0	5	0	3	6	5	0	8	7	6	0	3	0	1	1	5
Rad6-2 M3	0	0	1	4	0	0	0	0	0	0	0	8	0	7	0	4	0	0	0	5
Rad6-2 M5	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2	5	0

Colonization of pathogen on plants (1-12, degree 12 refers very highly infection) (Data was kindly provided by Dr. Mahmut Tör and obtained by his postdoc Dr. Kate Bailey).

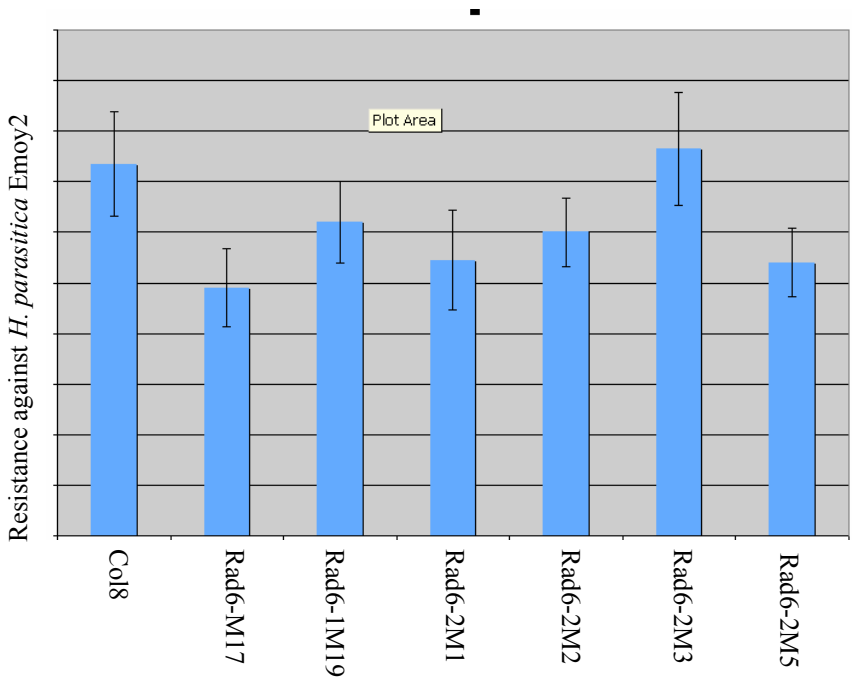


Figure 3.52 *H. parasitica* Emoy2 (avirulent) inoculation results wild type *A. thaliana* Col8, Δrad6-1 *A. thaliana*, Δrad6-2 *A. thaliana*. (Data was kindly provided by Dr. Mahmut Tör and obtained by his postdoc Dr. Kate Bailey).

Table 3.13 Score table of *H. parasitica* on Δrad6-2 *A. thaliana*

Emoy2	Resistance scores																			
Col-8	14	1	3	5	6	6	9	12	3	6	11	9	3	20	9	11	4	5	7	3
Rad6-1 M17	8	9	11	0	6	3	7	8	6	5	3	3	7	0	1	0	9	0	5	7
Rad6-1 M19	5	3	6	3	8	3	10	7	8	4	1	2	14	13	7	4	8	6	10	2
Rad6-2 M1	8	7	12	4	4	1	6	7	5	1	2	10	0	11	3	0	0	13	3	12
Rad6- M2	6	11	7	7	8	10	8	1	7	2	4	8	3	8	9	7	3	8	2	1
Rad6-2 M3	11	3	14	20	6	11	12	10	4	0	2	6	9	13	8	9	5	2	3	5
Rad6-2 M5	4	9	1	7	10	8	3	5	0	5	0	4	9	8	6	4	7	9	6	3

Resistance against pathogen in mutant and wild type plants (1-20, degree 20 refers very highly resistant plant, no infection) (Data was kindly provided by Dr. Mahmut Tör and obtained by his postdoc Dr. Kate Bailey).

Rad6-1 and Rad6-2 *Arabidopsis* mutants were inoculated with virulent *H. parasitica* Noks1. Control inoculation was applied on Col8 wild type *Arabidopsis*. Figure 3.51 and Table 3.12 represent that upon virulent inoculation the pathogen

successfully infects and grows on wild type plant, but does not infect and grow on the Rad6 mutant plants. Score table presents the inoculation results such as Rad6-1 M17 and Rad6-2 M3 plants show resistance against *H. parasitica* Noks1, but not to Col8 wild type. *H. parasitica* Emoy2 is avirulent on *Arabidopsis* Col8. Figure 3.52 and Table 3.13 show the result of avirulent inoculation on Rad6 mutants and wild type plant. Similarly with the barley silencing experiment, knock-down of *Rad6* gene copies does not effect on resistance level in resistant plant. Mutants are already resistant against their avirulent pathogen. As a result Rad6-1 and Rad6-2 mutant *Arabidopsis* plants behave as wild type plant against virulent pathogen but they show resistance upon inoculation of virulent pathogen.

3.5 Identification of differentially expressed genes of boron tolerant plant, *Gypsophila perfoliata* L.

3.5.1 Total RNA isolation from the samples for differential display analysis

Plants were grown under excess amount and natural abundance of boron concentrations in liquid nutritional media. The excess boron concentration was adjusted as 500 µM because according to Ture and Bell (2004) *Gypsophila perfoliata* can tolerate up to 35 mg/kg, which is approximately 500 µM boron. The toxic and normal boron levels in the soil were considered to be above 300 µM and 30 µM by Takano, respectively (Takano et al., 2002). Thus, the control plants were let grown in 30 µM boron concentration which was the only difference in the growth conditions of the two plant sets. The sample plant was treated with 1000 µM boron concentration to got information about our differentially expressed gene fragments.

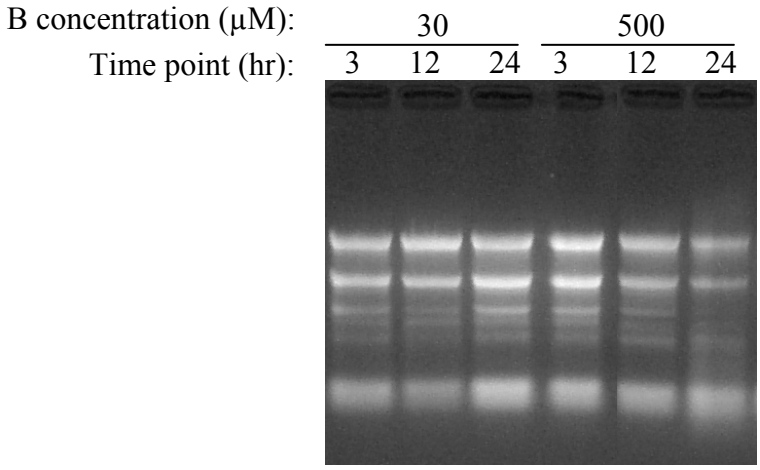


Figure 3.53 Total RNAs from the normal boron concentration (30 μM) and excess boron (500 μM) containing medium treated samples.

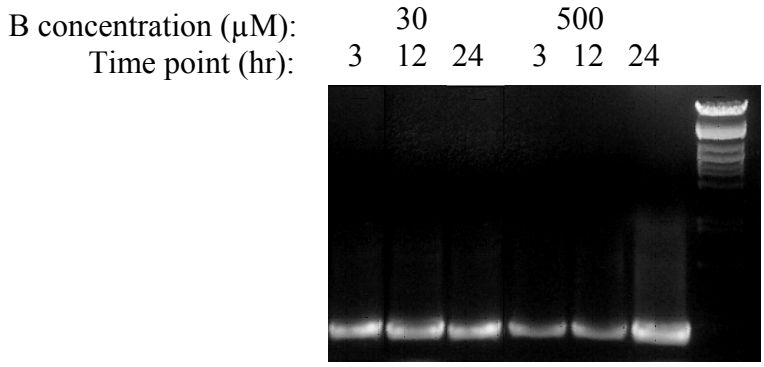


Figure 3.54 After cDNA synthesis of control and treated samples, Actin-1 confirmation PCR was evaluated. The expected complete DNA sequence size bands were generated this means that cDNA synthesis reactions were successfully done.

3.5.2 DDRT-PCR and evaluation of results obtained from DDRT-PCR analysis

To detect the differentially expressed transcripts or fragments upon treatment of high boron concentration, DDRT-PCR experiments were carried out using total of 90 primer combinations at combined RNAs of each time point (3, 12, and 24 hrs) of treated and control plant samples. The experiments combinations were carried out using custom made P and T primers from the Clontech's Delta DD method. Sequences of the primers used are listed in Table 2.2 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. By investigating the autoradiograph images, 40 differentially expressed bands were selected and excised from the dried gels. The selection was based on presence and absence of the bands at the same size of the treated and control samples. Of those, 10 transcript products were not re-amplifiable. The remaining were cloned after PCR re-amplification and sequenced. 18 well-read sequences were obtained; most showed homologies to the 5' ends and the 3' un-translated regions (UTRs) of the genes from *Arabidopsis thaliana* and *Oryza sativa* in the DNA sequence databanks. The characterized DDRT-PCR sequences were submitted to NCBI databank with the accession numbers presented in the table 3.14. The putative functions of the genes were predicted by sequence homologies of genes or proteins which are available in NCBI database.

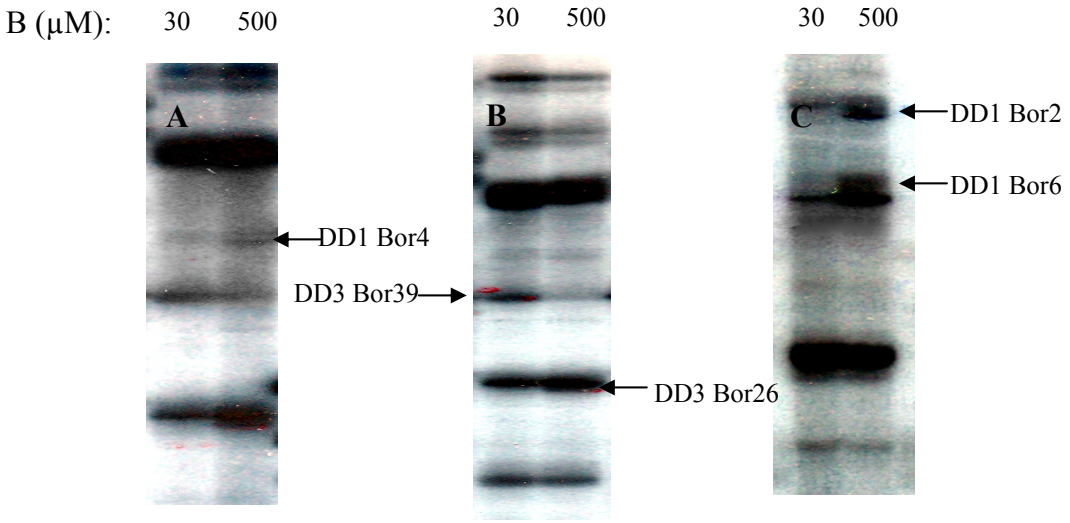


Figure 3.55 Some of the DD profiles are pictured. DD1 Bor4 is seen as more accumulated in the excess boron concentration but the DD3 Bor39 gene transcript gives the less amount of transcription in the high boron concentrated medium. A,B,C gels were provided from different types of DD RT-PCR reactions using combinations of different P and T primers such as in A the P1 and T2 primer pair was used. P2, T4 and P4, T7 were used respectively in B and C.

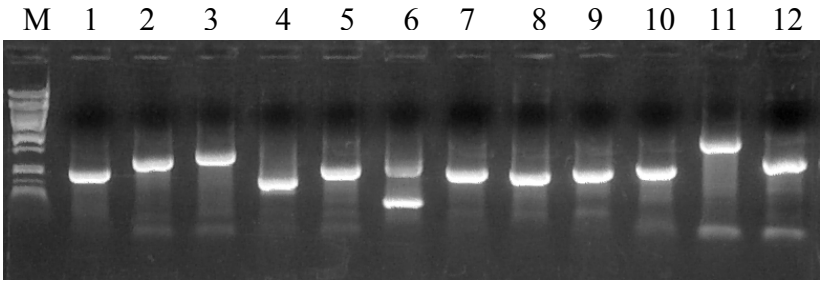


Figure 3.56 PCR re-amplification of the band profile excised from the DNA denaturing poly-acrylamide gel. Amplified DD fragments separated on 1% agarose gel. Lanes from 1-12 are reamplified that belong to, DD2 Bor13, DD3 Bor43, DD3 Bor80, DD3 Bor40, DD1 Bor4, DD3 Bor39, DD1 Bor6, DD3 Bor79, DD3 Bor72, DD3 Bor13, DD1 Bor2 and DD3 Bor5, respectively.

3.5.3 Cloning and sequencing of differentially expressed fragments

Cloning of the excised DNA fragments was achieved using pGEM-T Easy vector system and ligation products were transferred into competent *E.coli Dh5-α* cells. Colony PCR amplification from the selected right white and ampicilin resistant colonies was performed by using M13 primer mediated PCR 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 double digested with *EcoR*I and *Xho*I restriction enzymes and run on the 1% agarose gel, the restriction enzymes and M13 primers used for the experiments because of the reliability of that materials for pGEM-T Easy vector system.

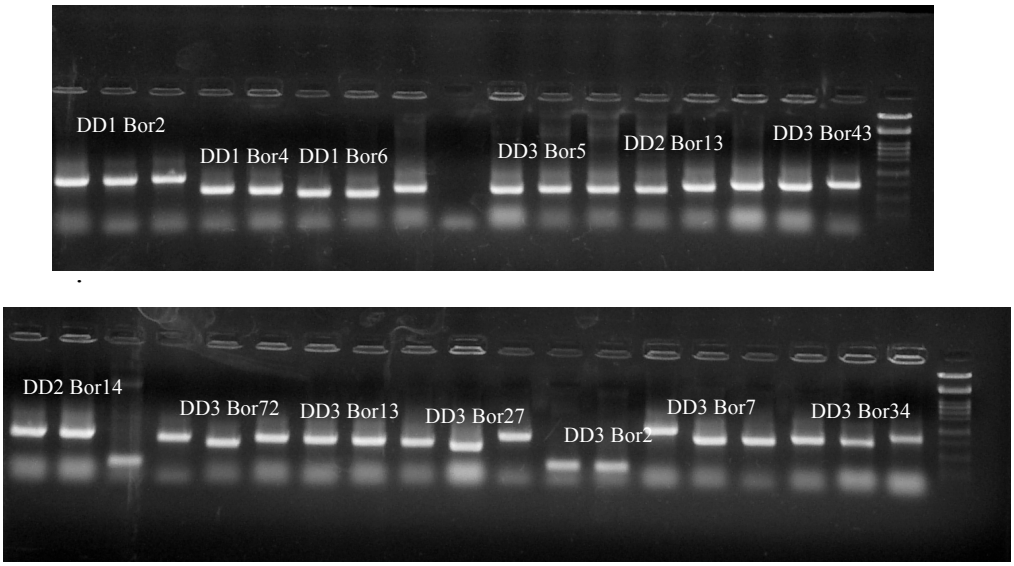


Figure 3.57 PCR amplifications of the colonies after transformation and cloning of the re-amplified DNA fragments. The expected colonies were selected after PCR gel fragment analysis through the size of DNA bands. DD colonies M13 reaction products were separated on 1 % agarose gel. Lanes from 1-12 are selected for plasmid isolation and sequencing that belong to labels on bands.

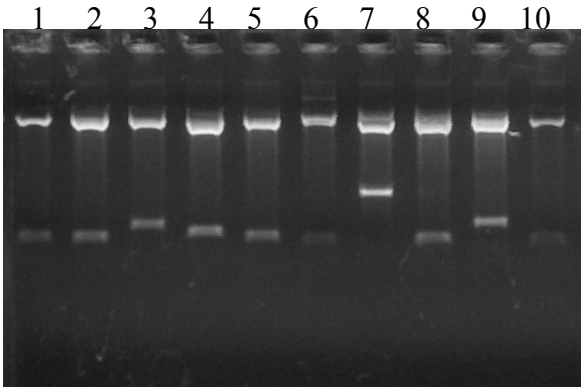


Figure 3.58 *EcoR*I and *Xho*I restriction double digestion of plasmids from the selected colonies. Lanes from 1 to 10 are clones DD1 Bor6, DD1 Bor4, DD3 Bor5, DD3 Bor13, DD3 Bor72, DD3 Bor34, DD1 Bor2, DD3 Bor36, DD3 Bor45, DD3 Bor44 respectively.

3.5.4 Results of the sequences and identities of gene fragments

By investigating the autoradiograph images, 40 differentially expressed bands were selected and excised from the dried gels. Of those, 10 transcript products were not re-amplifiable. The remaining were cloned after PCR re-amplification and sequenced. 18 well-read sequences were obtained; most showed homologies to the 5' ends and the 3' un-translated regions (UTRs) of the genes from *Arabidopsis thaliana* and *Oryza sativa* in the DNA sequence databanks. The putative functions of the genes were predicted by sequence homologies of genes or proteins which are available in NCBI database. Our identified clones mostly related with cell regulatory, cell proliferation based proteins, abiotic stress related and proteasome pathway components. Two of our clones showed homology with 26S proteosome system (DD3 Bor5, DD3 Bor36). One of them has homology to osmotic stress activated protein kinase (DD3 Bor40). Another one is a transcription factor homolog; zinc finger (DD1 Bor2), one of them is photosystem II protein (DD3 Bor7). Two of them showed homologies to epimerases (DD3Bor26, DD3 Bor 42) which are related to oxidative stress regulation. Five of the expressed ones (DD1

Bor4, DD2 Bor14, DD3 Bor34, DD3 Bor39, DD3 Bor41) showed homologies to functionally unknown proteins. Only one clone (DD3 Bor39) shows down regulation.

3.5.5 Confirmation of the results obtained from DD analysis

Transcripts were selected to be confirmed with qRT-PCR analysis. The 24th hr time point has been used as a template for each primer sets designed based on the sequence readings of the DDRT-PCR fragments (Table 3.14). Among 18 qRT-PCR performed only the three of them were observed to be unchanged under high 500 μM boron treatment; DD3 Bor2, DD3 Bor41 and DD3 Bor72 at the 24th hr time point. According to normalization with *18S rRNA* gene, 15 of the transcripts showed differences in expression levels (Table 3.15). Our identified clones mostly related with cell regulatory, cell proliferation based proteins, abiotic stress related and proteasome pathway components. Two of our clones showed homology with 26S proteosome system (DD3 Bor5, DD3 Bor36). One of them has homology to osmotic stress activated protein kinase (DD3Bor40). Another one is a transcription factor homolog; zinc finger (DD1 Bor2), one of them is photosystem II protein (DD3 Bor7). Two of them showed homologies to epimerases (DD3 Bor26, DD3 Bor 42) which are related to oxidative stress regulation. Five of the expressed ones (DD1 Bor4, DD2 Bor14, DD3 Bor34, DD3 Bor39, DD3 Bor41) showed homologies to functionally unknown proteins. Only one clone (DD3 Bor39) shows down regulation.

3.5.6 qRT-PCR Analysis

For the efficiencies of the target gene amplifications, gene specific standard curve analysis was generated for each target and calculated the PCR efficiency; 90% efficiency is considered to be qRT-PCR amplification success and this is used for relative fold change estimations. *18S rRNA* was used gene for normalizations as an

internal control. Representative qRT-PCR profiles of genes are presented in Figure 3.58 (using primers in the table 2.3), in which Metallo-dependent hydrolase (DD3 Bor13) was analyzed. According to 90% PCR efficiency, the 2.9 cycle difference equals to $1.90^{2.9}$ (6.43) fold induction in the level of DD3Bor13 mRNA level at 500 µM. H_3BO_3 (Figure 3.58 **a**), since the C_f values of the normalization control from both of the samples were identical in this specific case (Figure 3.58 **a**), there was no need to factor for comparisons, in other word, the amount of total RNA was considered to be very similar for each of this sample and the control. The folds of gene expression levels are presented in the table 3.12 for each gene.

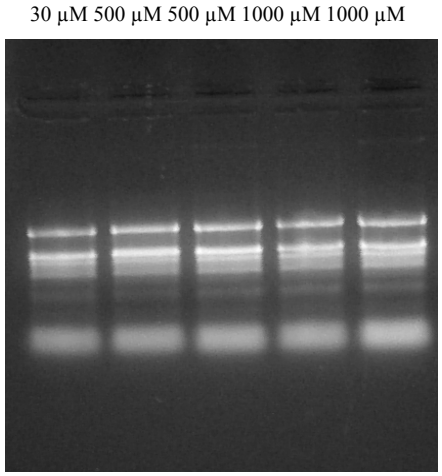


Figure 3.59 Before qRT-PCR analysis different types of treatments were applied and the RNAs of them were isolated. Those RNAs were treated with DNAse precipitated with lithium chloride and revers transcripted for quantitative Realtime RT-PCR analysis. Additionally a set of degenerate BOR1 primers was designed based on *Arabidopsis* BOR1 protein, to detect if there is a homolog gene expressed in our plant samples. However, the primer sets used were unable to provide any PCR products with cDNA of 24^{th} hr excess boron treated sample.

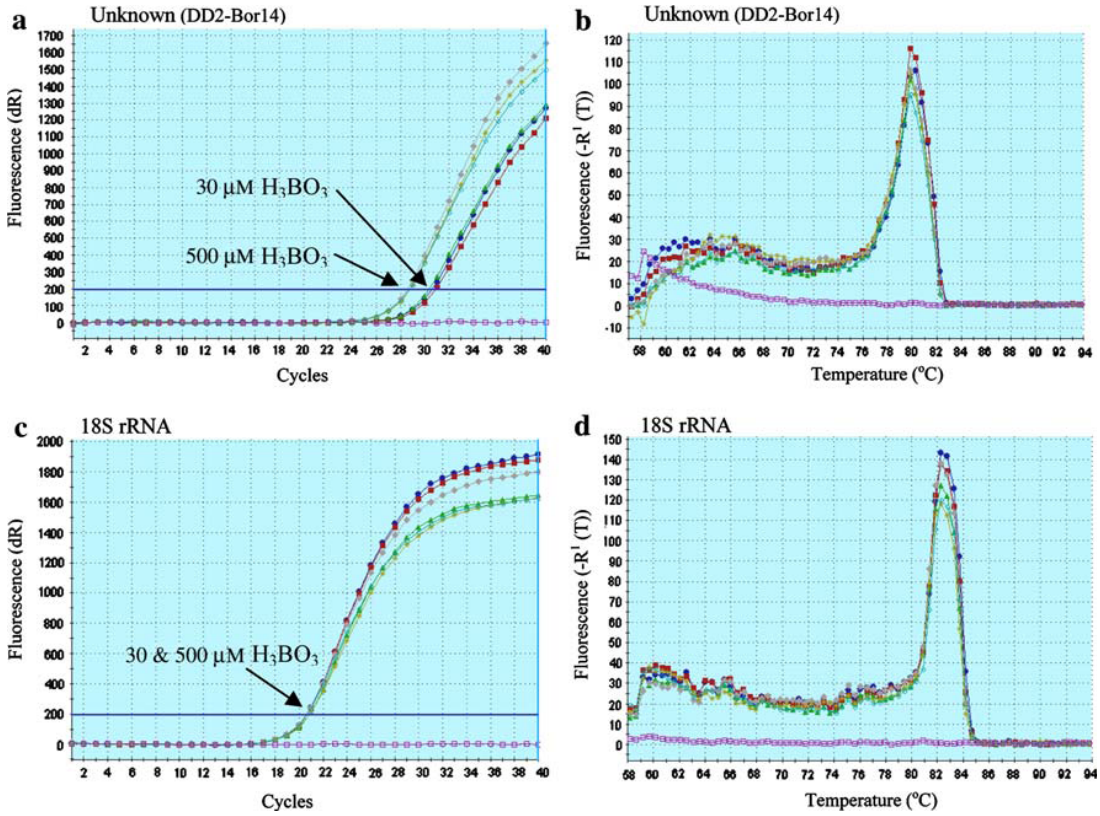


Figure 3.60 Real-time PCR (qRT-PCR) profiles for clone DD2 Bor14. The treatments were performed using 30 µM and 500 µM on plants. **a**: DD2 Bor14 amplification plot **b**: disassociation curve **c**:18S rRNA amplification plot **d**: disassociation curve. The first three amplification plots are boron treated sample triplicate reactions the second three indicates boron untreated control sample reactions.

In Figure 3.60c the normalization was done successfully all 6 reactions giving the same amplification profile so the equivalence of RNA levels is provided. Disassociation curves are seen in Figure 3.60b and 3.60d these are the proof for single band producing qRT-PCR reaction. According to 90% PCR efficiency the 2.9 cycle difference means 6.43 fold induction in the level of DD2 Bor14 mRNA level (Figure 3.60 **a**).

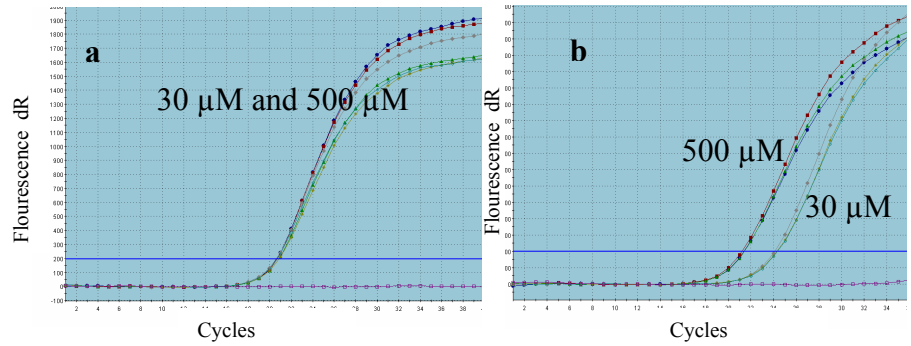


Figure 3.61 Real-time PCR (qRT-PCR) profiles for clone DD1 Bor6. The treatments were performed using 30 µM and 500 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD1 Bor6 clone (GTP-binding protein GB3) target between the treated and control samples, indicating 3.3 cycles corresponding to 8.31 fold expression level difference.

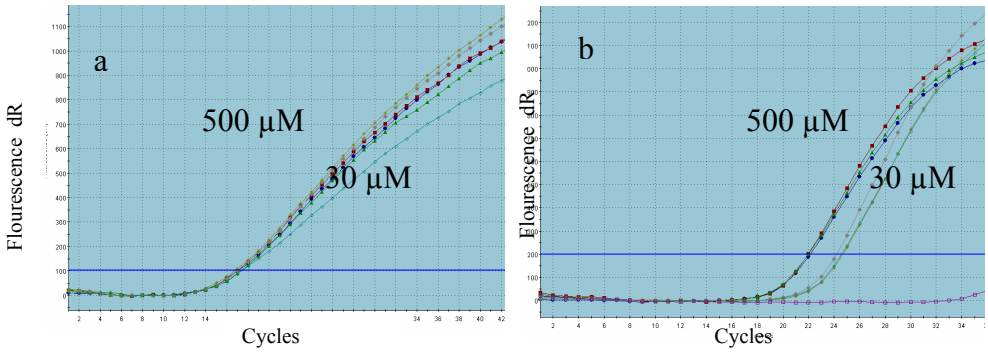


Figure 3.62 Real-time PCR (qRT-PCR) profiles for clone DD3 Bor40. The treatments were performed using 30 µM and 500 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD3 Bor40 clone (Putative osmotic stress-activated protein kinase) target between the treated and control samples, indicating 2.6 cycles corresponding to 5.31 fold expression level difference.

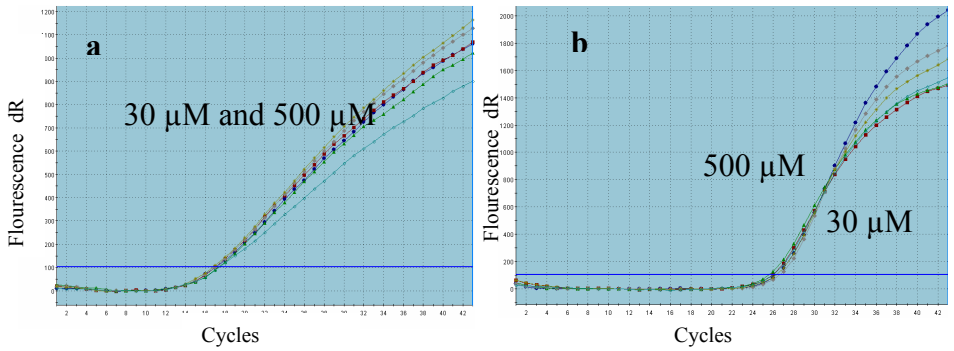


Figure 3.63 Real-time PCR (qRT-PCR) profiles for clone DD3 Bor2. The treatments were performed using 30 µM and 500 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD3 Bor2 clone (Speckle-type POZ protein-related) target between the treated and control samples, indicating 0 cycles corresponding to same expression level.

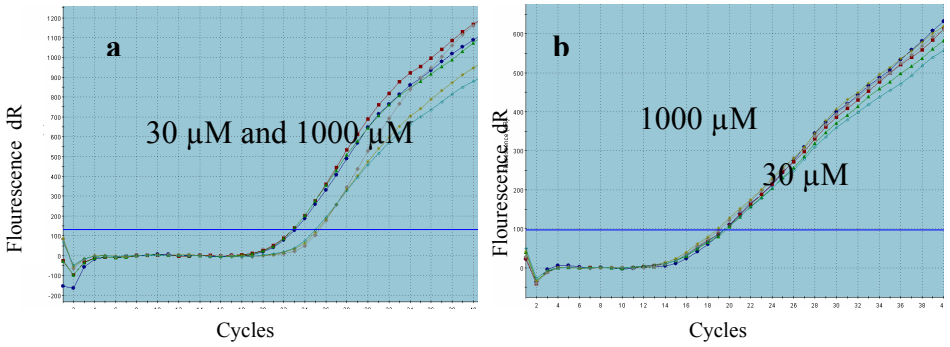


Figure 3.64 Real-time PCR (qRT-PCR) profiles for clone DD3 Bor2. The treatments were performed using 30 µM and 1000 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD3 Bor2 clone (Speckle-type POZ protein-related) target between the treated and control samples, indicating 2.38 cycles corresponding to 4.61 fold expression level difference.

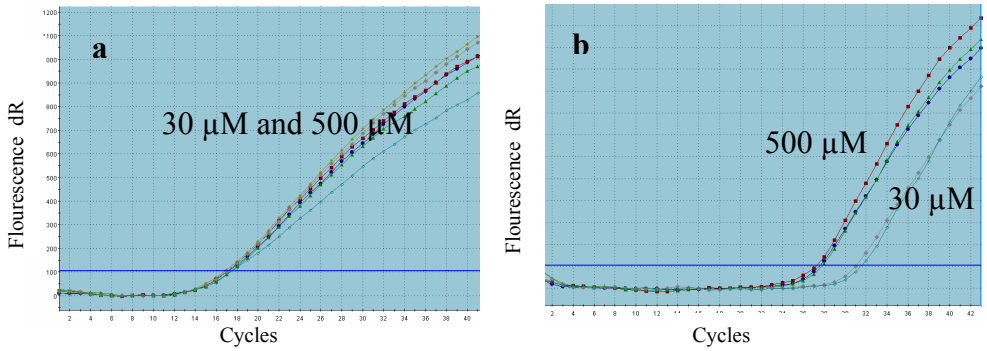


Figure 3.65 Real-time PCR (qRT-PCR) profiles for clone DD3 Bor2. The treatments were performed using 30 µM and 500 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD3 Bor5 clone (26S proteosome subunit RPN7) target between the treated and control samples, indicating 3.8 cycles corresponding to 11.46 fold expression level difference.

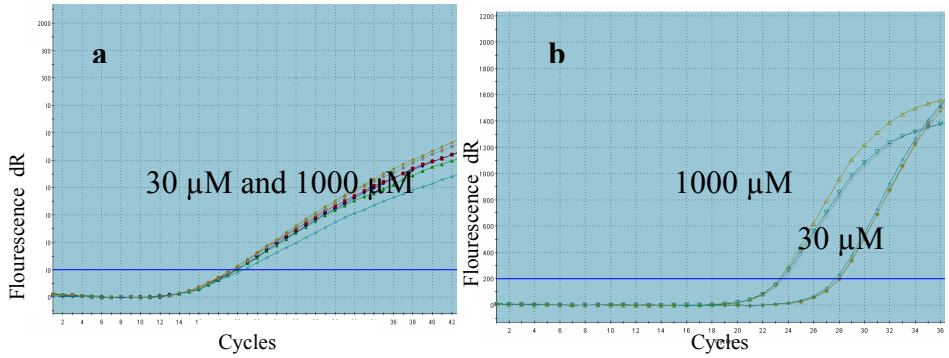


Figure 3.66 Real-time PCR (qRT-PCR) profiles for clone DD3 Bor2. The treatments were performed using 30 µM and 1000 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD3 Bor5 clone (26S proteosome subunit RPN7) target between the treated and control samples, indicating 4.5 cycles corresponding to 17.96 fold expression level difference.

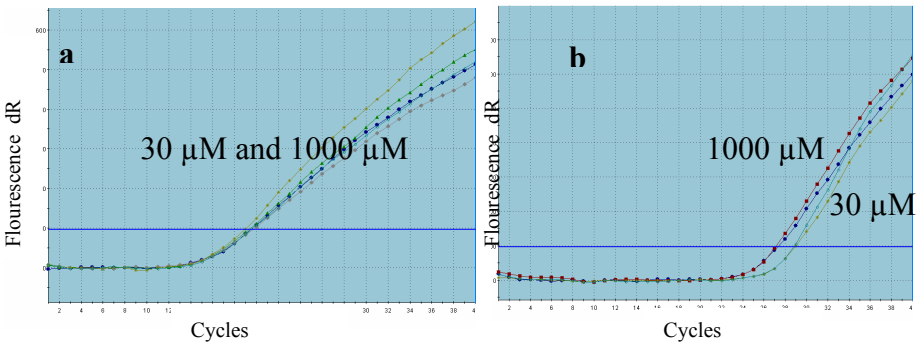


Figure 3.67 Real-time PCR (qRT-PCR) profiles for clone DD1 Bor2. The treatments were performed using 30 µM and 1000 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD1 Bor2 clone (ELF6 (Early flowering 6); transcription factor) target between the treated and control samples, indicating 1.5 cycles corresponding to 2.61 fold expression level difference.

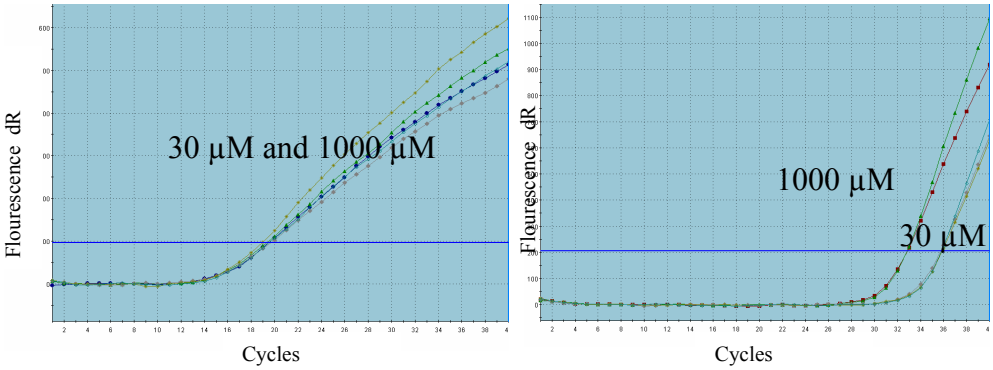


Figure 3.68 Real-time PCR (qRT-PCR) profiles for clone DD1 Bor2. The treatments were performed using 30 µM and 500 µM on plants. RNA samples are from 24th hour of post treatment. **a**: Normalization of mRNA levels using *18s rRNA* gene expression with triplicates for control normal condition and high-level boron treated samples. **b**: Expression level differences observed for the DD1 Bor2 clone (ELF6 (Early flowering 6); transcription factor) target between the treated and control samples, indicating 3 cycles corresponding to 6.72 fold expression level difference.

Table 3.14 DDRT-PCR transcripts identified with the sequence homologies found in NCBI sequence database.

DD clones	bp*	GenBank EST-Accessions	Blast hits	GenBank Accessions of hits	Blast scores	Positive scores (aa)	Positive scores (%)
DD1 Bor 2	566	FE905318	ELF6 (Early flowering 6); transcript.factor [*A. thaliana*]	NP196044.2	4e-24	98/164	59
DD1 Bor 4	339	FE905319	Expressed protein [*A. thaliana*]	NP190169.1	47	8/9	88
DD1 Bor 6	337	FE905320	GTP-binding protein GB3 [*A. thaliana*]	AAM66946.1	7.4	15/16	93
DD2 Bor 10	286	FE905321	Histidine kinase A, N-terminal [*M. truncatula*]	ABE94292.1	9e-31	67/73	91
DD2 Bor 13	428	FE905322	Putative arginyl-tRNA synthetase [*O. sativa*]	AAT07655.1	2e-58	120/142	84
DD2 Bor 14	598	FE905323	Unknown [*H. vulgare*]	AAL77110.1	2e-37	85/102	83
DD3 Bor 2	186	FE905324	Speckle-type POZ protein-related [*A. thaliana*]	NP566212.2	1e-21	62/76	81
DD3 Bor 5	430	FE905325	26S proteosome subunit RPN7 [*C. annuum*]	ABK42076.1	9e-66	131/138	94
DD3 Bor 7	525	FE905326	Photosystem II protein D2 [*T. aestivum*]	NP114244.1	2e-86	170/171	99
DD3 Bor 13	384	FE905327	Metallo-dependent hydrolase, composite [*M. truncatula*]	ABE77689.1	1e-54	108/117	92
DD3 Bor 26	102	FE905328	Putative D-ribulose-5-phosphate 3-epimerase [*S. demissum*]	CAB41122.1	5e-06	25/27	92
DD3 Bor 34	316	FE905329	putative leucine-rich receptor-like protein kinase [*O. sativa*]	BAD22997.1	4.3	18/28	64
DD3 Bor 36	306	FE905330	26S Proteosome regulatory particle chain RPT6-like protein [*A. thaliana*]	AAM62497.1	1.0	22/24	91
DD3 Bor 40	258	FE905332	Putative osmotic stress-activated protein kinase [*O. sativa*]	BAD15474.1	6.6		
DD3 Bor 39	160	FE905331	No significant hit				
DD3 Bor 41	315	FE905333	Copalyl diphosphate synthase 1; CPS1 [*C. maxima*]	AAD04292.1	3.2	18/35	51
DD3 Bor 42	99	FE905334	Putative D-ribulose-5-phosphate 3-epimerase [*A. thaliana*]	AAF03437.1	6e-07	28/33	84
DD3 Bor 72	357	FE968988	Ribosomal protein L13a, cytosolic [*A. thaliana*]	CAB41927	3e-51	113/119	94
DD3 Bor 27	349	FE968987	No significant hit	-	-	-	-
DD3 Bor 43	465	FE905335	No significant hit	-	-	-	-
DD3 Bor 44	302	FE905336	No significant hit	-	-	-	-
DD3 Bor 45	465	FE905337	No significant hit	-	-	-	-
DD3 Bor 77	799	FE905339	No significant hit	-	-	-	-
DD3 Bor 79	320	FE905341	No significant hit	-	-	-	-
DD3 Bor 81	320	FE905343	No significant hit		-	-	-
DD3 Bor 76	225	FE968986	DNA, TJ sequence [*N. tabacum*]	D84238.1	1e-51	148/164#	90#
DD3 Bor 78	285	FE905340	Acyl ACP thioesterase [*C. nucifera*]	EF222023.1	4e-125	228/228#	100#
DD3 Bor 80	485	FE905342	No significant hit	-	-	-	-
DD3 Bor 82	371	FE905344	No significant hit	-	-	-	-
DD3 Bor 28	226	FE905340	No significant hit	-	-	-	-

aa: homologies to amino acid sequences; * corresponding to the read sequence information; # similarities to nucleotide sequences. NC: not changed.

Table 3.15 Fold changes in expression levels confirmed by qRT-PCR when compared to 30 µM B treatment.

DD clones	Blast	qRT-PCR / fold changes			
		500 µM		1000 µM	Ratio
		1st biol. rep. triple tech. rep.	2nd biol. rep. triple tech. rep.	triple technical replicates	
DD1 Bor 2	ELF6 (Early flowering 6); transcript.factor [A. thaliana] / NP196044.2	6.72±0.36 ↑	5.65±0.29 ↑	2.61±0.35 ↑	-
DD1 Bor 4	Expressed protein [A. thaliana] / NP190169.1	NC	NC	5.58±0.54 ↑	+
DD1 Bor 6	GTP-binding protein GB3 [A. thaliana] / AAM66946.1	8.31±0.15 ↑	6.97±0.23 ↑	4.50±0.45 ↑	-
DD2 Bor 10	Histidine kinase A, N-terminal [M. truncatula] / ABE94292.1	3.47 ±0.15 ↑	3.61±0.43 ↑	3.92±0.19 ↑	NC
DD2 Bor 13	Putative arginyl-tRNA synthetase [O. sativa] / AAT07655.1	3.28 ±0.18 ↑	3.28±0.23 ↑	1.90±0.20 ↑	-
DD2 Bor 14	Unknown [H. vulgare] / AAL77110.1	6.86 ±0.16 ↑	5.65±0.13 ↑	7.08±0.35 ↑	NC
DD3 Bor 2	Speckle-type POZ protein-related [A. thaliana] / NP566212.2	NC	NC	4.61±0.10 ↑	+
DD3 Bor 5	26S proteosome subunit RPN7 [C. annuum] / ABK42076.1	11.46±0.20 ↑	11.60±0.23 ↑	17.96±0.35 ↑	+
DD3 Bor 7	Photosystem II protein D2 [T. aestivum] / NP114244.1	10.21±0.20 ↑	6.86±0.13 ↑	14.86 ±0.65 ↑	+
DD3 Bor 13	Metallo-dependent hydrolase, composite [M. truncatula] / ABE77689.1	8.58±0.21 ↑	8.30±0.43 ↑	4.97±0.30 ↑	-
DD3 Bor 26	Putative D-ribulose-5-phosphate 3-epimerase [S. demissum] / CAB41122.1	10.08±0.15 ↑	11.46±0.13 ↑	13.03±0.13 ↑	+
DD3 Bor 34	Putative leucine-rich receptor-like protein kinase [O. sativa] / BAD22997.1	4.98±0.15 ↑	3.84 ±0.73 ↑	3.28±0.24 ↑	NC
DD3 Bor 36	26S Proteosome regulatory particle chain RPT6-like protein [A. thaliana] / AAM62497.1	7.80±0.20 ↑	8.31±0.73 ↑	6.43±0.63 ↑	-
DD3 Bor 40	Putative osmotic stress-activated protein kinase [O. sativa] / BAD15474.1	5.30±0.30 ↑	4.97±0.15 ↑	1.90±0.15 ↑	-
DD3 Bor 39	No hit	7.04±0.30 ↓	7.08±0.43 ↓	4.14±0.10 ↓	-
DD3 Bor 41	Copalyl diphosphate synthase 1; CPS1 [C. maxima] / AAD04292.1	6.86±0.30 ↑	6.72±0.24 ↑	3.61±0.40 ↑	-
DD3 Bor 42	Putative D-ribulose-5-phosphate 3-epimerase [A. thaliana] / AAF03437.1	5.96±0.30 ↑	6.03±0.13 ↑	4.34±0.41 ↑	-
DD3 Bor 72	Ribosomal protein L13a, cytosolic [A. thaliana] / CAB41927	NC	NC	3.17±0.44 ↑	+

24th hour leaf samples; NC: not changed; ↑ up-regulated; ↓ down-regulated; (+) up- or down regulation increased at 1000 µM B compared to 500 µM B; (-) up- or down-regulation decreased at 1000 µM B compared to 500 µM B.

3.6 Discussion

3.6.1 *Rad6* gene expression decreases upon avirulent inoculation

Our initial experiments indicated that *Rad6* gene expression was suppressed upon avirulent yellow rust inoculation. *Puccinia striiformis (*Pst) 232E137 inoculation on Avo-Yr1 wheat plant caused 3.65 fold decrease in the level of *Rad6* gene expression as presented in Figure 3.2 (**d**). In addition, detected *Rad6* gene expression level suppression were detected upon inoculation with *Blumeria graminis* f. sp *hordei* Bgh103(64/01) which is avirulent agent of Pallas-01/Mla1 barley plant. The transcript levels of *Rad6* gene was quantified in inoculated and mock inoculated barley leaves at 24 hour after inoculation (hai). The same results were obtained in barley-pathogen interaction (see Figure 3.3**b** and 3.5**c**). The possible reason for suppression of that gene might be that *Rad6* gene has negative regulatory function for plant disease resistance as *Spl11* in *Oryza sativa*. *Spl11* gene is involved the same mechanism as *Rad6* in ubiqutylation, therefore our observation is supported in the literature (Zeng et al., 2004).

3.6.2 VIGS can be applied on wheat

VIGS is a revolutionary method for gene silencing. The targeted genes can be silenced and their functions might be observed. VIGS of monocots has been first reported by Holzberg et al. from LSBC in 2002. They have developed a gene silencing method constructing plasmids for transcription of three genomes by modifying the RNA virus, BSMV. *Pds* gene silencing of wheat was also optimized in our laboratory. *Pds* gene expresses Phytoene Desaturase (PDS) enzyme which is an important enzyme of biosynthesis of carotenoid pathway. PDS protects the chlorophyll pigment by converting phytoene with desaturase activity into ζ-carotene (Grünewald et al., 2000). Thus the suppression of PDS enzymatic activity leads to the photo-bleaching in leaves (Bartley and Scolnik, 1995). Therefore, white photo-bleaching symptoms are expected with the *Pds* gene silencing. The successful BSMV:PDS4As inoculations have been applied on different types of wheat plants;

in Figure 3.8 *Pds* gene silencing is apparent in both wheat lines tested. The *Pds* gene silencing was quantified at different time points in wheat plants by comparing BSMV:00 non-*Pds* silenced wheat plants (Figure 3.11 and Table 3.2).

To show the viral spreading in wheat cells GFP expression visualized using GFP constructed BSMV γ vector which was linearized and the equal amount transcripts were produced for BSMV:GFP inoculation. Viral spreading and GFP expression with BSMV system were pictured in Figure 3.7 **d-f**.

3.6.3 *Rad6* gene silencing of wheat *via* VIGS results HR: RAD6 is a negative regulator of cell death

With the loss of function of *Rad6* gene by 'reverse genetics approach' the function of this gene was aimed to indicate. Unexpectedly i came across with the HR-like symptoms on wheat plant leaves. Figure 3.15 **a-f** shows the indication of HR with different types of histochemical assays and auto-flourescence vision.

Rad6 gene encodes RAD6 ubiquitin-conjugating enzyme (E2) which has a high homology with *Oryza sativa* RAD6 protein at the protein level (98%). In the past 10 years scientists have shown that there is a linkage between ubiquitination and plant disease resistance. Recent studies suggest that ubiqutination of proteins may play important roles in the regulation of plant disease resistance. First indication was the cloning of *Sgt1* after noticing highly susceptible Arabidopsis mutant. SGT1 was described as a novel subunit of ubiquitin ligase complex (E3). It has been found that it interacts with SKP1 which is a component of SCF type E3 ubiquitin ligase complex (Kitagawa et al., 1999). The roles and importance of SGT1 were reported and the requirement of SGT1 in plant disease resistance in both host and non host resistance mechanisms has been also presented (Azevedo et al., 2002; Peart et al., 2002). Involvement of SCF-type E3 ligases (ubiquitin ligases) in pathogen responses has been suggested by Dinesh-Kumar's group (Liu et al., 2002). The RING finger E3 ligases were also determined and found to be required for plant disease HR response. RPM1 R (resistance protein in Arabidopsis against *Pseudomonas syringae*) protein needs RIN2 and RIN3 E3 ligases to be functional and to give HR response

(Kawasaki et al., 2005). CMPG1 is another type of E3 ligase, which contains a U-Box domain. In tomato and tobacco plants it has been shown to be a positive regulator of PCD in disease resistance (Gonza´ lez-Lamothe et al., 2006) Its Arabidopsis homolog, PUB17, is also essential for plant disease resistance (Yang et al., 2006). As explained, some positive regulators for plant gene for gene mediated and general defense responses are known at the moment. In this thesis, a negative regulator of ubiquitination for plant disease resistance is revealed.

3.6.4 Induction of *Rar1*, *Sgt1*, *Hsp90* gene expressions in *Rad6* gene silenced wheat and barley

To find the reasons of HR in *Rad6* silenced plants the levels of disease resistance related genes have been measured by Realtime qRT-PCR. In literature the up-regulation expression levels of some genes were determined upon pathogen attack. *Rar1, Sgt1,* and *Hsp90* genes are some of them (Caldo et al., 2004). The induction of mRNA of those genes has been also detected upon avirulant pathogen attack (Figure 3.34). Therefore, it was aimed to track the *Rar1*, *Sgt1,* and *Hsp90* gene expression regulation in *Rad6* silenced plants. BSMV:RAD6.2As inoculated plant mRNA pools were compared with BSMV:00 inoculated plant transcriptome. The results support the HR phenotype: *Rar1*, *Sgt1,* and *Hsp90* gene expression inductions were observed in *Rad6* silenced wheat and barley (different types of varieties were used) (Table 3.4-3.7 and Figure 3.18). These genes are induced subsequent to *Rad6* level decrease and this induction in those genes causes HR in plant leaves. Since these genes are known to be required for effective HR. So RAD6 may perhaps regulate the level of some genes for resistance mechanisms in plant cell *via* ubiqutination.

3.6.5 *Rar1* or *Sgt1* gene silencing do not effect the expression of *Rad6* gene

Rad6 expression level change regulates the expression of ubiquitination related *Sgt1, Rar1* and *Hsp90* genes. RAR1, SGT1, and HSP90 interact with each other. All of them required for disease resistance mechanisms, the importance of

those three genes was reviewed in Chapter I, *Rar1* gene and *Sgt1* gene was silenced in wheat separately, in order to understand if *Rad6* is controlled either *Rar1* or *Sgt1* or vice versa. The data of Table 3.10 and 3.11 show the silencing of *Rar1* and *Sgt1* genes. Differentiation of *Rad6* gene expression is not affected in *Rar1* or *Sgt1* silenced wheat plants. Data suggest claiming that RAD6 may regulate the RAR1, and SGT1 but RAR1 and SGT1 don't regulate the RAD6. RAD6 acts on the upstream mechanisms of RAR1 and SGT1. At the RNA level reason of HR symptoms in *Rad6* gene silenced wheat plant have been clarified.

3.6.6 Enhanced disease resistance was observed on *Rad6* silenced susceptible barley

Obviously the silencing of *Rad6* gene causes HR symptoms on wheat and barley as explained above. Then biologically test the *Rad6* silenced plants was aimed to asses if the pathogen growth on leaves is ceased. *Rad6* silenced wheat and barley physiologic records also support the production of HR generation. *Rad6* silenced barley plants were tested with avirulent and virulent pathogens. If HR generates on *Rad6* silenced barley, no fungal growth should be visible. So the Myc-tagged *Mla1* gene transgenic Golden promise (Gp) barley plants were inoculated with BSMV:RAD6.2As and BSMV:00. After that the plants have been infected with Bgh95(53/01) virulent powdery mildew pathogen. No fungal structures were detected on *Rad6* gene silenced Mla1-Myc barley. On the other hand fungal secondary hyphea structures have been detected on the BSMV:00 and FES mock inoculated Mla1-Myc barley plants as expected (Figure 3.20 and 3.21).

To confirm the above results in another R-Avr system which is Mla6-Bgh103(64/01). *Mla6* gene containing Golden promise barley plants were inoculated with BSMV:RAD6.2As and BSMV:00. Then the virulent powdery mildew inoculations have been performed by Bgh103(64/01). No fungal structures were detected on *Rad6* gene silenced Mla6 barley however, fungal secondary hyphea structures have been detected on the BSMV:00 and FES mock inoculated Mla6 barley plants (Figure 3.22 and 3.23).

Bülbül barley cultivar which has no *R* gene against powdery mildew fungal attacks (Bülbül, universal susceptible barley cultivar) was used for HR production in order to verify the effect of *Rad6* gene silencing. Without any viral inoculation just treating with FES solution Bgh95(53/01) pathogen infects the plant (Figure 3.25). BSMV:00 'virus alone' positive control inoculation was applied on Bülbül followed by inoculated with Bgh95(53/01) virulent pathogen inoculation, pathogen growth on FES mock and BSMV:00 inoculated Bülbül barley was successful (Figure 3.24). However, lastly the *Rad6* gene was silenced in Bülbül plants and inoculated with Bgh95(53/01) virulent pathogen, however there was no powdery mildew growth because of the HR (Figure 3.26). Our hypothesis has been supported with different types of barley inoculations. HR generation does not allow the pathogen growth in silenced plants. On the other hand, already resistant plant was still resistant to avirulant pathogen race when *Rad6* gene silenced and no pathogen growth was observed as expected. The additional data suggests this hypothesis. In the figure 3.27 and 3.28 avirulent powdery mildew inoculation can not be successful because of the HR generation of resistant plant. *R* dependent gene for gene resistance is shown in those Figures. Figure 3.26 and Figure 3.27- 28 give same HR responses when Myc-Mla6/Gp is inoculated with Bgh103(64/01) and Myc-Mla6/Gp is inoculated with Bgh95(53/01). The inoculations are avirulent so HR is performed and no pathogen growth is observed as pictured in the figure 3.27, 3.28, 3.30 and 3.31.

3.6.6.1 *Arabidopsis thaliana* Rad6 mutants show enhanced resistance against virulent *H. parasitica* (Oomycete)

RAD6 may play a negative regulatory role in plant disease resistance. The idea is presented for the reason that *Rad6* gene silencing causes induced HR (resistance symptom) in wheat and barley. Phytopathologic biological function tests also prove that the virulent powdery mildew can not infect and can not colonize on *Rad6* gene silenced susceptible barley plants. Additionally the model plant *Arabidopsis* was tested to support the case. Virulent (*H. parasitica* Noks1) and avirulent (*H. parasitica* Emoy2) inoculations were applied on Rad6-1, Rad6-2 and Col8 *Arabidopsis thaliana* plants. Virulent inoculations on *Arabidopsis* Col8 gave

expected disease consequences therefore pathogen growth was observed on susceptible *Arabidopsis* Col8 plants. Nevertheless Rad6-1 and Rad6-2 *Arabidopsis* mutants showed enhanced resistance and no fungal growth (Figure 3.51 and 3.52). These results confirm our results showed by VIGS. On the other hand, resistant plant *H. parasitica* inoculation did not cause disease in any of the cases meaning mutants and resistant wild type plants are still resistant against avirulent *H. parasitica* inoculation.

3.6.7 Proteomics approach supports the HR caused by *Rad6* silencing in wheat

In order to find the differentially expressed protein upon silencing of *Rad6* gene in wheat plants 2-D PAGE proteomics analysis was performed. Successfully *Rad6* gene silenced Avo-Yr10 wheat showing 85.5% suppression (in *Rad6* gene) expression was used. BSMV:RAD6.2As inoculated (silenced) Avo-Yr10 wheat plant proteome was compared with that of BSMV:00 (without any endogenous plant gene silencing) virus alone inoculated Avo-Yr10 wheat plants. To subtract the virus alone induced or silenced plant proteins BSMV:00 positive control inoculation on Avo-Yr10 plant have been applied for comparison.

Two-dimensional gel electrophoresis (2-DE) was utilized and a total of 47 protein spots were identified. 17 protein spots that are up-regulated by silencing of *Rad6* gene and 30 protein spots were found to be down-regulated. A total of 38 unique proteins from these 47 spots were identified by LC-MS/MS, and many of them were explained below.

Pathogenesis related (PR) proteins encoded by host plant are defined as proteins that are induced upon avirulent pathogen attacks or chemical treatments. PR proteins are grouped into categories such as PR-1, PR-2, PR-3 PR-5. Some of them play role as antifungal proteins such as PR-1 proteins (Niderman et al., 1995). Beta-1, 3 glucanase proteins are also PR-2 type proteins that are induced upon avirulent pathogen inoculation (Li et al., 2001). In literature is shown that beta-1, 3 glucanase protein is also up-regulated with fusarium head blight infection in the spikes of hexaploid wheat (*Triticum aestivum*) (Zhou et al., 2005). This datum confirms the

up-regulation of beta-1, 3 glucanase proteins upon activation of resistance mechanisms. Another study is related with the beta-1,3 glucanase over-expression which shows the fungal resistance of that transgenic plants (Lan et al., 2000). Therefore induction of that protein is the result of disease resistance activation so the *Rad6* silenced plant shows the HR response because of beta-1, 3 glucanase protein up-regulation.

Glutathione *S*-transferases play important roles in normal cellular metabolisms as well as in the detoxification of diverse xenobiotic compounds such as herbicide detoxification. GSTs are induced in response to oxidative stress to protect plant cell components from when plants are attacked by pathogens and challenged abiotic stresses (Levine et al., 1994). Plant GSTs are known as induced upon pathogen attack (Kim et al., 1994). One of our up-regulated proteins in *Rad6* silenced plant is GST. The increase in GST suggests us that plant cells behave as if they encountered with the pathogen. The proteomics experiments by Zhou et al. show the up-regulation of GST with fusarium head blight infection in the spikes of hexaploid wheat (Zhou et al., 2005). Additionally Lin et al. has proven the GST up-regulation with analysis of rice defense response induced by probenazole (PBZ) (Lin et al., 2007). Therefore GST up-regulation is the result of disease resistance induction so the *Rad6* gene silencing gives the resistance induction in plants.

In the literature there is an evidence for methionine synthase mRNA up-regulation after inoculation of powdery mildew in wheat. So the methionine synthase up-regulation can be used as indication of disease resistance induction (Bhuiyan et al., 2007). Our 2D-PAGE result presents us methionine synthase up-regulation in *Rad6* gene silenced wheat also. Therefore induction of disease resistance upon silencing of *Rad6* gene could be acceptable.

Another up-regulated protein is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in *Rad6* silenced wheat plant. The protein is shown to be up-regulated upon wound response in rice plant (Shen et al., 2003). Recent publication discusses about the Rubisco proteolysis protection seen for PCD in *Avena sativa* (oat) plants (Coffeen and Wolpert, 2007). So the down-regulation of

Rubisco protein is prohibited in resistance plant. Our silenced plant resembles resistance after plant pathogen activation pattern.

Some of the down-regulated proteins in *Rad6* gene silenced wheat plants are discussed below:

As mentioned in the introduction part H_2O_2 is the resistant plant product to come up with pathogen attacks. At the site of infection H_2O_2 is produced as reactive oxygen intermediate (ROI). ROIs maybe involved in pathogen elimination and subsequent signaling of downstream component for effective resistance mechanism activation or both of them (Dangl and Jones, 2001). Peroxidase enzymes catalyze the reaction of hydrogen peroxide H_2O_2 conversion into water. So the peroxidase enzymes should not be active for efficient resistance mechanism signaling to occur. Therefore it is logical that the peroxidase protein is seen to be down-regulated by resistance phenomena which *Rad6* silenced wheat mimics.

Germin-like proteins (GLP) have various roles in plant development and defense against pathogen attack. Some of the GLPs encode superoxide dismutase (SOD) and their suppression leads to super-susceptibility to powdery mildew infection in barley (Zimmermann et al., 2006). In another study one of the GLPs was found to be induced upon infection of *Erysiphe necator* in grapevine. This GLP3 is shown to be having SOD activity (Godfrey et al., 2007). So there is a link between resistance and GLPs but GLP was identified in silenced plant as down-regulated protein. The situation can be explained by the in-solubilization of GLPs in plant proteome so the down-regulation of that protein is logical. Vallelian-Bindschedler et al showed the insolubilization of GLP in stressed (powdery mildew inoculated) barley leaves (Vallelian-Bindschedler et al., 1998).

The other down-regulated protein is the ATP-dependent Clp protease. The down-regulation of ATP-binding subunit clpA was found when compared its protein levels between *Rad6* silenced wheat plant and BSMV:00 inoculated wheat plant proteome. Eukaryotic cells have many proteases playing essential roles in cellular activities. ATP-dependent Clp protease is one of them. It has been revealed that, in

general, ClpP paralogs are required for chloroplast development and plant viability (Kuroda and Maliga, 2003). But the exact role of the protease in plants is not known and in order to functionally analyze the role of Clp protease in plants, RNAi was applied by Lars et al. in 2006. ATP-dependent Clp protease encoding gene was silenced in Arabidopsis. After the *Clp6* gene silencing they have performed 2-D PAGE analysis. As a result they indicated six up-regulated proteins: fructose bisphosphate aldolase, ribose 5-phosphate isomerase, a putative RNA binding protein, uracil phosphoribosyl-transferase (UPRT), a cyclophilin and some of those proteins up-regulated after pathogen attacks in plants. So ATP-dependent Clp protease protein down-regulation is seen in *Rad6* silenced wheat. This considers the possibility that resistance response of plant may lead to suppression of ATP-dependent Clp protease.

3.6.8 Yeast two Hybrid analyses suggests the RAD6-SGT1 interaction

What is the main reason for HR production in *Rad6* silenced plants and why the *Rad6* gene expression is down-regulated upon avirulent inoculation are questions that were tried to be answered by our part by performing protein interaction experiments. *Rad6* gene silencing resulted in HR like phenotype in wheat and barley where no pathogen growth was allowed. Those results were confirmed at RNA and protein level by qRT-PCR and 2-D PAGE respectively. The main reason for HR phenotype might have come from the increased mRNA levels of *Rar1* and *Sgt1*. The expression levels of these genes were measured upon avirulent pathogen inoculations in wheat and the up-regulation of *Rar1* and *Sgt1* expression were observed. This record is also consistent with literature (Wise et al., 2004). Wheat and barley plants, with suppressed *Rar1* and *Sgt1* levels *via* VIGS, are susceptible to pathogen attack (Scoffield et al., 2005; Hein et al. 2005). Supplementary data in the experiment show that the virus inoculation causes *Rar1* and *Sgt1* gene expression levels to decrease so that it would spread more efficiently. Therefore it is obvious that the induction of *Rar1* and *Sgt1* expression leads to HR in plants. Furthermore *Rad6* silencing causes

Rar1 and *Sgt1* expression stimulation therefore HR is generated upon silencing of *Rad6* gene. (Appendix-C)

How does RAD6 regulate the levels of SGT1 and RAR1? To find logical elucidation for this question one to one protein-protein interaction studies were performed. RAD6 protein was used as bait and questioned for interaction with SGT1 and RAR1. As a result it is observed that there is an interaction between RAD6 and SGT1, but no interaction takes place between RAD6 and RAR1. *In vivo* Y2H and Coimmunoprecipitation experiments have proven that RAD6 and SGT1 interact with each other (Yamamoto et al., 2004).

The SGT1 and RAR1 interaction is also well known (Boter et al., 2007). Therefore RAD6 interacts with SGT1 and may possibly regulate resistance *via* this interaction or ubiquitination. RAD6 may also regulate the RAR1 level through the SGT1-RAR1 interaction. The scientists have shown that ubiquitination pathway plays essential roles in disease resistance mechanism in plants. There are some positive regulators, as ubiquitination pathway components, of plant disease resistance such as U-box type E3 ubiquitin ligase CMPG1. Up to this point the positive regulators of cell death were exemplified but there are also ubiquitination pathway members playing role in disease resistance regulation as negative regulators. One of them is RIN1 (Holt et al., 2002) found in Arabidopsis which is a RPM1-interacting RING finger E3 ligase. Another example is SON1 F-box containing SCF type E3 ligase component. This protein is also a negative regulator because its *son1* mutant phenotype shows resistance to pathogen infections (Kim and Delaney, 2001). The other interesting example is Spl11 U-box type E3 ligase in rice. *Spl11* mutant of rice generates HR and shows resistance to blight rice pathogen (Zeng et al., 2004). Our *Rad6* gene is a negative regulator of plant cell death as *Spl11* (Appendix-C represents the RAD6 story in plant disease resistance).

3.6.9 Differential display analysis of high level boron tolerant *Gypsophila perfoliata* plant

The genes found allowed us to speculate on their possible involvements in boron tolerance mechanism based on the available literature information as presented here. Recently, Patterson and his colleagues applied proteomics approach in boron tolerant barley plants for identifying proteins differentially expressed for understanding the tolerance mechanism. They have described the protein level changes under low (50 μM) and high (5 mM) levels of boron concentrations both in root and shoot (Patterson et al., 2007). Our results are consistent with Patterson's table of involved proteins for boron tolerance mechanism. Some of the genes are the same as presented here, such as GTP-binding protein, 26S proteosome subunit protein, hydrolase, putative D-ribulose-5-phosphate 3-epimerase, 26S proteosome regulatory particle chain RPT6-like protein, ribosomal protein L13a. Our DD3Bor5 and DD3 Bor36 clones showed high similarity to 26S Proteosome regulatory particle non-ATPase subunit of rice and 26 S Proteosome Regulatory Particle Chain RPT6-Like Protein of *Arabidopsis thaliana*, respectively. Among various types of protein degradation processes, ubiquitin/26S proteosome turnover system controls the level and the activity of proteins. This system is involved in development, growth and defense processes in plants (Sullivan et al., 2003; Hellmann and Estelle, 2002; Hershko and Ciechanover, 1998). In general, genes of 26S proteosome system are highly induced upon stress conditions. In this study too, the induction level is quite high (11.46 fold) upon high boron concentration treatment, putatively indicating that the plant is coping with boron stress by controlling protein degradation, since misfolded proteins, the proteins of the unused pathways or switching pathways are subjected to protein degradation. Under excess boron concentration, the tolerant plant may try to change its pathways to cope with toxicity. Our clone DD3Bor26 shows amino acid sequence similar to Ribulose-phosphate 3-epimerase protein, which is another induced protein in tolerant plant sample found in the proteomics study (Patterson et al., 2007). This gene was also shown to be differentially expressed in *Pisum sativum* plant (Savenstrand et al., 2002) under low levels of UV-B and ozone stress conditions. These findings support our result, so that Ribulose-phosphate 3-epimerase protein may be differentially expressed under boron stress

condition as well. DD3Bor72 is another clone showing induction in its transcription level at high boron concentration. The protein sequence similarity search detects it as ribosomal protein L13a (RPL13a), which is also found as induced protein in boron tolerant Barley study (Patterson et al., 2007). Same gene transcript was also found to be induced 24 hour post inoculation of abscisic acid (ABA) treatment in lupine (*Lupinus luteus* L) (Cherepneva et al., 2003). A connection with abiotic stress tolerance with ABA treatment was shown on the myb transcription factor (*AtMYB44*), which is also activated under several abiotic stresses including high salinity, overexpressed transgenic Arabidopsis plant (Jung et al., 2007). Thus, RPL13a may most likely be involved in the upstream by controlling the stress responsive elements such as ABA inducing transcription factors.

DD1 Bor2 clone shows very high level of homology with *Arabidopsis thaliana* ELF6 (Early Flowering 6); transcription factor (Noh et al., 2004) which belongs to the Jumonji (Jmj) family proteins (Toyoda et al., 2003). In the excess amount of boron, its expression is induced almost 10 times, which is a significant change. ELF6 is characterized as repressor of photoperiod in *Arabidopsis thaliana* (Noh and Amasino, 2003). Additionally, the Jmj domain was shown to be a negative regulator of cell proliferation (Toyoda et al., 2000) with proposed transcriptional repressor roles (Toyoda et al., 2003). Jmj proteins (Jmj C domain) were also shown to have histone demethylation activity with a role in histone modification where Jmj domain itself is responsible for this activity (Tsukada et al., 2006). Thus, Jmj proteins have roles in development, transcription, and/or chromatin regulation (Takeuchi et al., 2006). The sequence information of the DD1Bor2 corresponds to a region beyond the Jmj domain of ELF6 and in spite of the high level of homology between the two the former's function is still unknown. Considering the available literature information, it may be putatively postulated that the identified gene *via* the clone DD1 Bor2 may have roles in responding to the excess boron *via* repressing photoperiod and in regulating developmental processes. It is possible to speculate the function of boron such as regulating the control of histone demethylation activities. Recent study reports critical involvement of boron in light induction activity by being part of the complex as an autoinducer (AI-2) in a bioluminescent marine bacterium (Chen et al., 2002). Thus, it would be very interesting to seek if

such an autoinducer can be isolated in ELF6 over expressing Arabidopsis since it would effect the photoperiod. DD2 Bor10 clone has a 91% similarity with histidine Kinase A at amino acid sequence level. Histidine kinases play roles in signal transduction in plants, fungi, protozoa and prokaryotes. In an earlier study, it is reported that under salt stress (nickel toxicity) four types of histidine kinases perceived and transduced the salt signal in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Marin et al., 2003). There are also other reports about histidine kinase involvement in the osmotic stress response, which shows fungal histidine kinase responses to osmotic stress and fungicide (Motoyama et al., 2005). Previously Peiguo Guo et al. (2007) indicated that histidine kinase may facilitate aluminum (Al) tolerance because this gene is highly expressed under Al toxicity in wheat (Guo et al., 2007). So the DD2 Bor10 clone showing homology with histidine kinase A may take role in response to boron stress tolerance similarly as in other metal ion stresses.

DD2 Bor13 shares homology with Arginyl-tRNA Synthetase. Arginyl-tRNA Synthetases are essential for all cells. Their primary role is attaching the amino acids to transfer RNAs for protein synthesis (Szymanski et al., 2000). They are also involved in other cellular processes such as synthesis and turnover of diadenosine tetraphosphate where the latter is involved in many kind of stress conditions in eukaryotic and bacterial cells (Jakubowski, 1983; Lee et al., 1983). The clone also may play roles in stress response in plants because it is up regulated under excess boron concentration. DD3 Bor7 clone has homology to Photosystem II protein D2. It was documented that the reaction center complex assembly needs the accumulation of the D2 protein (Komenda et al., 2004). The photosystem II has been shown prone to abiotic stresses such as light and heat stress (Kamata et al., 2005). Boron toxicity may affect the Photosystem II as being an abiotic stress factor. DD3 Bor7 clone mRNA induction was detected under high concentration of boron in the tolerant plant and this might be a response of tolerance caused from stress conditions. Clone DD3 Bor26 shows amino acid sequence similarity to Ribulose-phosphate 3-epimerase protein. In a study, it has been shown that this gene is differentially expressed in *Pisum sativum* plant (Savenstrand et al., 2002), under low levels of UV-B and ozone stress conditions. So this gene may be differentially expressed under boron stress condition. The only down regulated gene transcript found annotated as

DD3 Bor39 has shown no homology to any known gene. This gene indeed may play role as an important negative regulator by de-repressing toxic effects of high boron concentration. Although only a short sequence of information is available, the gene can still be cloned via 5'RACE and functional gene analysis experiments can be further conducted in *planta*.

CHAPTER 4

CONCLUSIONS

Plants are exposed different kinds of stresses during their life times. Stress factors can be categorized into two groups, biotic and abiotic. The objective of this thesis was functionally characterizing the pathogen stress regulated *Rad6* gene in different aspects to get hints about the plant-pathogen molecular interactions. Functional genomics and proteomics approaches have been applied for this purpose. The excess level of boron micronutrient concentration is one of the abiotic stress factors for plants. Another objective of this thesis was to identify the genes involved in boric acid stress tolerance toxic level of boron treated tolerant plants were carried out.

Prior to the VIGS mediated RNA degradation of specific wheat genes for functional analysis BSMV mediated gene silencing system was optimized. Due to the white photobleaching phenotype of *Pds* silenced plants, *Pds* gene silencing was used to be phenotypic marker. Additionally GFP transient expression by BSMV system has been achieved in wheat in the content of thesis.

Rad6 gene has been found decreased upon race specific pathogen attack in wheat and barley plants. This expression level suppression might be the reason to protect plant itself against the invaders. To functionally characterize the *Rad6* gene BSMV mediated VIGS system have been used on wheat and barley plants. Silencing of that gene (mRNA expression level of gene decrease) was confirmed by qRT-PCR. In every silenced plant HR like symptoms were observed phenotypically. To figure that out silenced plants transcriptome and proteome were analyzed. *Rar1*, *Sgt1* and *Hsp90* gene expressions of BSMV:RAD6.2As (*Rad6* gene silenced) and BSMV:00 inoculated wheat and barley plants were relatively quantified. 2-D PAGE proteomics analysis was performed on silenced and positive control wheat plants. Totally 47

protein spots were analyzed which are differentially synthesized in silenced plant leaves. 9 of the protein spots give no hit within the aminoacid database. 30 protein spots were detected down-regulated or suppressed in the silenced Avo-Yr10 wheat plant according to non-silenced but viral inoculated Avo-Yr10 wheat leaves and 17 protein spots were detected as up-regulated in the silenced plant proteome. The silenced wheat and barley plant transcriptomes confirm the HR because the expression level of some genes are up-regulated in silenced plants relative to non-silenced BSMV:00 control virus inoculated plants. 2-D PAGE has verified the HR phenotypes of *Rad6* silenced plants. Some proteins such as β-1,3-glucanase (PR-2) and Glutathione S-transferases are up-regulated in *Rad6* gene silenced wheat plant. These proteins were also detected as up-regulated proteins in the proteome of resistant plant upon pathogen attacks (Li et al., 2001; Zhou et al., 2005). Some of proteins have been shown down-regulated such as ATP-dependent Clp protease and peroxidase in *Rad6* silenced wheat plant relative to viral inoculated wheat plant. These proteins are known as decreased proteins during avirulent pathogen infection.

Expression levels of two important disease resistance related genes which are *Rar1* and *Sgt1* genes, are increased in *Rad6* silenced wheat and barley plants. Possibly *Rad6* gene regulates the *Rar1* and *Sgt1* mRNA levels. These two gene expressions are also up-regulated upon avirulent pathogen attack. *Rad6* silencing reasons same responses as avirulent pathogen inoculation performed in wheat and barley (Coldo et al., 2004). *Rar1* and *Sgt1* genes have been silenced in wheat by using VIGS system. Decrease of the gene expressions *via* silencing were confirmed by qRT-PCR. *Rad6* mRNA has been relatively quantified in *Rar1* and *Sgt1* silenced wheat plants compared to BSMV:00 inoculated wheat plant transcriptomes. As a result *Rar1* and *Sgt1* have been separately and successfully silenced but the expression level of *Rad6* gene is not changed in *Rar1* and *Sgt1* silenced plants. Therefore *Rad6* is not regulated by *Rar1* or *Sgt1* but the reverse occurs.

To perform fungal pathogenesis tests different kinds of inoculations have been applied on *Rad6* silenced barley plants. Silencing of *Rad6* gene causes HR in both resistant and susceptible barley plants. Therefore subsequent to silencing of *Rad6* gene, the virulent powdery mildew inoculation could not be successful, no

pathogen growth has been observed on susceptible plants. Physiological pathogenesis test results have proven our prior suggestions also. In avirulent powdery mildew inoculation the *Rad6* silenced barley plants behave as BSMV:00 inoculated barley.

To clarify HR produced *Rad6* silencing observable fact one to one protein-protein Y2H interaction experiments were performed. RAD6 protein has been used as bait and examined to find if it interacts with RAR1 or SGT1. As a result RAD6 does not interact with RAR1 but does interact with SGT1 as published previous experiment (Yamamoto et al., 2004).

Additionally Arabidopsis *Rad6* gene mutants (*Rad6.1* gene copy and *Rad6.2* gene copy mutants) (Δrad6-1 and Δrad6-2 Arabidopsis) are found to be show enhanced resistance against virulent *Hyaloperonospora parasitica* (downy mildew Oomycete). The susceptible wild type Arabidopsis plants are acted as resistant phenotype once one of the *Rad6* gene copies is mutated. So the *Rad6* gene has a negative regulatory role in plant disease resistance. E2 (Ubiquitin conjugating enzyme, RAD6) takes role in plant disease resistance.

Very recently some of the species of *Gypsophila* genus collected from the boron rich soils in Turkey were shown to be remarkably tolerant to high levels of boron. Boron at a very limited amount is necessary for the normal development of plants; however, high level abundance of boron is toxic in general. Nevertheless, adaptability of plant species allows them withstanding the extreme amounts of metal ion presence in the soil by various strategies. This study is conducted on highly boron tolerant *Gypsophila perfoliata* L. located from the boron mining area. The first preliminary molecular level study in this organism was reported in here, attempting to identify genes related in boron tolerance. The plant was transferred into plant nutritional medium in the presence high extreme concentration of boron of 35 mg/kg (~500 μM) boric acid for finding of genes responsible for the tolerance. The mRNA transcriptome in the excess level boron treated plant was compared with the transcripts of the plants grown under normal boron concentration using differential display PCR method (DDRT-PCR). Thirty different bands corresponding to

expressed genes were excised from the DNA sequencing gels and were sequenced. Among which 18 of them were confirmed *via* quantitative reverse transcription real time PCR (qRT-PCR) (three biological and technical replicates have been performed for 500 μM boron concentration treatment and extra 1000 μM boron concentration treatment were also analyzed). The gene fragments thought to be involved in boron tolerance have homologies to transcription factors, proteosome subunit, photosystem II D2 and an unknown protein (Unver et al., 2008).

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APPENDIX-A

DNA sequences of the differentially expressed gene fragments from *Gypsophila perfoliata* L. (boron stress related).

1- DD1 Bor 2 ELF6 (Early flowering 6); transcript.factor [A. thaliana] FE905318

CATTATGCTGAGTGATATCTTTTTTTTTCACATTTACCAATGCAGATTACGAAAAG
ATTAAGGCATACGCTGCCGCAATTGCAGAAGAAATCAGAACGTCCTTCAATTATAA
AGAGATCCAGCTTGAGTCTGCCACCGAGGAAGAATTAAAACTTATAGATACTGCTA
TCGATGCTGAACAACTTTCTGACGTCCAAGAAGACTGGACATCGAAACTCGGGATT
AACTTACGTTATAGTGCTAAGAAAGGTTGTCTGTCAAAGAAGATAGAGCATACATT
GAATCCCGACCATTTACTCTCGGCTATGGCCCCTGTTTCAAGAATCTCAAGTATCG
AGTGGTGGTCTAGAAAAGCTCGGTCTAAATCCGAGTCAAATCGTCCGGAGCAGAAC
GTATTTAATGACCATCCACCTAAAACAGACGAGGTTAAGCTTAAATCTGATGGTCA
GCTGGGTAAAGGTGGAAAGGTGCTTCTCCAATACTATAGGAAGAAGTTTAAAAACC
AATTGAAGACAATTTAGTGTAGATCTCCGTTTCAGGTTAGCTCTTAGACTGCGGCC
GCTCAA

2- DD1 Bor 4 Expressed protein [A. thaliana] FE905319

ATCCTCTTAGACTGCGGCCGCTCAGCCTACAAGGTTACATCCTAGGTTCTAGATAC
CGGGATCTCGGAAAATGACATAACATGAACAGTGATGCTGAATAAATATCGATTTC
AGTACTACCTTCTTACATGAACATTATAAGTCCTGAAATTAGGGGACGAAGTTTAA
ATTCATGTGAGACAGTACTGAAAGTCAATACGACCCGACAATATTGCCAAGCACAC
ACGTTCTCACGGGAAGAACATAAATCCGGGCAAATACCATTTGATTAAGAGATTGA
ATTTAGAACAAACTATTTAAATTTAAGAGGAAAAAAAAAGATATCACTCAGCATAA
TG

3- DD1 Bor 6 GTP-binding protein GB3 [A. thaliana] FE905320

TCCTCTTAGACTGCGGCCGCTCAGGATATCCCAGCTAAGACTGCTTCCTGCTGTAG
TTCCTGAAAACTGTTTTGCTCCAGGCCTCACCGTTTCTGGCTGTCTGCGTTAACTC
TATCTCCCACGACTGGGATGGGTGTCTGTCGGCTACATTTGGTATGTAAAAAACGA
GTGCTCTGTCTTGGTTTGAAAAGAAGAATAGCGAGGGATTTATGTTAGAATTGTGT
GACTAAATTCAGGAGTTTATACGGCGTACATGGATTTTTTTCTACCACGTTGAAAT
TCATGTGTAAAATGGACATTATTTTTACGAAAAAAAAAGATATCACTCAGCATAA

4- DD2 Bor 10 Histidine kinase A, N-terminal [M. truncatula] FE905321

AGCGGCCGCAGTCTAAGAGAAGCTAGATCAAGGATTGAGGAAGCGTTAGGAACATG
TCTCCTTCCTTCTTTACAGTTAGTGCCTGCAAATCCTGCTGTTGGACAGGAGATTT
GGGAAGTCATGAGTCTCCTTCCCTATGAGGTGAGATTTCGTCTTTATGGTGAATGG
GAAAGAGATGATGAGCGGATACCAATGATTTTGGCAGCTAGACAAACAGCAAAGCT
GGAAACTCGACGGATCCTAAAGCGGCTTGCCAAAAAAANANAGATATCACTCAGCA
TAATGA

5- DD2 Bor 13 Putative arginyl-tRNA synthetase [O. sativa] FE905322

TGAGCGGCCGCNNTTTAAGAGGCCTTAGAAGAGCTGGAGCAGCTCCAAGTCTTTAA
AAGCAATTTAATGTCGCCGCAGGTTGACATCCGGAGGACCTCCAATACCGTCCCAA
AGTCGCCAATGAGGGGCCAACCCTCGCCGCGGCGCTCTCTTGGAGCGATGGCCCGC
CGTCACCGGCANTTAGGACCGCGCAAGTGCNCTAGAAGCACAGCTTAGAAGGGGGA
GAGAGATGTATCCGTACGGCTGTACATTTGGTGGCGAGCTGTGTAATAGTTGTTTN
TAGTGATGCTTCTTGCTCATTTGTAGGGTTTGGCCCGGAATATGANTACGGCACTT
TGATGAATTGTTTTTCAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTC
TGCGTTGTTACCACTCTTAGACTGCGGCCGCTCAA

6- DD2 Bor 14 Unknown [H. vulgare] FE905323

CATTATGCTGAGTGATATCTTTTTTTTTCGACATTAAGAGACCAAATAACACAGGT
CATGCTTGTTTTTATCGGCTAAAAATTAAACACGACGGCAAAGCTTCCAGAAAAAA
GCGTAACTTTCGAGTCAGTCGACATTTAAGCTATTTATTCAAAGTGCTATTTTGCA
GCTTCTGAAGAAGCGAATTCTCCATCTAGAATGAAAATCCATCGCCCAGTTATCGA
CAATTGACTGCAATCTAGCTGTGCTTATGAGCAGAAGTAGCAATTACGGTGCTCTC
TGCTCAACATATGAACCAGAAATCCCAGAAGTTCCCAAGACTGTGACCTTCCCCCA
CTCTGAGCCAAGGTGACTGCATCTCATCTTCAAATGAATCATCCTTGTTCCCAACT
CTGTCTCGACTTTCTTCCTTAAGTAGAACTCATCAGGACCAAATGAATCCAAATAC
TTGGTATACCGATCATAAGCTGTTCTTAATGCCTGGCCATAGTATTCAGGATACGG
CGTTGCATTCTCGCCAGAAATTCCTCCGTGGTCATCAATGGCCATTCTCAAGGACT
CAAGGGTGCCTATGAACTCTTAGACTGCGGCCGCTCAA

7- DD3 Bor 2 Speckle-type POZ protein-related [A. thaliana] FE905324

TATTAACCCTCACTAAAGATCTNGNNNTGGTTAATTTCAAAGTTGCTGGCAGCATC
GGATAAATATGAGCTTGTGAGGCTCAGGCAGTTGTGTGAATCTTTGTTGTGCGAGA
AGATAGATGTAACTCTGGTCGGAGAGTTTCTAGCATTAGCAGACTGCTACCATGCC
ACTGACTTGAAGGCTATTAGTCTAAGATATGCTGCTGAAAATCTTGCAGCTGTTAT
GCGTTCTGATGGTTTTGAATACCTCAAGGAGCACTGCCCAGCGTTGCAGTCAGATC
TTTAGTGAGGGTTAAT

8- DD3 Bor 5 26S proteosome subunit RPN7 [C. annuum] FE905325

GTTTAGCGGGATCAAAGTGCATTCTTTGCATCTGGGCGATTTGTCTCCAAAACTCC
AGCAACTTTGTCGATTTTGCAGTGCAGTTTCCCAGCTGCAATAAAGCGAGATAGCT
CCAAATCGATGAACTCAACAGTAACACCAAATGCCTTTGCCGTCGCCTCAATGGTG
ACACTTTTGTAAGGTTCAAGGAACTGAGAATAAACAACAGTCCTGACCTCCCTCAT
GTAATAACGGAAGTGTCGTTGTAAGTAGCGATCAAACTTTATTTGTTCTGATAGGC

CAGCAAATGCCGAGAAGAACGACTTGTACTGACAATCATAAAGAGAATTCATGAAC
TCGGACAGATACGGAATTTTTCCAATCACGGTCAAAATTTCAGGAGCATCTACTAC
CTTTTGTTTGAGCGACACTCTATCAAGGGATATGATGA

9- DD3 Bor 7 Photosystem II protein D2 [T. aestivum] FE905326

ATGCTGAGTGATATCTTTTTTTTTCAAGGATTTCATAATTGGACGTTGAACCCATT
TCATATGATGGGAGTTGCCGGAGTATTAGGTGCGGCTCTGCTATGCGCTATTCATG
GAGCGACCGTAGAAAACACTCTATTTGAGGACGGTGATGGTGCAAATACCTTCCGT
GCTTTTAACCCAACTCAAGCTGAAGAAACTTATTCAATGGTCACTGCTAACCGCTT
TTGGTCCCAAATCTTTGGTGTTGCTTTTTCCAATAAACGTTGGTTACATTTCTTTA
TGCTATTTGTACCCGTCACCGGTTTATGGATGAGTGCTATTGGCGTAGTTGGCTTG
GCTCTGAACTTACGTGCCTATGACTTTGTTTCCCAGGAAATCCGTGCAGCGGAAGA
TCCTGAATTTGAGACTTTCTACACCAAAAATATTCTTTTAAACGAGGGTATTCGTG
CGTGGATGGCAGCTCAGGATCAGCCTCATGAAAATCTTATATTCCCTGAGGAGGTT
CTACCACGTGGAAACGCTCTT

10- DD3 Bor 13 Metallo-dependent hydrolase, composite [M. truncatula]
FE905327

GTTTAGCGGGATCAAAGTGCTGCTTTAGCAGGCGGAACAACCATGCATATTGACTT
TGTCATACCTGTTAATGGAAGTTTATCAGAAGGCCTGAAGTCTTACCGGAAGAAGG
CACAAAAATCTTGTATGGATTACGGTTTCCATATGGCAATTACTAAATGGGACGAA
GTTGTTTCTAAGGAGATGGAGACCATGGTGAAGGGGGAAGGTATTAATTCGTTCAA
GTTCTTCATGGCGTACAAGGGGTCCTTTATGGTAAACGATGAACTTTTGATCGAGG
GGTTCAAAAAATGCAAGTCCTTGGGTGCCTTACTGATGGTTCATGCTGAAAATGGC
GATGCTGTTTTCGAAGGGCAAAAAAAAAGATATCACTCAGCATAATGA

11- DD3 Bor 26 Putative D-ribulose-5-phosphate 3-epimerase [S. demissum]
FE905328

AGACGAAGTCTCGCTCTGTCGCCCAGGCTGGGTGCAGAGGTACAATCTNAGCTCAC
TGCAACCTCCACCTCCTGGATTCAAGAGATTCTCCTGCCTCAGCCTCCCCAGTAGG
TGGGATTACAGACATGCACCACCACATCTGGCTAATTTTTGTATTTTTAGTGGAG
ACAGGGTTTCACCATGTT

12- DD3 Bor 34 putative leucine-rich receptor-like protein kinase [O. sativa]
FE905329

CATTATGCTGAGTGATATCTTTTTTTTTGAGGTAGTGCTATTGTTTCCTCTTGCAT
AGGTTATCTATCCATTATTATCAACATGTGATGGTGTGAAAATTTACTGTTCTACA
CTCATTAAAAATGTGCCTCCTTTCTTCCTTGGCTGCTACTGAATTTCCACAAACCT
TTTCCTCAACCAATTGGGAAATGTAATCATTTTGTGGCTAGGGAATACCGAATACC
TGAAATTCAAAGATATTTCCTCCAACCCACAATGTTTGTCTCATTCACTATTTGTA
CGGTCTCCAACNGCGGCACTTTGATCCCGCTAAACA

13- DD3 Bor 36 26S Proteosome regulatory particle chain RPT6-like protein [A. thaliana] FE905330

CATTATGCTGAGTGATATCTTTTTTTTTGACATGATGAGCAAAAAACTGATTGACA
AATTACACAGACTTGATCCACAAAATAATAAATTGAAGTAGTAAGAAGAGATAAAC
TTACATAATACTCGGAAATTCCCACCAAAAACCTGTCGTCTAAGAACATTTCCATA
TCCAGCTACATTTTCTGCCTTGTTGGTTAGATGCAGCGACTCTAAAAGTTTAACCG
TCTTTACTATCAGCTTGTAGATTAAGTATTTGGGAAACAACAAGCTTTGAAAGCTC
GTTGANTGCACTTTGATCCCGCTAAACA

14- DD3 Bor 40 Putative osmotic stress-activated protein kinase [O. sativa] FE905332

GTTTAGCGGGATCAAAGTGCTGCTTTAGCAGGCGGAACAACCATGCATATTGACTT
TGTCATACCTGTTAATGGAAGTTTATCAGAAGGCCTGAAGTCTTACCGGAAGAAGG
CACAAAAATCTTGTATGGATTACGGTTTCCATATGGCAATTACTAAATGGGACGAA
GTTGTTTCTAAGGAGATGGAGACCATGGTGAAGGGGGAAGGTATTAATTCGTTCAA
GTTCTTCATGGCGTACAAGGGGTCCTTTATGGTAAACGATGAACTTTTGATCGAGG
GGTTCAAAAAATGCAAGTCCTTGGGTGCCTTACTGATGGTTCATGCTGAAAATGGC
GATGCTGTTTTCGAAGGGCAAAAAAAAAGATATCACTCAGCATAATGA

15- DD3 Bor 41 Copalyl diphosphate synthase 1; CPS1 [C. maxima] FE905333

GTTTAGCGGGATCAAAGTGCCGCGTTGGAGACCGTACAAATAGTGAATGAGACAAA
CATTGTGGGTTGGAGGAAATATCTTTGAATTTCAGGTATTCGGTATTCCCTAGCCA
CAAAATGATTTCATTTCCCAATTGGTTGAGGAAAAGGTTTGTGGAAATTCAGTAGC
AGCCAAGGAAGAAAGGAGGCACATTTTTAATGAGTGTAGAACAGTAAATTTTCACA
CCATCACATGTTGATAATAATGGATAGATAACCTATGCAAGAGGAAACAATAGCAC
TACCCCAAAAAAAAAAGATATCACTCAGCATAATGA

16-DD3 Bor 42 Putative D-ribulose-5-phosphate 3-epimerase [A. thaliana] FE905334

GTTTAGCGGGATCAAAGTGCATAGTCGCGGGGAGTTCAGTATTTGGAGCACCAGAT
CCAGCCCAAGTTATCAAAGTATTGAGGCAGAGCGTGGAGGAAG

17-DD3 Bor 43 No significant hit FE905335

ATCGACGGGNCCGGGANCCGATTCNTTATGCTGAGTGATATCTTTTTTTTTGGGAA
AAAATTTTGTTGTGTATTGTAACTTTATGATCCAAAAGACAGGAGTTATTTGCTAA
TTGCTAGTCTATGATCTCTATGCAACGCGCTTTACAAAACATGTTTTCAACAACAA
CATCATCAAATCCTTACAAACAGGAAGAACAGACTAATAAAAGATTGGGGAAGAGG
AAATATCTATCTCCGAATCTCTGATGCTTCAAACCGACATTCACCATTGCAGCAAG
TGCTTGTATAGCAGACATCAATGACCCTATTTGATAGAATTGATACAGCCTACAAG
AAATCCATGGCGCTCCTCGTACGAAATTGTGAAGTTAAATAATAATAGATGAATAA
CAGCCTCAACGATCCAGTCAGATCTTTAGTGAGGGTTAATAATCTAGATGCATTCG
CGAG

18-DD3 Bor 44 No significant hit FE905336

TCANNNNNCNTANNNNNNTNTTNNGTGNNTGGTNNNTANTCNNNCAAGTCNTNNNA
GAATAGTGTATGCGGCGNCCGANTNNNNTNCCNGGCNTCAATACGGGANATNCNNN
CNNCATAGCAGAACTTTAAAAGTGNTCAGCGAATGCATCTAGATTAAGCAGTGGTA
ACAACGCAGAGTACGCGGGCTGAGGATGGAGGTGCCGGCCAGGGGGGTTGTTGGGA
GGCAGAGGGGTGCTCATCGCCCCGGTCACGAATTCAATCGGNTCCCGGGCCCGTCG
ACNNCAG

19- DD3 Bor 45 No significant hit FE905337

TCGACGGGNCCGGGANCCGATTCNTTATGCTGAGTGATATCTTTTTTTTTGGGAAA
AAATTTTGTTGTGTATTGTAACTTTATGATCCAAAAGACAGGAGTTATTTGCTAAT
TGCTAGTCTATGATCTCTATGCAACGCGCTTTACAAAACATGTTTTCAACAACAAC
ATCATCAAATCCTTACAAACAGGAAGAACAGACTAATAAAAGATTGGGGAAGAGGA
AATATCTATCTCCGAATCTCTGATGCTTCAAACCGACATTCACCATTGCAGCAAGT
GCTTGTATAGCAGACATCAATGACCCTATTTGATAGAATTGATACAGCCTACAAGA
AATCCATGGCGCTCCTCGTACGAAATTGTGAAGTTAAATAATAATAGATGAATAAC
AGCCTCAACGATCCAGTCAGATCTTTAGTGAGGGTTAATAATCTAGATGCATTCGC
GA

20- DD3 Bor 77 No significant hit FE905339

GCGTCGANGGGCCCGGGATCCGATTGCGGACGGTGCTTTAGTGAGGGTTAATAAGG
NCNTTATGCTGGTGATATCTTTTTTTTTGCGGACGGTGCTTTANTGANGGTTAATA
NGGTCATTATGCTGAGTGATATCTTTTTTTTTTGCNGANGGTGCTTTAGTGAGGGNT
ANTAANGTCNTTNTGCTNAGTGATNTCTTTTTTTTTGCGGACGGTGCTTTAGTGAG
GGTTAATAGGGTCATTATGCTGANTGATATCTTTTTTTTTTGCNGACNNNGCTTTAN
TGANNGCTGANNGATATCTTTTTTTGCGGACNNTGCTTTANTGAGGGTTAATATGG
TCATTATGCTGANTGATATCTTTTTTTTTTGCTGNNANNGTGCTTTANTGNNGNTAA
NTAGGGTCATTATNCTNANTGATACCTTTTTTTTTTGCGGGCGGNCCTTAAGGGNGG
GTTAATAGGANNNNTAGANNNNNNNGATANCNNNTTTTNNACCATNTGAGCATTAG
GGNGGNTNAATAAGGNNANCNANAANCANNNNNGAGGTACCNANCNNNCANTCCCC
TGCCCCCGTTTGACNNNNGTNNGAAAGNNNNCNNNCNGNGANNNCNNANTTNNNCC
NNCNNAANTACAGCGATANTANANGNGCACACCNNAATNTAAGAGNGNGCNNNNNG
NTCNNTNNNCAANGGTTAGGGAANNGGTGTGGGGGNGCAGNTTTGNNNNGACAANN
TNNTNTCCNNANNTGTGNTGAAATTNNGTAAAANGAGGCGCNAA

21- DD3 Bor 72 Ribosomal protein L13a, cytosolic [A. thaliana] FE968988

CGCTCTTACAGATTAACTCGAGGTTCGAAGAGAAGTCGTTGGAAAAGCCGGAACTG
GGAGTTAAACCAATCGACGAAGAAGGAAAGTATACGTTGGACCCTAGAGAGGAAAG
AGAAGAGAAGGTCGAGGGACTACCATAGTATCACCAAGGTCGGCTGAAGGAACCTC
TCTGCCGGATCGTTCGTCATGAACACTGGGAAAACCTCGGAGTTGTGGAAGTTTCG
TAGCCCCTGCTGGTAGGAGAACTAGAACAGTATTCCTCCTCCCTATGGGAGCATGT
GGAAGTTGGATCGGTTACGTCGTCGAGGAGAGAACCAGAACACGCCTTAGTAGGGT
GCCTGCCACGAAATCACTCCC

22- DD3 Bor 79 No significant hit FE905341

GNNCTCTGNNTCNNNGGGNNNNGGNNCCGATTNNNTNGGNNNANNGAGAACTNTNT
NNGTNNNNNNNNGNANNNNTTNANNNTGTNCANGATGNNGAGGNTGCTGCNTATAC
TATCAAAGNTGTNACGTGTNCNCCTTGTAATGNTTTNATCTTNACNTCGTCACTTG
ACGATNTNATTGTNNNCNNNTTGNANNCCTNATANGTNATACCTCTGATANTTTAN
GCANCTGCCTGCTTNCTCNCGTCCNTNNCNTTCTTGTNGNNCNGCTNANCAACNNA
NATATTCNTCTGCATATTGNATCGNATCCCG

23- DD3 Bor 81 No significant hit FE905343

ACTAAAGCACCGTCTAGACGCACACTGTCACCAGAGGTATTAACCTCACTAAAGCA
CCGTCCTTAGACGCACACTTGTCACCAGATATTAACCCTCACTAAAGCACCGTCCA
GACGCACACTTGTCACCAGATATTAACCCTCACAAAGCACCGTCCTAGACGCACAC
TTGTCACCAGATATTAACCCTCACTAAAGCACCGTCCAGACGCACACTTGTCACCA
GATATTAACCCTCACTAAAGCACCGTCCAGACGCACACTTGTCACCAGATATTAAC
CCTCACTAAAGCACCGTCCTTAGACGCACACTTGTCACCA

24- DD3 Bor 76 Nicotiana tabacum DNA, TJ sequence FE968986

GTCCTACCCAATCTAGATGCATTCGCGAGGTACCGAGCTCGAATTCACTGGNCGTC
GTTTTACAACGTCGTGACTGGGAAACCCTGGCGTTACCNACTTAATCGCCTTGCAG
CACATCCCCTTCGCAGCTGGCGTATAGCGAAGANGCCGCACGATCGCCTTCCACAG
TGCGCAGCTGATGCGATGAATTGTAGCGTTATATNNGTTANNTCGCGTAATTTGTA
A

25- DD3 Bor 78 Cocos nucifera acyl ACP thioesterase FE905340

ATCACTTAAGCGCCGGCGGACGTCCAGCTGGTATACCCTCTCGAGGGTTGCGCAAC
CTACGTATCGAACTCATAAGATATCACAGTGGATTTATCGAACCGCATTAGTACCA
GTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGTGTGTTGTATGCT
CGGCCTTCGTATTTCACATTTCGGACCCCACGGATTACTCACTCGATTGAGTGTAA
TTAACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGACAGCACGGTCGACG
TAATTACTTAGCCGGTTGCGCGCCCCTCTCCGCCAAACGCATAACCCGCGAGAAGG
CGAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGACGCCGCTCGCCATAG
TCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGNCCCCTATTGCGNNNTT
CTTGTACACTCGTTTTCCGGTCGTTTTCCGGTCCTTGGCATTTTTCCNGCGCAACG
ACCGCAAAAAGGTANCNGAGGCGGGGGGACTGCTCGTAGTGTTTTTAGCTGCGAGT
TCAGTNNCNNCGCTTTGGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACCT
TCGANGGNGCACGCNAGAGNCAAGGNTGGGANGNNGAATGGCNTATGGACNGGCGG
AAANAGGAAGCCCTTCGCANNNGAAAGAGTATCGANNNCGACANNNTA

26- DD3 Bor 80 No significant hit FE905342

TAGATTGGGTAGGACTGGTCATCTGGACGGTGCTTTAGTGAGGGTTAATAGCTGGA
CGGTGCTTTAGTGGGGGTTAATAGCTGGACGGTGCTTTAGTGAGGGTTAATAGCAC
TGGTCATCTGGGGGTAGGACTGGTCATCTTGGACGGTGCTTTAGTGAGGGTTAATA
CTGGTCATCTGGGGGTAGGACGGTGCTTTAGTGAGGGTTAATAGCACTGGTCATCT
GGGGGTAGGACTGGTCATCTGGGGGTAGGACTGGTCATCTGGGGGTAGGACTGGTC
ATCTGGGGGTAGGACTGGTCATCTGGGGGTAGGACTGGTCATCTGGGGGTAGGACT

GGTCATCTGGGGGTAGGACTGGTCATCTATAGGACGGTGCTTTAGTGAGGGTTAAT
ACTGGTCATCTGGGGGTAGGACTGNTNNATCTATAGGACGGTGCTTTAGTGAGGGT
TAATAATCGGNTCCNGNCCGTCGA

27- DD3 Bor 82 No significant hit FE905344

ATTCGAGCTCGGTACCTCGCGAATGCATCTAGATTATATGCTGAGTGATATCTTTT
TTTTTAGGACGGTGCTTTAGTGAGGGTTAATAGTGATCATTATGCTGAGTGATATC
TTTTTTTTTGGGACGGTGCTTTAGTGAGGGTTAATAGTGATCATTATGCTGAGTGA
TATCTTTTTTTTTTAGGACGGTGCTTTAGTGAGGGTTAATATCGCTCATTATGCTGA
GTGATATCTTTTTTTTTTGGGACGGTGCTTTGTGAGGGTTAATATTCATTATGCTGA
GTGATATCTTTTTTTTTTAGGACGGTGCTTTAGTGAGGGTTAATATCACTCNNTATG
CTGAGTGATATCTTTTTTTTTTGGGACGGTAATCGGNTCCCGGGCCCGTCGAC

28- DD3 Bor 28 No significant hit FE905340

AAAGATATCACTCAGCATAATGATATTAACCCTCACTAAAGCACCGTCCCAAAAAA
AAAGATATCACTCAGCATAATGAGTATTAACCCTCACTAAAGCACCGTCCCAAAAA
AAAAGATATCACTCAGCATAATGATATTAACCCTCACTAAAGCACCGTCCCAAAAA
AAAAGATATCACTCAGCATAATGAATCGAATTCCCGCGGCCGCCANGGCGGCCGGA
GCN

29- DD3 Bor 39 No Hit FE905331

GTTTAGCGGGATCAAAGTGCCATTGGTCTCAACCTCACGTTACTAGTTTCTTTGTT
ATCATTTAAACTATGAACACTTAACGCGACTACTTGTACTAGCTGTTCTTCTGAAT
GGGTAATTTTGTGCCACGAAAAAAAANAGATATCACTCAGCATAATGA

APPENDIX-B

Biotic stress related DNA and protein sequences with alignments

1-RAD6 FE900153

Nucleotide sequence:

ATGTCGACTCCTTCAAGGAAGAGGCTGATGAGGGACTTCAAGCGGCTGATGCAGGA
CCCTCCTGCGGGCATAAGCGGGGCGCCGCAGGACAACAACATAATGCTGTGGAATG
CTGTGATTTTTGGCCCTGACGATAGCCCGTGGGATGGAGGCACGTTTAAGCTGACT
CTCCAGTTTAATGAAGAATATCCTAATAAGCCACCAACAGTTCGGTTTATTTCTCG
GATGTTTCACCCTAACATTTATGCTGATGGAAGCATATGCTTAGATATTCTACAGA
ATCAGTGGAGCCCAATATATGATGTAGCTGCTATACTTACATCTATCCAGTCGCTG
CTGTGTGATCCTAACCCAAATTCGCCTGCTAACTCAGAAGCTGCCCGCATGTTCAG
TGAGAACAAGCGAGAGTACAACCGCAAAGTGCGGGAGATTGTTGAGCAGAGCTGGA
CGGCAGACTAA

Protein sequence:

MSTPSRKRLMRDFKRLMQDPPAGISGAPQDNNIMLWNAVIFGPDDSPWDGGTFKLT
LQFNEEYPNKPPTVRFISRMFHPNIYADGSICLDILQNQWSPIYDVAAILTSIQSL
LCDPNPNSPANSEAARMFSENKREYNRKVREIVEQSWTAD.

2-BSMV α genome sequence:

GTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCACATAATCGTAATCGAT
TCTTCTTGATCTCTAAACAACACTTTCCCGTTAGCATGGCTAGCGATGAGATTGTC
CGCAATCTGATCTCCCGTGAGGAGGTGATGGGTAATTTGATTAGCACAGCTTCTAG
CTCAGTAAGGTCACCCTTACATGACGTACTGTGCTCGCACGTAAGGACCATCGTCG
ATTCCGTGGATAAGAAAGCGGTCAGTCGCAAGCATGTTGATGTACGGCGCAACATC
TCCTCTGAAGAGTTACAGATGTTGATAAATGCATATCCTGAATATGCCGTTTCATC
CTCAGCTTGTGAATCTGGTACTCATAGCATGGCGGCTTGTTTTCGATTTCTGGAGA
CAGAATACCTCTTAGATATGGTTCCAATGAAAGAGACTTTTGTTTATGACATTGGT
GGTAACTGGTTTTCTCATATGAAGTTTCGTGCTGATAGAGAAATTCATTGTTGCTG
TCCGATCTTATCTATGAGAGATTCTGAAAGACTGGAAACACGCATGATGGCAATGC
AAAAATATATGCGTGGATCGAAAGACAAACCGTTACGCTTGTTAAGCCGTTATCAA
AATATCCTGCGTGAACAAGCGGCGAGAACAACTGCCTTTATGGCAGGTGAGGTGAA
TGCGGGTGTTCTCGATGGAGATGTGTTTTGTGAGAACACTTTTCAAGACTGTGTGA
GACAGGTGCCCGAAGGTTTTTTGAAGACAGCTATAGCAGTTCATAGCATCTACGAT
ATCAAAGTGGAAGAATTTGCGTCTGCATTGAAAAGAAAAGGTATAACACAGGCTTA
TGGGTGCTTCCTGTTTCCTCCTGCTGTATTGATAGGTCAGAAGGAAGGTATTTTAC
CTTCCGTGGACGGTCATTACTTGGTGGAGAATGGCAGGATTAAGTTCT

TCTTTGCGAATGATCCGAATGCCGGTTACTCTCATGACCTTAAGGATTATCTGAAG
TATGTGGAAAAAACCTACGTGGATATAAAGGATGGAGTGTTTGCTATTGAGCTGAT
GCAAATGCGAGGTGATACCATGTTCTTTAAGATCACGGATGTCACCGCAGCAATGT
ATCATATGAAATACAGAGGTATGAAACGTGATGAAACATTCAAATGCATTCCGTTG
CTAAAAAATTCATCCGTTGTCGTACCTCTATTTTCGTGGGACAACCGTTCTTTAAA
GATCACAAGTGGTTTATTACCACGAACTTTGGTCGAGCAAGGTGCGGCGTTTATTA
TGAAAAACAAGGAGAAGGACTTGAACGTTGCTGTGTTGAAGAACTATCTTTCCGCT
GTGAACAACTCATACATTTTCAACGGATCCCAGGTTAGAGATGGTGTGAAAATTGC
CCCGGATTTAATCTCCAAATTGGCAGTGACTCTGTACCTGAGAGAAAAGGTCTATC
GACAAAGAGAAAATTCAATCATAAGTTATTTCGAGCAAGAAATGCTTCACGATCCC
AACTTGAAAGCCATGTTTGGAGACTTTCTGTGGTTTGTTCCAAATACTCTCTCGAG
TGTCTGGAAGAACATGCGAAAATCACTGATGGAATGGTTTGGCTACGCAGAATTTG
ACTTGACTACTTTTGATATTTGCGATCCCGTTCTCTACGTAGAGATAGTGGATCGG
TATAAGATCATTCAAAAAGGGCGAATTCCACTTGGTGAGTTTTTTGATTGTCATGA
AGAATGCGAGAATTACGAACTGCGTGAGAAGGAGAAAAATGACCTAGCGGTGAAAA
TGGCCCAGAAGGTAACAGGGACGGTGACCGAATGCGAGAAGGACCTGGGACCTCTT
GTTCAACCGATAAAACAGATATTGGTTCAACTTGTGATGCCCAATTTGGTCAGAGC
GCTGTGTAGACCTCGTAGCCCAACGTCTCCTTTGGACTTAAATATCCCAGGGTCAA
CTCCATCACACTCAAGTTCAGATTCTGAACAATCTATGACTGAAGAAGCGAGCTGC
GCCATTGCGGGTAGCGTACCAACATGGGAAATTGCGACTAAGAAAGATCTAACCTT
TCAGCGAATTGATGAAGATATGTCTCGACGAACTGGTATGCCTCCAAGACCAAAAG
TAACTTCTAGTTACAACATGAATGCCAGAGCTGAGTTTCTCTACTATCAACTGTGT
AGCGTGATTTGTGAAAGGGCTCAGATTTTGAGTGTCATCGAAGACTTTCGTCAGAA
TTTGATATTCTCAGATAAAGTGGCCGTTCCATTGAACGCTAGATTCTACAGTTTTC
AGTCATTGCAACCCGGATGGGTGTTCAAGACTCCATCGCATAGTGAAGTAGGCCAC
AGTTATGCAGTACATTTTGACTTCAAGACAGTTGGAACCGATTTGGAAGAGAGCCT
AGCTTTTTGCCGAATGGTACCGATTTCATGGGATAAAAGCGGCAAATACATCGCGA
CAACTCCTCATTTTCCCGAGAGACATGGTTACTACGTGATTTGTGACAACACTAAA
TTGTGTAACAATTGGCTTATTTACAATAAGTTAGTTGATGTCTACGCACGAGTGGC
TGATAGACCTCTGAGATTTGAGTTGATTGACGGAGTTCCTGGCTGCGGAAAGTCAA
CCATGATTTTAAACAGCTGTGATATTCGACGCGAAGTTGTTGTTGGTGAAGGAAGG
AATGCAACTGATGACTTAAGGGAGAGGTTCAAGCGTAAGAAAAATTTGAATAGTAA
GACTGCTAATCATAGAGTTCGAACGCTTGACAGCTTATTACTTGCTGAAGGACCTT
GTGTACCGCAAGCTGATAGGTTTCATTTTGATGAAGCTCTAAAAGTTCATTACGGC
GCCATAATGTTCTGTGCTGATAAGCTTGGTGCCTCAGAAATTCTCGCTCAGGGAGA
TAGGGCTCAACTGCCGATGATCTGTCGTGTAGAAGGTATTGAACTTCAATTTCAAT
CTCCTGATTACACGAAGACGATCATAAATCCTAAGCTACGATCATACCGTATCCCT
GGAGATGTTGCCTTCTATTTGAGTGCTAAGGAATTTTACAAAGTTAAAGGAATACC
TCAAAAGGTTACAACTTCTAACAGTGTGAAACGTTCCCTGTACGCTAGAGGCGAAA
CAACTCCGGAAAGATTCGTGAGTTTGCTTGATGTTCCGGTGAGAAAAAACACCCAT
TATCTAACCTTCTTACAAGCTGAGAAGGAAAGTTTGATGAGTCATTTGATTCCAAA
GGGTGTGAAGAAAGAGTCTATTTCAACGATTCATGAGGCGCAGGGTGGTACCTATG
AAAATGTGATTCTGGTCCGTTTGCAACGGACGCCCAATGAAATTTATCCGGGTGGA
CCTAGGTCCGCCCCTTACATTGTGGTTGGGACTTCAAGGCATACAAAAACTTTCAC
TTATTGTAGTGTTACGGACGATAAGTTGCTTTTAGATATCGCCGACGTCGGTGGTA
TTGCACATACACCTATTCGTACTTTTGAATCTCATATAGTTTAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAATGTTTGATCAGATCATTCAAATCTGAT
GGTGCCCATCAACCATATGATGGGAGTGTTTGCAAGTCCACTATAATCGAACTTGA
AAACGATGCCTGAATTGGAAACCATGAATCTTAACGGATTCTGGAGAGAAAATTTA

GGAATTGGTATGTAAGCTACAACTTCCGGTAGCTGCGTCACACTTTAAGAGTGTGC
ATACTGAGCCGAAGCTCAGCTTCGGTCCCCCAAGGGAAGACCA

3-BSMV β genome sequence:

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGC
AGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGA
CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACC
TGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGG
TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCC
CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGG
TAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCG
AGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACAC
TAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA
GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTT
GTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGAT
CTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG
TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGT
TTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTT
AATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCT
GACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGT
GCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA
CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCA
TCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGT
TTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGG
TATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCA
TGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAG
TTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGT
CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCT
GAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGG
GCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTC
GTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCA
AAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTG
AATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTC
TCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG
CGCACATTTCCCCGAAAAGTGCCACCTGAAATTGTAAACGTTAATATTTTGTTAAA
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AACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTT
TGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGATGCCCCGATTT
AGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAA
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CACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTG
CGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGC
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CACGACGTTGTAAAACGACGGCCAGTGAATTAATACGACTCACTATAGTAAAAGAA
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AGATCGTGATCTACCTGCTGATGTAGACAGACGGTTTGCAGGTGCTAGAGGACAAA
TTGGTTTACCCAATTATCTTCCTGCGCCAAAATTCTTTCGTCTCGATAAGCGAACT
ATCGCTGAACTGACTAGACTCTCTCGTCTTACGGATCAGCCGCACAACAATCGTGA
TATAGAGCTTAACCGAGCGAAAAGAGCCACAACTAACCCATCTCCCCCGGCGCAGG
CACCGTCGGAGAATCTTACTCTTCGTGATGTTCAACCGTTAAAGGATAGTGCGTTG
CATTATCAATACGTGTTGATTGACCTACAGAGTGCGAGACTCCCAGTGTATACCAG
GAAGACTTTCGAACGTGAACTCGCTTTGGAATGGATCATTCCAGATGCCGAGGAAG
CGTGACCTGCTGTTGAAGCGGTAAAAGGATGTACATATGTATCTTATTTATTTTGT
TTATCTATTTTCTTTTACTTTTAGTTTTTGCTTTTTACGCGTTAACTAGATGTATT
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TAGGCCCAATGACCTTAAAGGCGGGACTAAGGGATCATCTCAAGTGGGTGAAAATG
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CCCAAGTCTGTGAAAATGCAAAGCAATCTGGCCGATAAGTTCAAAGCGAATGATAC
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AATCTGATTTTGAGTACACTGGTCGACAGTATTTCAGAGCTAGATCAAATTTCTTT
GAAATGATTAAGCTCGCATCCTTGTATGACAAACATCTAAAGGAATGTATGGCGCG
AGCCTGCACCCTAGAACGAGAACGATTGAAGCGTAAGTTACTCCTAGTACGAGCTT
TGAAACCAGCAGTTGACTTCCTTACGGGAATCATCTCTGGAGTTCCTGGCTCAGGA
AAATCAACCATTGTGCGTACTTTGCTCAAAGGTGAATTTCCGGCTGTTTGTGCTTT
GGCCAATCCTGCCTTAATGAACGACTATTCTGGTATTGAAGGCGTTTACGGGTTAG
ATGACCTGTTGCTTTCTGCAGTTCCGATAACGTCTGATTTATTGATCATAGATGAA
TATACACTTGCTGAGAGCGCGGAAATCCTGTTGTTACAACGAAGACTCAGAGCCTC
TATGGTGTTGTTAGTCGGGGATGTAGCTCAAGGAAAAGCCACCACTGCTTCCAGTA
TTGAGTATTTAACTCTGCCGGTGATCTACAGATCAGAGACGACTTATCGTTTGGGA
CAAGAGACTGCTTCGCTTTGCAGCAAGCAGGGTAACAGAATGGTTTCAAAGGGTGG
AAGGGACACAGTGATCATTACTGATTACGATGGCGAAACAGATGGAACGGAGAAAA
ATATCGCTTTTACTGTCGATACAGTTCGAGATGTGAAAGATTGCGGGTACGATTGT
GCCCTGGCAATTGATGTGCAAGGGAAAGAATTCGATTCAGTGACTTTATTCCTAAG
GAACGAAGACCGGAAAGCTTTAGCAGATAAGCATTTGCGTTTAGTCGCTTTGAGCA
GACATAAGTCGAAGTTAATCATCAGGGCCGACGCGGAAATTCGTCAAGCATTCCTG
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TACGGAATCCGGCGATAATATTCACAAATTCGCCAACGGAGGTAGTTACAGGGACG
GGTCAAAGAGTATAAGTTATAATCGTAATCATCCTTTTGCCTATGGCAATGCCTCA
TCCCCTGGAATGTTGTTGCCCGCAATGCTTACCATCATCGGAATCATTTCCTATTT
ATGGCGAACAAGAGATTCCGTGCTCGGAGACTCAGGCGGAAACAATTCCTGCGGAG
AAGACTGTCAGGGCGAATGTCTTAACGGACATTCTCGACGATCATTACTATGCGAT
ATTGGCTAGTCTTTTTATCATTGCTCTATGGTTATTGTATATATATCTAAGCAGTA

TACCTACGGAGACTGGTCCCTACTTCTATCAAGATCTGAACTCTGTGAAGATCTAT
GGAATAGGGGCTACGAATCCAGAAGTTATTGCGGCCATCCACCATTGGCAGAAGTA
CCCTTTTGGGGAATCTCCGATGTGGGGAGGTTTAGTCAGTGTTTTGAGCGTTCTTC
TTAAACCGCTGACGTTAGTTTTTGCGTTAAGCTTTTTTCTCTTACTTTCTTCAAAA
AGGTAAAAAAAAAAAAAAAAAAAATGTTTGATCAGATCATTCAAATCTGATGGTGC
CCATCAACCATATGATGGGAGTGTTTGCAAGTCCACTATAATCGAACTTGAAAACG
ATGCCTGAATTGGAAACCATGAATCTTAACGGATTCTGGAGAGAAAATTTAGGAAT
TGGTATGTAAGCTACAACTTCCGGTAGCTGCGTCACACTTTAAGAGTGTGCATACT
GAGCCGAAGCTCAGCTTCGGTCCCCCAAGGGAAGACCACTAGTCATGCAAGCTTTC
CCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTG
TGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTC
TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCAC
TGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAA
CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCT

4-BSMV γ genome sequence:

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGC
AGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGA
CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACC
TGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGG
TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCC
CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGG
TAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCG
AGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACAC
TAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA
GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTT
GTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGAT
CTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG
TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGT
TTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTT
AATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCT
GACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGT
GCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA
CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCA
TCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGT
TTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGG
TATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCA
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TTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGT
CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCT
GAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGG
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7-pγpds4As sequence:

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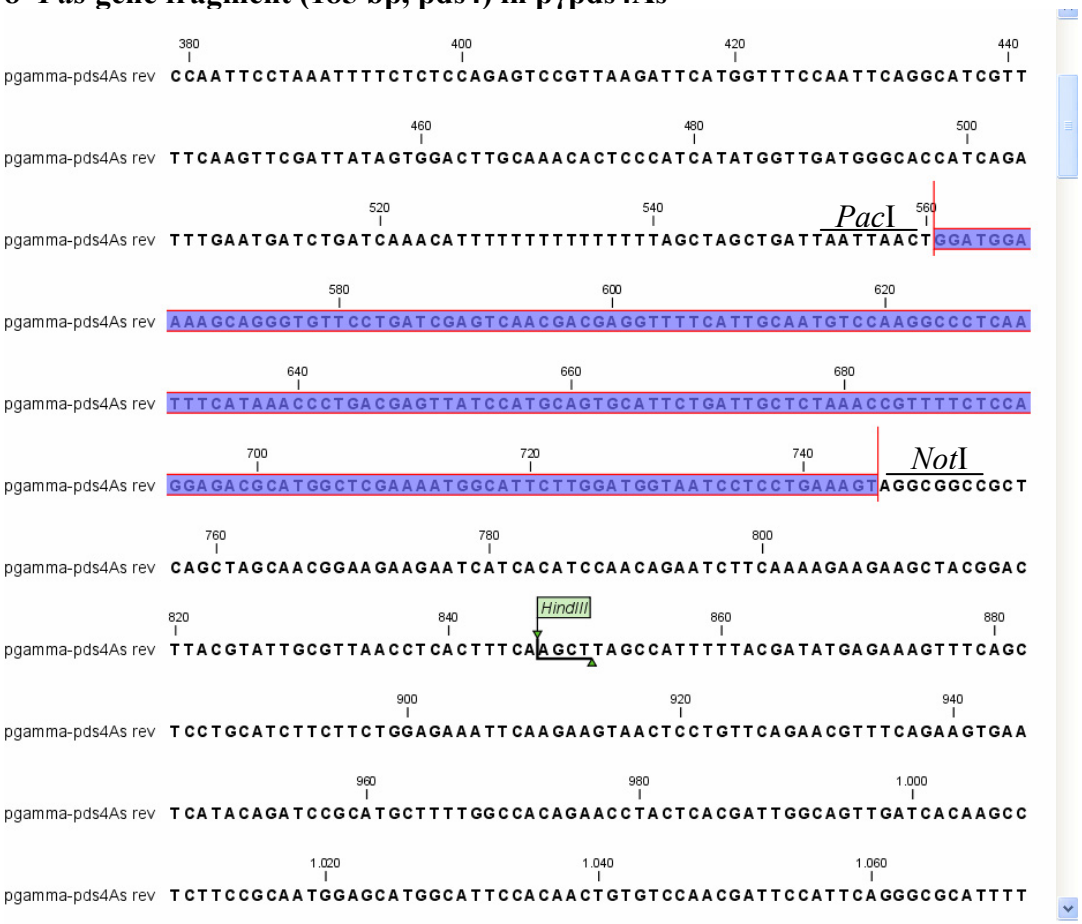
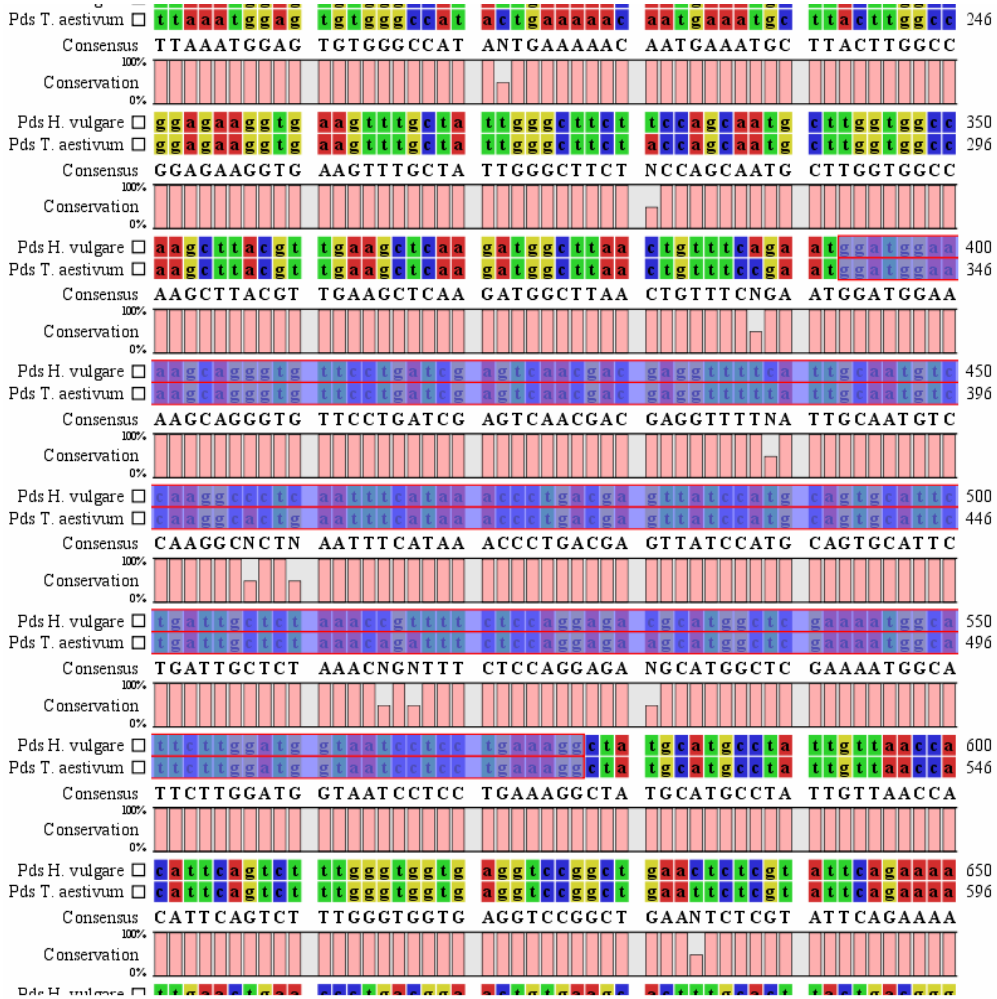
8- *Pds* gene fragment (185 bp, pds4) in pγpds4As



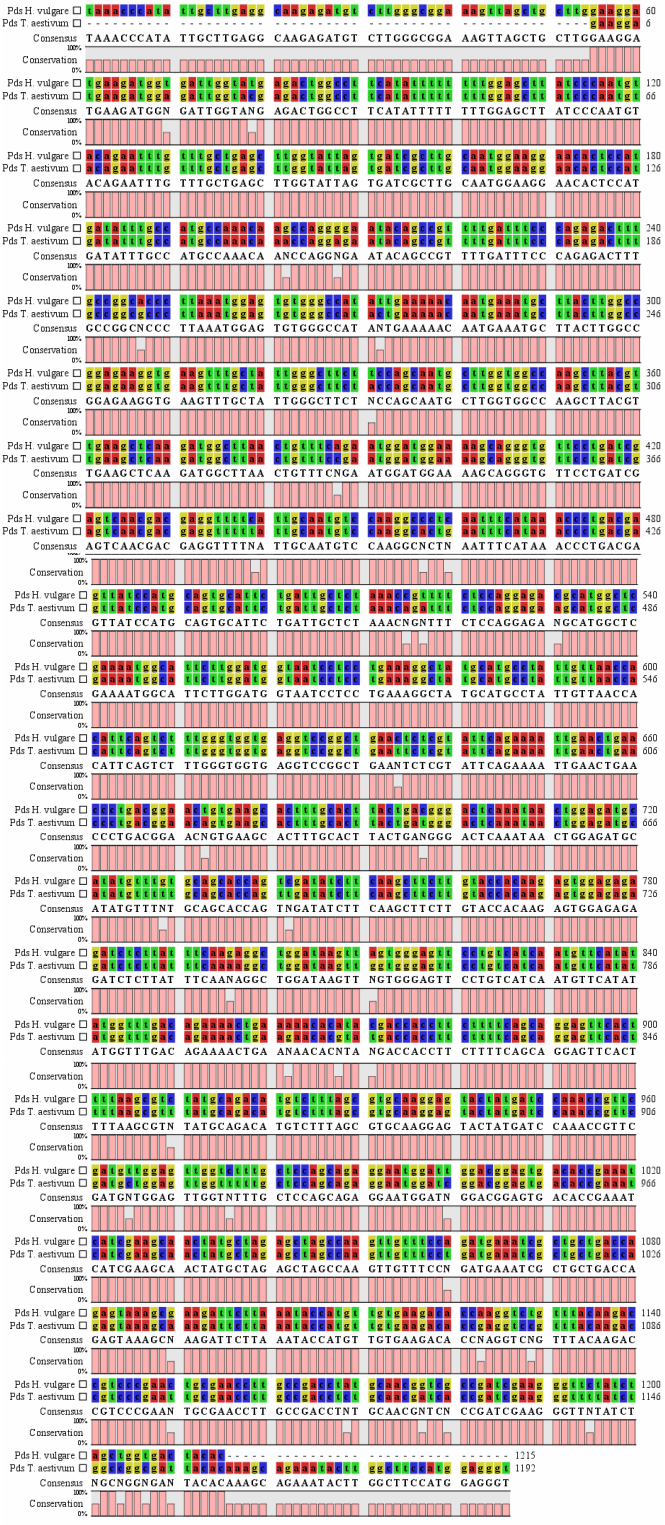
The colored region represents the *Pds* gene fragment (185 bp, pds4) in pγpds4As vector

9- pds4 region for both *T. aestivum* and *H. vulgare*



Blue colored region is the sequence of *Pds* gene used for cloning (pγpds4) for silencing construct preperation. It has been indicated that the *T. aestivum* pds4 region has 97% homology with *H. vulgare* pds4 region**.**

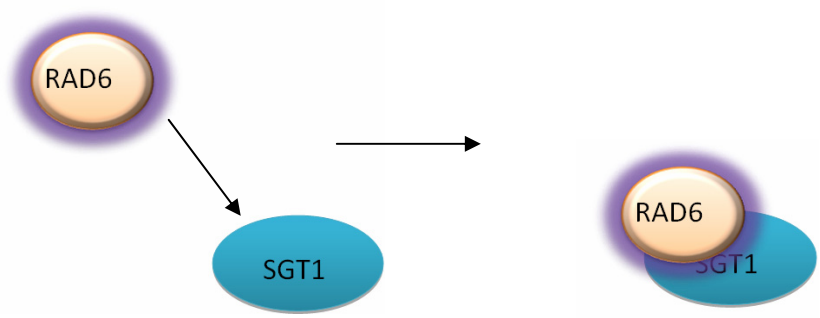
10-Alignment of *Pds* gene of *H. vulgare* and *T. aestivum*



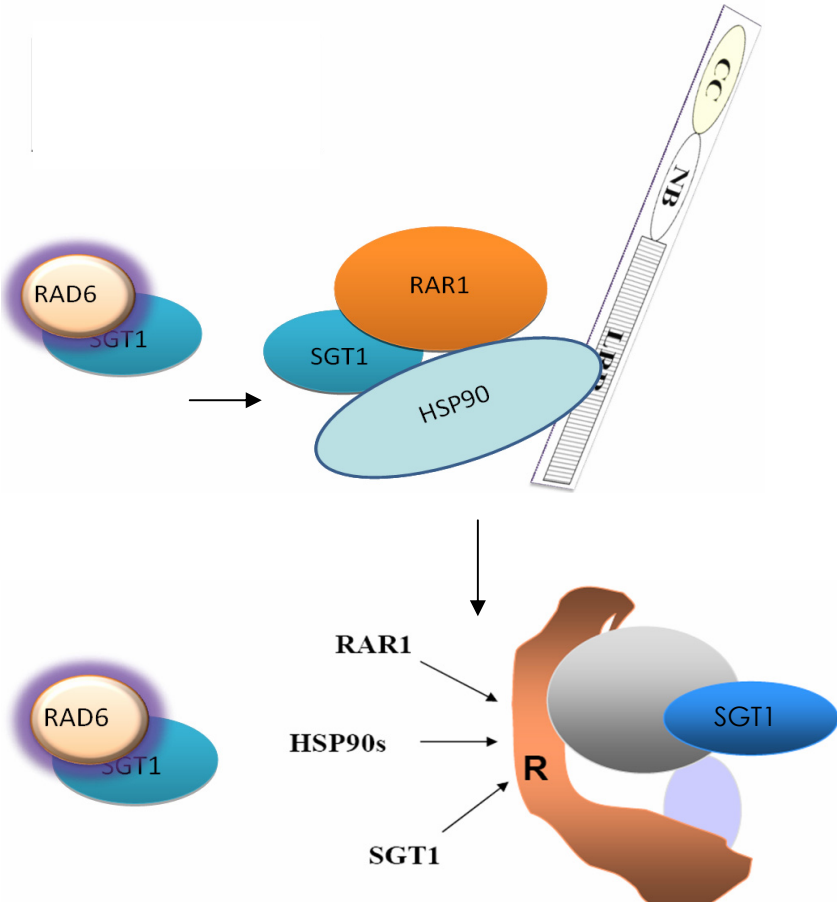
Alignment of *Pds* gene of *H. vulgare* and *T. aestivum*, there is 96% homology between two sequences.

APPENDIX-C

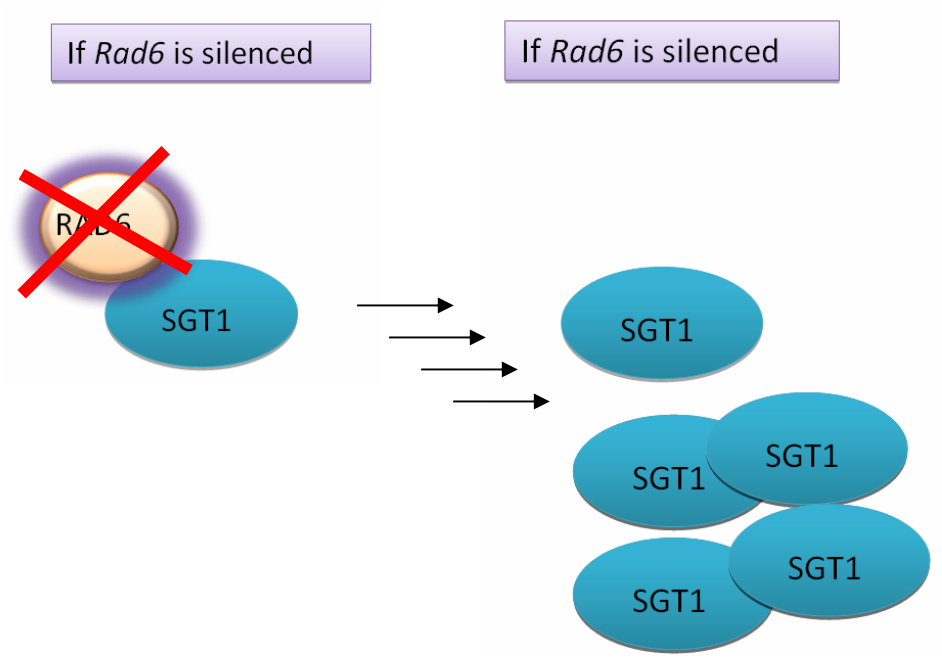
RAD6 interacts and controls the SGT1



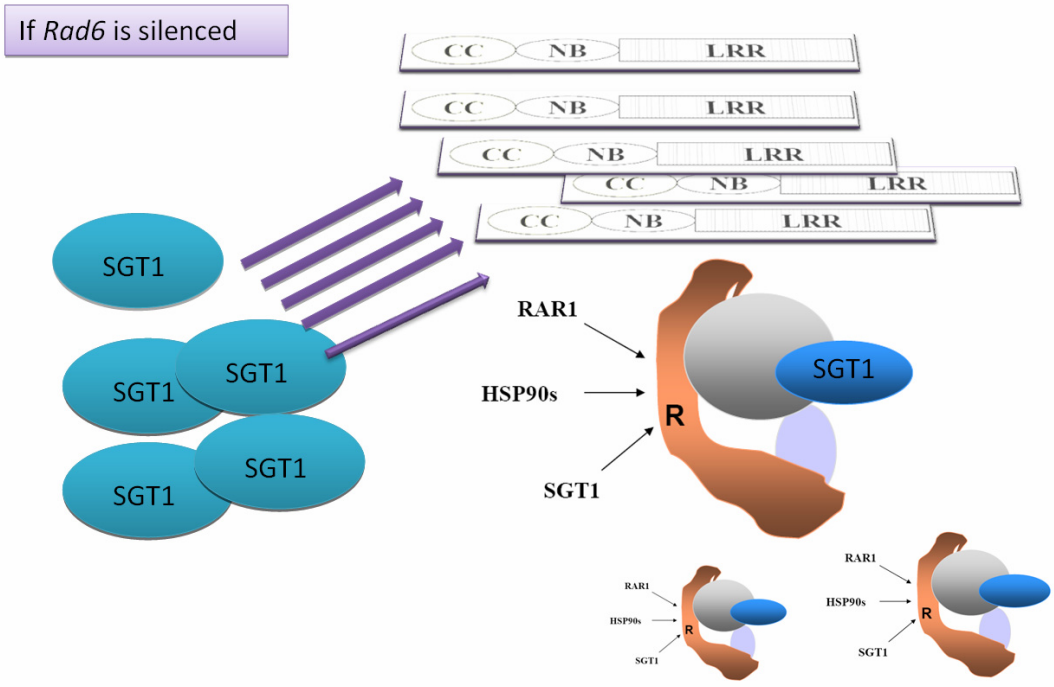
RAD6 interacts with SGT1 and controls the level of SGT1.

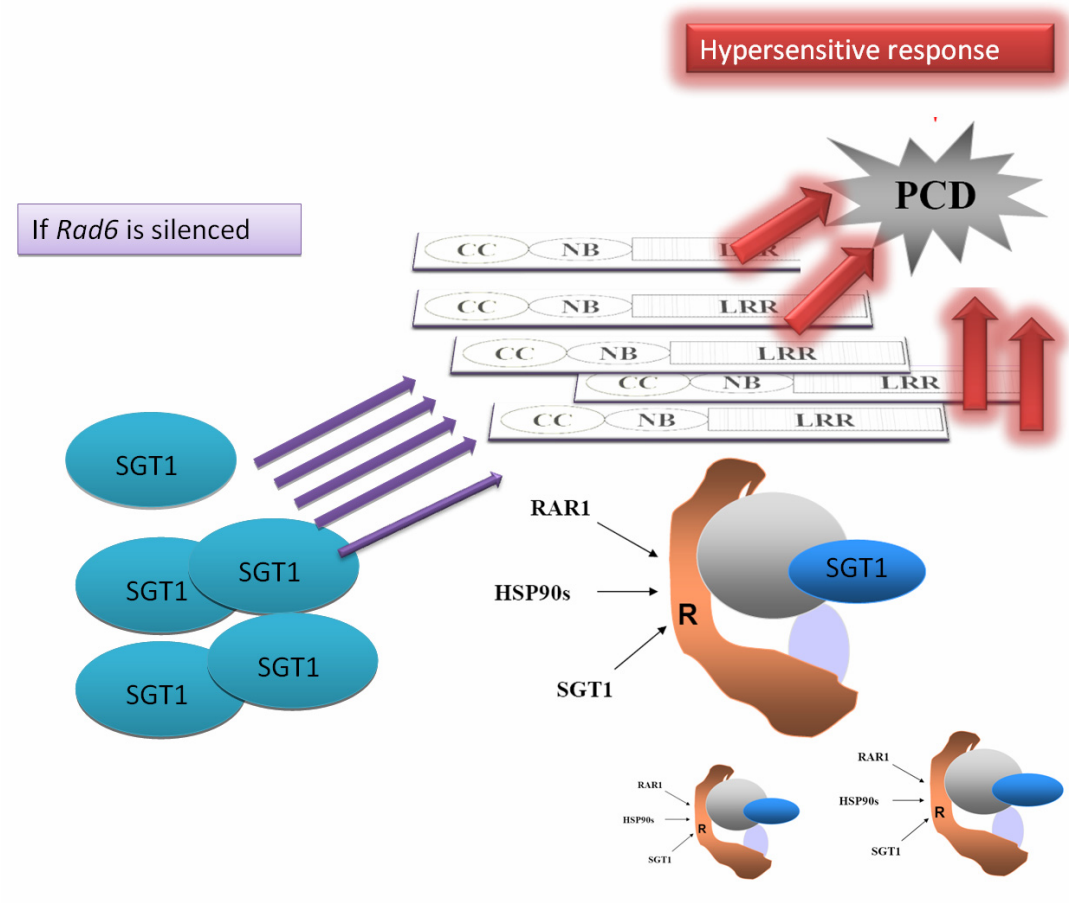


SGT1 inter acts with RAR1 and HSP90. The triple interaction is required for R protein stability, level and folding therefore, for functionality.



If *Rad6* gene is silenced, more SGT1 will be accumulated.





Since more R (resistance) protein will be functional, SGT1 over-expression leads the plant cell to programmed cell death in *Rad6* gene silenced plant.

APPENDIX-D

1 *Yr10* gene silencing in wheat plants

1.1 LRR (Leucin rich repeat) part of *Yr10* gene amplification for VIGS construction and BSMV inoculations

LRR part of *Yr10* gene (GeneBank# AF149114) gene was cloned from Avo-Yr10 wheat plant cDNA to make a construct for VIGS. using *NotI* restriction cleavage site containing forward 5' ATAGCGGCCGCGCCGTCTCTGCATGACCTCT 3' and *PacI* site containing reverse primers 5' ATATTAATTAA AAATCAATGAAGCCGCAACC 3' 159 bp length *Lrr* fragment was amplified from wheat (*Triticum aestivum*) plant's cDNA clone. Annealing temperature for PCR amplification of those primers was the 59 ^{o}C. 30 µL of final volume and following components were mixed; 2 µl diluted plasmid DNA, 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25^{o}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), 1 U of *Taq* DNA polymerase (Bioron), 10 pmol forward primer and 10 pmol reverse primer sterile distilled water up to 30 µl volume. PCR cycling conditions were 94 °C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min. Amplified PCR products were loaded on 1% agarose gel and analyzed. PCR products were purified and then ligated with pGEM-T easy vector for amplification and sequencing analysis. Using same procedures with 2.6.3.2 to 2.6.3.6 pγLrr1.S clone was constructed and linearized. α, βΔβa, γ, pγLrr1.S *in vitro* transcriptions were done and Avo-Yr10 plants were inoculated with BSMV:LRR1.S.

1.2 qRT-PCR and measurement of *Yr10* gene expression level changes in BSMV:LRR1.1As silenced wheat

The measurements of *Yr10* gene expression of Lrr part of *Yr10* gene silenced and BSMV:00 wheat plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs of the 15 dpi leaf samples have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample/primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA* (GenBank accession no: X16077.1). *Yr10* qRT-PCR analyses were performed by the Yr10 qRT-PCR Forward 5' TGATTTTCAATTTGGGCTGGA 3' and Yr10 qRT-PCR reverse 5' CGTCAGTGTGGGC TTGTTG 3' primers.

1.3 *Yr10* gene silencing *via* BSMV in wheat

To detect the silencing of Yr10 gene with the BSMV:LRR1.S, *Yr10* gene expression was measured after the BSMV:LRR1.S inoculation. Systemic RNA silencing of *Yr10* gene was performed.

1.4 *Yr10* gene silencing Lrr part construct preparation

Prior to silencing experiment Lrr part of *Yr10* gene has been cloned from wheat transcriptome. Then obtained sequence information and clone have been used for VIGS construct preparation. Using gene-specific primers harboring appropriate (*Pac*I and *Not*I restriction enzyme) cleavage restriction enzyme sites at their 5' ends VIGS construct has been engineered. 159 bp lengths Lrr part of *Yr10* gene specific amplification was performed.

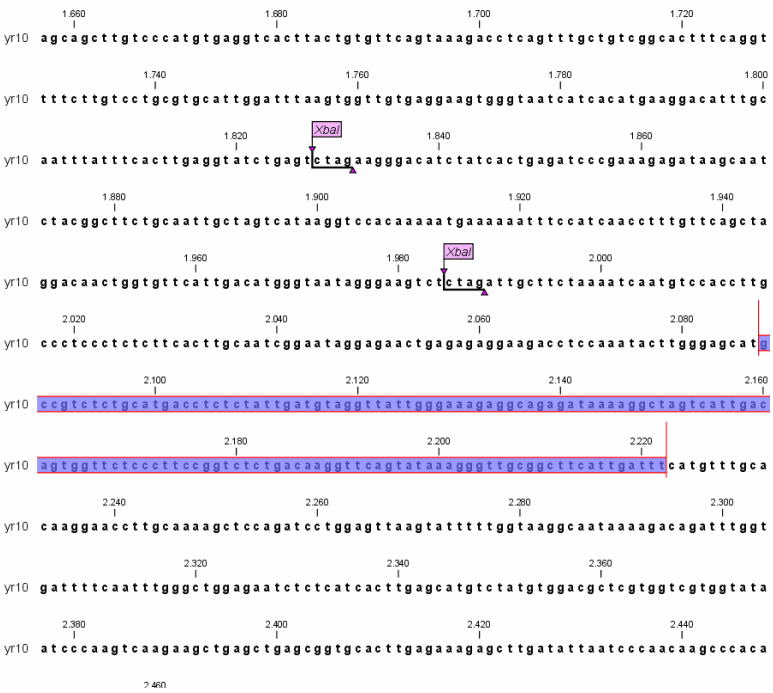


Figure 1 Lrr part of Yr10 gene for VIGS construct preparation.

Amplified fragment was ligated with silencing pγ.As and pγ.S vector then this ligation product was transformed into *E. coli* competent cells. Using colony PCR with specific primers, colonies with insert were selected. (Figure 2 **a**).

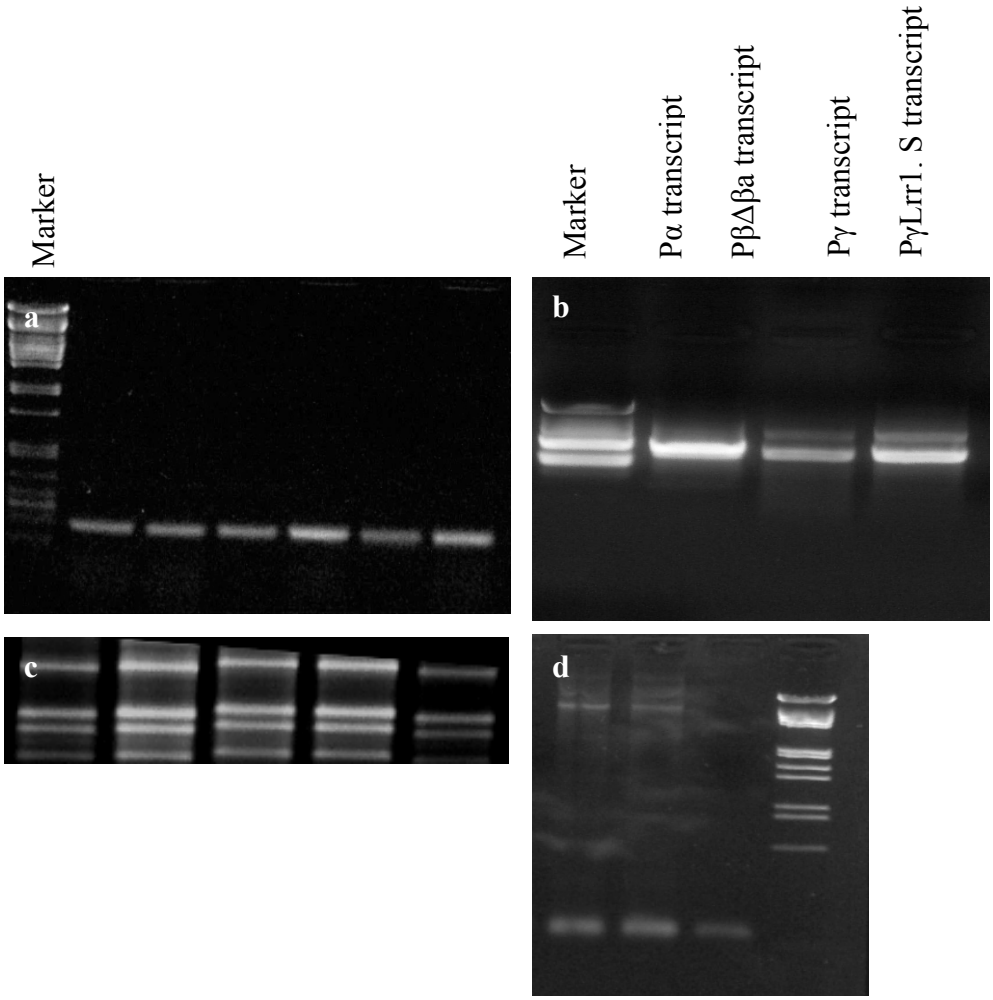


Figure 2 *Yr10* gene silencing of wheat. **a**: pγ sense and anti-sense vectorse were engineered with *NotI* and *PacI* then cloned (first three states sense and the other states anti-sense). **b**: BSMV:LRR1 Sense construct linearized plasmid version with linear pα, pβΔβa, pγ plasmids. **c**: RNA isolations from (BSMV:LRR1.S) Lrr silenced Avo-Yr10 (first three, 15 dpi), BSMV:00 (forth, 15 dpi) and FES control (last, 15 dpi) Rar1.1 145 bp **d**: *NotI* and *PacI* enginered fragment was amplified from Avo-Yr10 wheat plant cDNA Lrr part of Yr10.

1.5 qRT-PCR analyses for *Yr10* gene expression level determination in silenced wheat lines

Gene expression of *Yr10* in BSMV:LRR1.S silenced Avo-Yr10 and BSMV:00 inoculated Avo-Yr10 were compared by using qRT-PCR. It has been shown that *Yr10* gene silencing was efficiently performed (Figure).

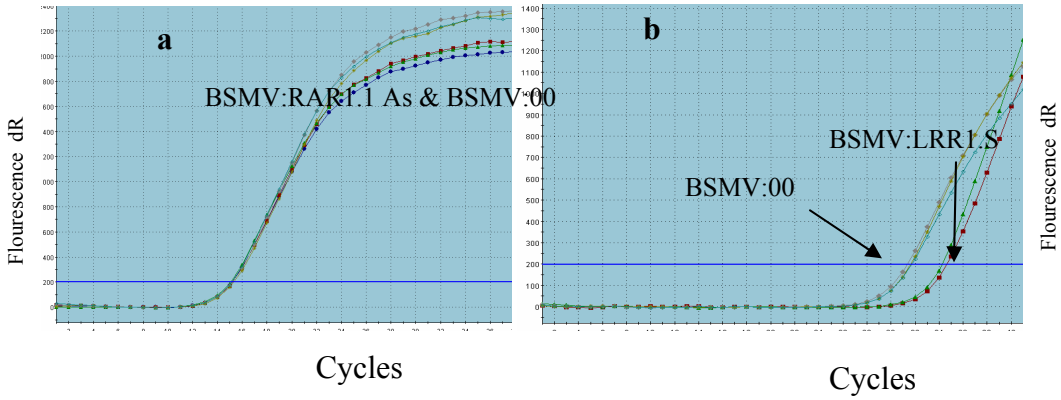


Figure 3 *Yr10* gene silencing **a**: BSMV:LRR1.S inoculated and BSMV:00 inoculated Avo-Yr10 wheat plants RNAs normalized by *18s rRNA* gene level equalization. **b** BSMV:LRR1.S silenced Avo-Yr10 wheat plant, *Yr10* gene expression level was decreased by approximately 6.85 fold relatively to BSMV:00 inoculated Avo-Yr10 wheat plants.

APPENDIX-E

RAN analysis of RNAs used for *Rad6* gene silencing determination for Figure 3.17

Overall Results for sample 2 : Sample 2

RNA Area:	644.3	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	1,421 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 2 : Sample 2

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.38	43.23	138.3	21.5
rRNA 1	46.10	50.12	98.1	15.2
28S	50.21	50.57	0.9	0.1

Overall Results for sample 3 : Sample 3

RNA Area:	147.8	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	326 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 3 : Sample 3

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.33	43.23	33.8	22.9

Overall Results for sample 4 : Sample 4

RNA Area:	125.8	RNA Integrity Number (RIN):	6.1 (B.02.03)
RNA Concentration:	277 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 4 : Sample 4

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.41	43.09	22.2	17.6

Overall Results for sample 5 : Sample 5

RNA Area:	130.4	RNA Integrity Number (RIN):	6.0 (B.02.03)
RNA Concentration:	288 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 5 : Sample 5

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.35	43.31	20.9	16.0
rRNA 1	45.29	48.14	17.7	13.6

Overall Results for sample 6 : Sample 6

RNA Area:	534.5	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	1,179 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 6 : Sample 6

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.22	43.06	120.2	22.5
28S	50.07	50.44	0.1	0.0

Overall Results for sample 7 : Sample 7

RNA Area:	408.6	RNA Integrity Number (RIN):	7.4 (B.02.03)
RNA Concentration:	901 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 7 : Sample 7

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.13	42.88	62.9	15.4
rRNA 1	45.35	47.83	52.6	12.9

Overall Results for sample 8 : Sample 8

RNA Area:	925.1	RNA Integrity Number (RIN):	6.1 (B.02.03)
RNA Concentration:	2,040 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.6	Result Flagging Label:	All Other Samples

Fragment table for sample 8 : Sample 8

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.92	42.96	288.9	31.2
28S	44.98	48.84	177.6	19.2

Overall Results for sample 9 : Sample 9

RNA Area:	288.4	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	636 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.9	Result Flagging Label:	All Other Samples

Fragment table for sample 9 : Sample 9

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.98	42.71	70.9	24.6
28S	45.89	47.85	61.7	21.4

Overall Results for sample 10 : Sample 10

RNA Area:	295.0	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	651 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 10 : Sample 10

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.00	42.79	66.8	22.7
28S	49.38	50.60	1.4	0.5

Overall Results for sample 11 : Sample 11

RNA Area:	1,217.5	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	2,685 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 11 : Sample 11

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.89	42.59	254.3	20.9
28S	48.50	48.88	0.5	0.0

Overall Results for sample 12 : Sample 12

RNA Area:	1,159.5	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	2,557 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	All Other Samples

Fragment table for sample 12 : Sample 12

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.83	42.54	230.2	19.8

RAN analysis of RNAs used for *Rad6* gene silencing determination for Figure 3.19

Overall Results for sample 1 :

RNA Area:	214,7	RNA Integrity Number (RIN):	6,3 (B.02.03)
RNA Concentration:	3.039 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,0	Result Flagging Label:	RIN: 6.30

Fragment table for sample 1 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,09	41,44	24,4	11,3
28S	45,72	48,47	24,3	11,3

Overall Results for sample 2 :

RNA Area:	234,4	RNA Integrity Number (RIN):	7,4 (B.02.03)
RNA Concentration:	3.317 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,2	Result Flagging Label:	RIN: 7.40

Fragment table for sample 2 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,08	41,38	25,0	10,7
28S	45,00	48,62	29,4	12,5

Overall Results for sample 3 :

RNA Area:	144,6	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	2.047 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,0	Result Flagging Label:	RIN N/A

Fragment table for sample 3 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41,48	43,89	21,1	14,6
28S	50,48	51,19	0,7	0,5

Overall Results for sample 5 :

RNA Area:	231,1	RNA Integrity Number (RIN):	6,7 (B.02.03)
RNA Concentration:	3.270 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,2	Result Flagging Label:	RIN: 6.70

Fragment table for sample 5 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39,85	41,31	26,0	11,3
28S	45,27	48,28	30,2	13,1

Overall Results for sample 6 :

RNA Area:	160,9	RNA Integrity Number (RIN):	8,0 (B.02.03)
RNA Concentration:	2.277 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,0	Result Flagging Label:	RIN:8

Fragment table for sample 6

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,93	42,73	34,3	21,3
28S	49,07	49,63	0,3	0,2

Overall Results for sample 7

RNA Area:	217,2	RNA Integrity Number (RIN):	7,1 (B.02.03)
RNA Concentration:	3.074 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,0	Result Flagging Label:	RIN: 7.10

Fragment table for sample 7 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41,02	43,13	27,6	12,7

Overall Results for sample 8 :

RNA Area:	499,6	RNA Integrity Number (RIN):	6,5 (B.02.03)
RNA Concentration:	7.071 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,1	Result Flagging Label:	RIN: 6.50

Fragment table for sample 8 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39,48	40,91	55,2	11,0
28S	44,05	47,62	61,3	12,3

Overall Results for sample 9 :

RNA Area:	261,0	RNA Integrity Number (RIN):	6,7 (B.02.03)
RNA Concentration:	3.694 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,0	Result Flagging Label:	RIN: 6.70

Fragment table for sample 9 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39,44	40,69	31,3	12,0
28S	45,10	47,27	31,3	12,0

Overall Results for sample 10 :

RNA Area:	192,8	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	2.729 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,9	Result Flagging Label:	RIN N/A

Fragment table for sample 10 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,55	42,90	23,6	12,3
28S	44,46	47,74	20,6	10,7

Overall Results for sample 11 :

RNA Area:	121,2	RNA Integrity Number (RIN):	4,0 (B.02.03)
RNA Concentration:	1.716 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,5	Result Flagging Label:	RIN:4

Fragment table for sample 11 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,54	41,83	5,0	4,1
28S	45,00	47,03	2,4	2,0

Overall Results for sample 12 :

RNA Area:	210,5	RNA Integrity Number (RIN):	N/A (B.02.03)
RNA Concentration:	2.979 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0,8	Result Flagging Label:	RIN N/A

Fragment table for sample 12 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,40	42,50	38,5	18,3
28S	44,75	46,80	32,3	15,3

APPENDIX-F

Sequences of the clones prepared for Y2H experiments in pDONR/Zeo

RAR1 (*Triticum aestivum*):

GGGGACAAGTTTGTACAAAAAAGCATGTCGGCGGAGACGGAGACGAGCGCCGCCGCGCCCGC
GCCCGCGCCCATGCGGTGCCAGCGAATAGGCTGCGACGCCATGTTCACCGACGACGA
CAACCCCGACGGCTCCTGCCACTACCACCCCTCCGGACCTATGTTTCATGATGGCAT
GAAAGAGTGGAGCTGTTGCAAGCAAAGAAGCCATGATTTTAGCTTATTTTTAGCTAT
TCCTGGATGTGCCACAGGGAAGCATACAACTGAGAAACCAGTCACAAAAGCTGTTTC
TCTTAACTCAAAGGCAACCCCACCAAAGTTAGCTCCAATCCAGTCTTCTAAGCAGGG
TGTGGAAACCGAGGCCTGCTCCAGGTGCCGTCAGGGTTTCTTTTGCTCCGACCATGG
ATCACAGCCCAAGGCACAAAAACCAGTTGCTGTAAATGGTACAAATACGGAACCTGT
CGAAAAATGCTCAGTTCCACAGCCCAGGAAAAAAGTTGTTAATGCTTTCTTGTACAAAGTG
GTCCCC

SGT1 (*Triticum aestivum*):

GGGGACAAGTTTGTACAAAAAAGCATGGCCGCCGCCGCCGCGTCGGATCTGGAGAGCAAGGC
CAAGGAGGCCTTCGTCGACGACGACTTCGAGCTGGCCGCCGAGCTCTACACCCAGGC
CATCGAGGCGGGGCCGGCTACCGCGGAACTCTACGCCGACCGAGCCCAGGCGCACAT
CAAGCTGGGCAGTTACACTGAGGCTGTAGCTGATGCCAACAAAGCAATTGAACTTGA
TCCTTCGATGCATAAAGCATACCTTCGGAAGGGCTCTGCTTGCATCAAGCTGGAGGA
ATACCAAACTGCAAAGGCTGCTCTTGAAGTGGGTTCTTCTTATGCATCTGGTGACTC
GAGGTTTACTCGTCTGATGAAGGAGTGTGATGATCGTATTGCTGAGGAGGCTAGCCA
GGTGCCAGTAAAGAATGCCGCTGCGGCTGTTGCTTCAGCTACATCTTCGGGGGCATC
TTCCGGGGCTACAACTGTGGCTACTGAAGCTGAGGACCAGGATGGCGCAAATATGGA
GAATGCACAGCCAACGATAGAAGTGCCAAGCAAGCCCAAATACAGGCATGACTACTA
CAATACTCCTACAGAAGTGGTACTGACTATATTTGCTAAGGGTGTTCCAGCTGACAG
CGTGGTTGTTGACTTTGGTGAACAGATGCTGAGTGTCTCAATTGAACTTCCCGGTGA
GGAACCATACCATTTTCAGCCTCGTCTGTTTTCAAAGATCGTCCCAGATAAGTGCAA
GTATACTGTGTTGTCTACAAAGGTTGAAATGCGCCTTGCAAAAGCTGAGCCAGTAAC
TTGGACATCATTGGATTATACTGGTAAACCAAAGGCTCCTCAGAAGATAAATGTACC
AGCTGAATCAGCCCAGAGGCCATCTTATCCATCATCAAAATCCAAAAAGGACTGGGA
TAAGCTTGAAGCTGAAGTGAAAAAACAGGAGAAGGATGAAAAACTTGATGGCGATGC
TGCATTGAACAAATTTTTCCGTGAAATTTACAGTGATGCTGATGAAGATATGCGTAG
AGCAATGATGAAGTCTTTTGTGGAGTCTAATGGAACCGTTCTCTCAACCAACTGGAA
GGATGTCGGGAAAAAGACGGTTGAAGGAAGCCCTCCTGATGGAATGGAGCTCAAGAA
GTGGGAGTATTAATACTCCCACTTGAGCTCGCTTTCTTGTACAAAGTGGTCCCC

YR10-1506 (*Triticum aestivum*):

GGGGACAAGTTTGTACAAAAAAGCATGGAGGTCGTGACCGGGGCGATGAGCACGCTCCTGCC
CTTGCTTGGCGACCTGCTTAAGGAGGAGTACAACCTGCAGAAGAGCACCAAGGGTGA
GATCAAGTTCCTCAAAGCAGAGCTGGAGAGCATGGAGGCTGCCCTCATCAAGATCTC
GGAGGCACCCTTGGATCAGCCACCTAACATTCAGGTCAAGCTCTGGGCGAGGGACGT
CAAGGACCTGTCCTATGAGATCGAAGATGGCATCGACAAATTCCGGGTGCACCTTGA
GTGCCGGCAACAAAAGAAGCCACACAGCTTTATGGGTTTCATCCACAAAAGCATGGA
CATGCTGACAAAGGGCAAGATCCGACACAAAATAGGCATCGATATCAAAGACATCAA
GAGCCGCATCAAGGAGGTCAGTGACAGGCGTGAAAGGTACAAGGTTGATAGCGTTGC
GCCCAAGCCCACCGGCACAAGTACTGATACACTTCGCCAGTTAGCCTTGTTCAAAAA
GGCGGAAGAGCTTATTGGCACCAAAGAAAAGAGCCTTGACATAGTCAAGATGCTGAC
GGAAGGAGATGAGGTCTTCAAGAAACATCTTAAGATGGTCTCTATTGTTGGCTTTGG
AGGCTTAGGGAAGACAACTCTTGCTAACGTGGTATATGAGAAGCTTCGCGGGGACTT
TGATTGTGCAGCTTTTGTCTCTGTGTCTCTTAATCCTGACATGAAGAAGCTTTTCAA
GTGTTTGCTCCATCAGCTTGACAAAGGCGAGTACAAGAACATCATGGACGAGTCAGC
GTGGAGCGAAACACAACTCATAAGTGAGATAAGAGATTTCCTTCGAGACAAGAGGTA
CTTCATTCTCATTGATGACATATGGGATAAATCTGTGTGGAATAATATTAGATGTGC
TCTGATTGAGAATGAATGTGGTAGTAGAGTAATCGCAACAACTCGCATTCTAGATGT
TGCCAAAGAAGTTGGTGGTGTTTATCAGCTTAAACCTCTTTCTACTAGTGACTCAAG
ACAATTATTCTATCAAAGAATATTTGGAATTGGCGACAAGCGTCCTCCTATTCAGTT
GGCTGAAGTAAGTGAGAAAATTTTAGGGAAATGTGGCGGAGTACCATTAGCTATCAT
TACACTGGCTAGTATGTTGGCTGGTAAAAAGGAACATGAAAATACATATACTTATTG
GTACAAGGTGTACCAATCTATGGGTTCTGGGCTAGAAAATAATCCTGGCCTGATGGA
CATGAGGAGGATACTACATGTCAGTTACTATGACCTACCTCCAAATCTGAAGACTTG
TTTACTGTATCTCAGTTTGTATCCAGAGGATTATAATATTGAAACCAAAGAGTTGAT
ATGGAAATGGATAGGCGAAGGATTCATTCATGAAGAGCAAGGGAAGAGCTTGTATGA
AGTAGGAGAGGATTACATTGCTGAGCTCATTAGCAAAAGCTTGGTCCAACCAATGTA
TATCAATATTGCTAATAAGGCGAGCTCTGTCCGTGTACACGACGCTTTCTTGTACAAAGTG
GTCCCC

YR10-1562 (*Triticum aestivum*):

GGGGACAAGTTTGTACAAAAAAGCGGTAGTAGAGTAATCGCAACAACTCGCATTCTAGATGT
TGCCAAAGAAGTTGGTGGTGTTTATCAGCTTAAACCTCTTTCTACTAGTGACTCAGG
ACAATTATTCTATCAAAGAATATTTGGAATTGGCGACAAGCGTCCTCCTATTCAGTT
GGCTGAAGTAAGTGAGAAAATTTTAGGGAAATGTGGCGGAGTACCATTAGCTATCAT
TACACTGGCTAGTATGTTGGCTGGTAAAAAGGAACATGAAAATACATATACTTATTG
GTACAAGGTGTACCAATCTATGGGTTCTGGGCTAGAAAATAATCCTGGCCTGATGGA
CATGAGGAGGATACTACATGTCAGTTACTATGACCTACCTCCAAATCTGAAGACTTG
TTTACTGTATCTCAGTTTGTATCCAGAGGATTATAATATTGAAACCAAAGAGTTGAT
ATGGAAATGGATAGGCGAAGGATTCATTCATGAAGAGCAAGGGAAGAGCTTGTATGA
AGTAGGAGAGGATTACATTGCTGAGCTCATTAACAAAAGCTTGGTCCAACCAATGTA
TATCAATATTGCTAATAAGGCGAGCTCTGTCCGTGTACACGACATGGTGCTTGACCT
TATCCCTTCCTTGTCAAATGAGGAGAACTTTCTCGCAACATTGGGTGGTCAGCAGAC
CAGGTCACTGCCAAGTAAGATCCGTCGACTGTCTCTCCAAAGCAGTGATGAAGAGGA
TGTCCAGCCAATGCCAACCATGAGCAGCTTGTCCCATGTGAGGTCACTTACTGTGTT
CAGTAAAGACCTCAGTTTGCTGTCGGCACTTTCAGGTTTTCTTGTCCTGCGTGCATT

GGATTTAAGTGGTTGTGAGGAAGTGGGTAATCATCACATGAAGGACATTTGCAATTT
ATTTCACTTGAGGTATCTGAGTCTAGAAGGGACATCTATCCCTGAGATCCCGAAAGA
GATAAGCAATCTACGGCTTCTGCAATTGCTAGTCATAAGGTCCACAAAAATGAAAAA
ATTTCCATCAACCTTTGTTCAGCTAGGACAACTGGTGTTCATTGACATGGGTAATAG
GGAAGTCTCTAGATTGCTTCTAAAATCAATGTCCACCTTGCCCTCCCTCTCTTCACT
TGCAATCGGAATAGGAGAACTGAGAGAGGAAGACCTCCAAATACTTGGGAGCATGCC
GTCTCTGCATGACCTCTCTATTGATGTAGGTTATTGGGAAAGAGGCAGAGATAAAAG
GCTAGTCATTGACAGTGGTTCTCCCTTCCGGTCTCTGACAAGGTTCAGTATAAAGGG
TTGCGGCTTCATTGATTTCATGTTTGCACAAGGAACCTTGCAAAAGCTCCAGATCCT
GGAGTTAAGTATTTTTGGTAAGGCAATAAAAGACAGATTTGGTGATTTTCAATTTGG
GCTGGAGAATCTCTCATCACTTGAGCATGTCTATGTGGACGCTCGTGGTCGTGGTAT
AATCCCAAGTCAAGAAGCTGAGCTGAGCGGTGCACTTGAGAAAGAGCTTGATATTAA
TCCCAACAAGCCCACACTGACGGTGAAGGTAACTCCACGCTGAGCTTTCTTGTACAAAGTG
GTCCCC

RAD6 (*Triticum aestivum*):

GGGGACAAGTTTGTACAAAAAAGCATGTGATATGATACAACATTACAAACTCACACAAGACG
TTACAAAATGTTTGCGTTCCAGATACAGACAGTGTAGACACAGGCCATGACAGAGCA
ATCTCCAACCGTCCAGCCAAACGATGCAACAAAGCAAGTTGCTGTTTCAGGTGGTAA
CGACAGAGTTCAGTCATTTTAGTTACAAACATTTTGGTACAGAAACCGCATGATAAC
ACATGGTGAGCTCAACTTATTAGTCTGCCGTCCAGCTCTGCTCAACAATCTCCCGCA
CTTTGCGGTTGTACTCTCGCTTGTTCTCACTGAACATGCGGGCAGCTTCTGAGTTAG
CAGGCGAATTTGGGTTAGGATCACACAGCAGCGACTGGATAGATGTAAGTATAGCAG
CTACATCATATATTGGGCTCCACTGATTCTGTAGAATATCTAAGCATATGCTTCCAT
CAGCATAAATGTTAGGGTGAAACATCCGAGAAATAAACCGAACTGTTGGTGGCTTAT
TAGGATATTCTTCATTAAACTGGAGAGTCAGCTTAAGCTTTCTTGTACAAAGTGGTCCCC

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PUBLICATIONS

A Published:

1-Khawar Khalid, **Turgay Unver,** Ozcan S., *In vitro* Induction of Crown Galls by *Agrobacterium tumefaciens* Super Virulent Strain A281 (pTiBo 542) in *Salvia sclarae and S. pratensis. Biotechnol & Biotechnol Eq.17/2003/2 (peer-reviewed)*

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3-Osman Bozkurt, **Turgay Unver**, Mahinur S. Akkaya Genes differentially expressed in wheat associated with resistance to yellow rust disease caused by *Puccinia striiformis* f.sp. *tritici Vol 71 issue 4-6 page 251-259, 2007* ***Physiological and molecular plant pathology*** (doi:10.1016/j.pmpp.2008.03.002) (sci)

4-**Turgay Unver** Mahinur S. Akkaya Identification of differentially expressed genes of a boron tolerant plant, *Gypsophila perfoliata* L., under toxic boron levels *Vol 27 issue 8 page 1411-1422 2008* ***Plant Cell*** *Rep (*doi:10.1007/s00299-008-0560-7) *(sci)*

B.1 Submitted Papers:

5-Osman Bozkurt, **Turgay Unver**, Lesley Boyd, Yasin Dagdas, Mahinur Akkaya, Genome wide transcription and miRNA expression analysis of wheat upon compatible and incompatible stripe rust infections (Molecular Plant Microbe Interaction)(sci)

B.2 MS ready for submittion

6- Mahinur S. Akkaya, **Turgay Unver,** Osman Bozkurt, Semra Hasancebi, Nese Ozgazi RAD6, a Negative Regulator of Plant Cell Death and Defense, Encodes E2 Ubiquitin conjugating Enzyme (Plant Physiology)(sci)

C Proceedings and Abstracts:

1-Osman Bozkurt, **Turgay Unver**, Mahinur S.Akkaya Buğdayda Sarıpas Hastalığına Karşı Dirençlilik Mekanizmalarında Rol Alan Genlerin ve Gen Ürünlerinin işlevsel Genomik Yaklaşımlarla Saptanması. 14. Biyoteknoloji Kongresi 31 Ağustos - 2 Eylül Eskişehir 2005-national

2-Mahinur S. AKKAYA, Figen Yildirim, Osman Bozkurt, Mine Turktas, Yasemin Aktas, Mehmet Somel, **Turgay Unver**, Adnan El-Asbhi "Bitklerde Hastalik Direnclilik e cinko alimi mekanizmalarinda rol alan genlerin bulunmasina yonelik stratejiler, XIII. Biyoteknoloji Kongresi, 25-29 Agustos 2003, Canakkkale, Turkiye.- national

3-Mahinur S. Akkaya, Xianming Chen, Osman Tolga Bozkurt, Figen Yildirim, **Turgay Unver**, Mehmet Somel "Isolation of RGAs and disease related gene fragments from wheat stripe rust resistant differential lines" 11th Internatioal Cereal Rusts and Powdery Mildews Conference, 22-27 August, 2004, Norwich, UK, Poster A2.1-**international**

4-Mahinur S.Akkaya, Osman Bozkurt, **Turgay Unver**, Figen Yildirim "Determination of differentially expressed wheat genes upon infection with avirulent races of Puccinia striiformis" 3rd Plant Genomics European Meetings, 22-25 September, 2004, Lyon, France, P 173.- **international**

5-Mahinur S.Akkaya, Osman Bozkurt, **Turgay Unver**, Figen Yıldırım, Senay Vural Korkut, Banu Avcıoğlu Dundar, Adnan Al asbahiBitkilerde biyotik ve abiyotik Faktorler sonucu uyarılan genlerin saptanması 14. Biyoteknoloji Kongresi 31 Ağustos - 2 Eylül Eskişehir-national.

6-Semra Hasancebi, **Turgay Unver**, Seda Yılmaz, İbrahim Cakır and Mahinur S. Akkaya Farklı Bacillus thuringiensis suşlarında Cry genlerinin saptanması 14. Biyoteknoloji Kongresi 31 Ağustos - 2 Eylül Eskişehir.-national

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8-Osman Bozkurt, **Turgay Unver**, Mahinur S. Akkaya, "Q-PCR determination of the genes induced by yellow rust infection in resistant wheat lines." ." Plant Genomics European Meetings Amsterdam, Nederlands P8-007 page 189.- **international**

9-Bülent Uzun, Mahinur S. Akkaya, **Turgay Unver**, M. Ilhan Çagirgan Development of SCAR markers linked to closed capsule mutant trait in sesame (sesamum indicum l.) FESBP XV 17-21 july 2006, Lyon, France.- **international**

10- **Turgay Unver**, Osman Bozkurt, Mahinur S. Akkaya Virus İndüklemesiyle Gen Susturma, VİGS, Yöntemi Kullanılarak Buğdayda (*Triticum aestivum*) Gen Susturma. XV Biotechnology congress, Antalya, Turkey, 2007-national

11- Mahinur S. Akkaya **Turgay Unver** Virus induced gene silencing of wheat Rad6: 26S proteosome ubiquitinylation system gene with a putative role in plant disease resistance pGEM-6 Meeting, 2007, Spain- **international**

12-Aslıhan Günel, Neşe Özgazi, **Turgay Unver** Mahinur S. Akkaya The identification of differentially expressed proteins of wheat Rad6 gene silenced plants using 2D-PAGE and Mass Spectroscopic Analysis XV Biotechnology congress, Antalya, Turkey, 2007.-national

13-Mahinur S. Akkaya, Yasin Dağdaş, Erhan Astarcı, Turan Demircan, Gülay Gök, Asude Çalla, **Turgay Unver**, Burak Demiralay Identification of miRNAs putatively involved in responding to the abiotic and biotic stresses in wheat, barley, and in spores of their disease agents XV Biotechnology congress, Antalya, Turkey, 2007.-national

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15-Mahinur Akkaya, Osman Bozkurt, **Turgay Unver**, Senay Korkut, İlay Celik Strategies Towards Understaanding of Yellow Rust Disease resistance in wheat,Proteomics protein structure and function workshop, 20-23 september Antalya, Turkey 2006-national

16- **Turgay Unver** Osman Bozkurt Lesley Boyd Mahinur S. Akkaya Genome wide expression analysis of the compatible and the incompatible yellow rust pathogen inoculated wheat line. pGEM-6 Meeting, 2007, Spain- **international**

AWARDS

1. TUBITAK(The scientific and technologic council of Turkey)scholarship for foreign country research program, **2007**
2. OYP(Faculty Development Program) Research assistantship, **2002-2008**
3. Best poster award XIV. Biotechnology congress, Eskisehir, Turkey **2005**
4. Article publication award (both METU and TUBITAK The scientific and technologic council of Turkey, **2007** and **2008**

MEMBERSHIPS

Biotechnology association 2003 – present