BIO-FUNCTIONAL ANALYSIS OF *RAD23* GENE USING VIRUS INDUCED GENE SILENCING METHOD IN RESPONSE TO POWDERY MILDEW ATTACK IN BARLEY

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ABSTRACT

BIO-FUNCTIONAL ANALYSIS OF *RAD23* GENE USING VIRUS INDUCED GENE SILENCING METHOD IN RESPONSE TO POWDERY MILDEW ATTACK IN BARLEY

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Blumeria graminis f.sp. *hordei* is an obligate biotrophic parasite causing powdery mildew disease in several cereals, including barley. Virus induced gene silencing (VIGS) is a widely used powerful tool in plant functional-genomic studies to determine the functions of genes in disease resistance.

In this thesis study, the function of *RAD23* gene in barley in plant resistance was investigated. The RAD23 is a ubiquitin (Ub) binding protein involved in programmed protein degradation by transferring of ubiquitylated substrates to the proteasome. It was identified as an induced protein responding to compatible powdery mildew-barley interaction in a previous proteomics study performed in our laboratory. Pallas-01 and Pallas-03 barley line seedlings were inoculated with *Blumeria graminis* f.sp. *hordei* race (*Bgh*103). Curiously, RAD23 was

observed in compatible interaction (Pallas-03/*Bgh*103) with 4 fold induction at 12 dpi, while in incompatible interaction RAD23 was absent.

Thus, it is thought that *RAD23* may play a role in disease resistance as a negative regulator. In order to confirm this hypothesis, BSMV mediated virus induced gene silencing (VIGS) was performed. To determine the effects of the silencing of *HvRAD23* gene in disease resistance mechanism, BSMV:*HvRAD23* treated (silenced group) and BSMV:00 treated (control group) Pallas-03 seedlings were inoculated with a virulent (*Bgh*103) and avirulent (*Bgh*95) pathogen races. The pathogen growth levels at 3 and 5-days post inoculation were analyzed under light microscope and then, hyphae lengths were measured to detect the difference in pathogen development between silenced and control groups. As a result, 21 % difference in primary hyphae lengths, 22 % difference in secondary hyphae lengths and 16 % difference in the longest hyphae lengths between two groups were observed. Then, quantitative real time PCR was performed to determine the *HvRAD23* gene silencing level. The *HvRAD23* silencing was only seen in 3 of the 6 samples. The average silencing of the samples is 17 %. Despite of low silencing level, microscopic analyses indicated significant difference in pathogen growth between silenced and control groups. Therefore, the results of the initial studies support that the *RAD23* is a negative regulator of plant disease resistance.

Keywords: Barley (*Hordeum vulgare*), powdery mildew (*Blumeria graminis* f. sp. *hordei*), 'Radiation-23' (*RAD23*), 'Virus induced gene silencing' (VIGS), 'Barley stripe mosaic virus' (BSMV), qRT-PCR.

ÖZ

ARPADA KÜLLENME HASTALIĞINA KARŞI VİRÜS İNDÜKLEYİCİ GEN SUSTURMA YÖNTEMİ İLE *RAD23* GENİNİN BİYO-FONKSİYONEL ANALİZİ

Aksoy, Yağmur

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Blumeria graminis f.sp. *hordei*, arpa gibi çeşitli tahıllarda külleme hastalığına neden olan bir çeşit zorunlu parazittir. Virüs İndüklenen Gen Susturma yöntemi (VIGS), bitki fonksiyonel-genomik çalışmalarında, genlerin hastalık direncine karşı fonksiyonlarını belirlemede yaygın olarak kullanılan güçlü bir araçtır.

Bu tez çalışmasında, arpadaki *RAD23* geninin bitki direnç mekanizmasındaki rolü araştırılmıştır. RAD23, ubikuitin proteinleri proteozoma taşıyarak programlanmış protein degredasyonuna yol açan bir ubikuitin bağlayıcı proteindir. Laboratuvarımızda gerçekleştirilen bir proteomiks çalışmasında RAD23, küllenme hastalığı ile uyumlu etkileşime karşılık olarak indüklenmiş bir protein olarak tanımlanmıştır. Pallas-01 ve Pallas-03, *Blumeria graminis* f.sp. *hordei* ile

inoküle edildikten sonra ilginç bir şekilde, uyumlu olmayan etkileşimde (Pallas-03/*Bgh*95) RAD23 proteini bulunmazken, uyumlu etkileşimde (Pallas-03/*Bgh*103) inokülasyondan 12 saat sonra 4 kat arttığı gözlenmiştir. Bundan dolayı, *RAD23* geninin hastalık direncinde negatif düzenleyici olarak rol oynadığı düşünülmektedir. Bu hipotezi doğrulamak için BSMV aracılı VIGS metodu uygulanmıştır. *HvRAD23* geninin susturulmasının hastalık direnç mekanizmasındaki etkilerini saptamak için BSMV:*HvRAD23* (susturulmuş grup) ve BSMV:00 (control grup) ile muamele edilmiş Pallas-03 fideleri, *Bgh*103 (virulan) ve *Bgh*95 (avirulan) patojen ırkları ile inoküle edilmiştir. 3. ve 5. günlerdeki patojen büyüme seviyeleri ışık mikroskobu aracılığıyla analiz edilmiştir. Uyumlu etkileşimde (Pallas-03/*Bgh*103) susturulmuş ve control grupları arasındaki patojen büyüme farkının belirlenmesi için hif uzunlukları ölçülmüştür. Sonuç itibariyle iki grup arasında, birincil hif uzunluklarında % 21, ikincil hif uzunluklarında % 22 ve en uzun hif boylarında % 16 fark gözlenmiştir. Daha sonra da, *HvRAD23* geninin susturulma seviyesini saptamak için qRT-PCR uygulanmıştır. *HvRAD23* gen susturulması 6 örnekten sadece 3 tanesinde gözlenmiştir ve ortalama susturma oranı % 17'dir. Ancak, düşük susturulma oranına rağmen, mikroskobik analizler, iki grup arasında patojen büyüme seviyesinde belirgin bir fark olduğunu göstermektedir ve başlangıçtaki bu çalışmalar *HvRAD23* geninin bitki hastalık direncinde negatif düzenleyici olarak rol oynadığını desteklemektedir.

Anahtar kelimeler: Arpa (*Hordeum vulgare*), küllenme hastalığı (*Blumeria graminis* f. sp. *hordei*), 'Radyasyon-23' (*RAD23*), 'Virüs indüklenen gen susturma' (VIGS), 'Arpa şerit mozaik virüsü' (BSMV), qRT-PCR.

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LIST OF ABBREVIATIONS

Avr:	Avirulence gene
Bgh:	*Blumeria graminis* f.sp. *hordei*
BSMV:	Barley Stripe Mosaic Virus
CC:	Coiled-coil
cDNA :	Complementary DNA
cds:	Complete coding sequence
CP:	Coat-protein
Cp:	Crossing point
Ct:	Treshold cycle
dpi:	Days post inoculation
DEPC:	Diethyl pyrocarbonate
dsRNA:	Double-stranded RNA
EF:	Elongation Factor
ETI	Effector-triggered immunity
FAO:	Food and Agriculture Organization
GAPDH:	Glyceraldehyde 3-Phosphate Dehydrogenase
hpi:	Hour post inoculation
HR:	Hypersensitive Response

LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
MAMP:	Microbial Associated Molecular Patterns
miRNA:	Micro RNA
Mlo:	Powdery mildew resistance gene / Mildew Locus O
mRNA:	Messenger RNA
NBS:	Nucleotide binding site
NB-LRR:	Nucleotide-binding and leucine-rich repeat
NER:	Nucleotide excision repair
PAMP:	Pathogen Associated Molecular Patterns
PDS:	Phytoene desaturase
bPDS4:	Barley phytoene desaturase 4
PR:	Pathogenesis-related proteins
PRR:	Pattern-Recognition Receptor
PTGS:	Post-transcriptional Gene Silencing
PTI:	PAMP-triggered immunity
PVX:	Potato virus X
qRT-PCR:	Quantitative Real-Time Polymerase Chain Reaction
RAD23:	Radiation-23
RdRP:	RNA dependent RNA Polymerase
R-gene:	Resistance gene

RISC:	RNA Induced Silencing Complex
RNAi:	RNA interference
ROI:	Reactive oxygen intermediates
siRNA:	Small interfering RNA
ssRNA:	Single-strand RNA
TGS:	Transcriptional gene silencing
TLR:	Toll-like receptors
TMV	Tobacco mosaic virus
U:	Unit
Ubi:	Ubiquitin
VIGS:	Virus Induced Gene Silencing

CHAPTER 1

INTRODUCTION

1.1 Powdery mildew disease of barley

1.1.1 Barley

Barley (*Hordeum vulgare* ssp. *vulgare*), diploid with 14 chromosomes, is one of the most important members of the grass family. It has various usage areas such as human food, animal feed and malting industry. It is also used to produce starch, utilized for food and the chemical industry (OECD, 2004). In addition to these, barley is used as a model crop for the various studies such as plant breeding methodology, cytogenetics, genetics, virology, pathology and biotechnology (Hockett and Nilan 1985).

The production rate of barley is the forth in the world after wheat, rice and maize (Zeybek et al., 2008). In the world, Turkey is the 9^{th} country in production of barley according to Food and Agriculture Organization (FAO, 2010) (Figure 1.1).

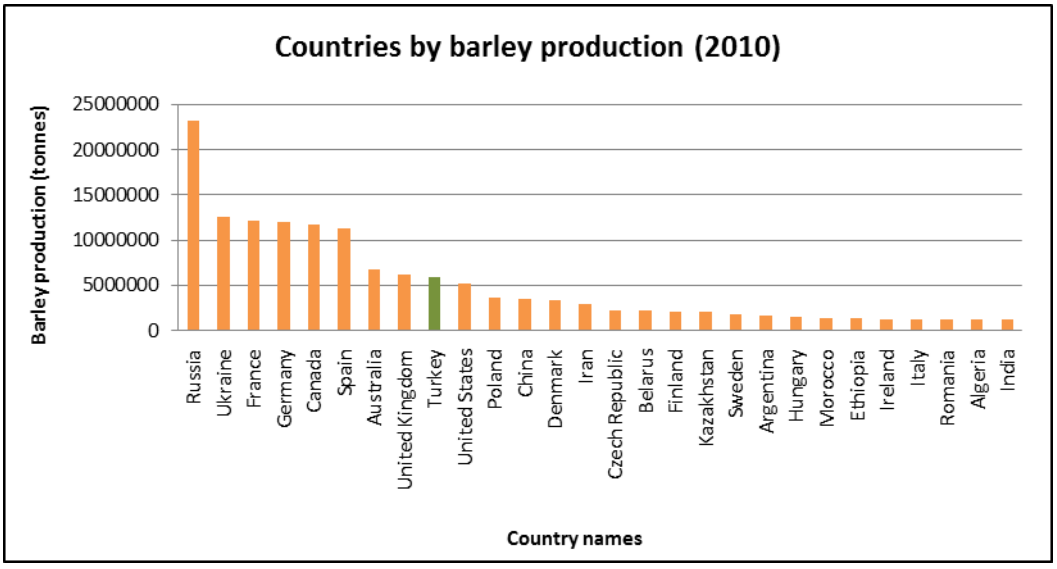

Figure 1.1 The countries by barley production in 2010 according to FAO (Production by country >1.000.000 tonnes).

1.1.2 Powdery mildew disease

Powdery mildew is the serious fungal disease, induced by *Blumeria graminis* (Dean, 2012). This disease influences a wide range of plants including vegetables, weeds, fruit trees, forest trees, ornamentals, shrubs, cereals and grasses.

Powdery mildew disease can be easily identified. The most characteristic symptom of it is white spots, occurring on a leaf surface. In addition to the leaf surface, this disease also infects other side of the leaf, bud, flower, young stem and young fruit. Then, the spots form the whitish-powder like structures. These tiny, soft, and airy structures, which are initially white and later turn to yellow-brown, may appear singly or in a group. After powdery mildew infection, the colour of the leaf may turn to yellow with green patches as in Figure 1.2 and the leaf may become deformed and fall prematurely. The degree of the disease rests on many factors such as the host plant variety, age of the host and weather during the growing season (Edmunds and Pottorff, 2012).

Figure 1.2 Powdery mildew disease in *Hordeum vulgare* ssp. *vulgare*. (http://www.shouragroup.com/f_BARLEY_e.htm)

1.1.3 *Blumeria graminis* f.sp. *hordei*

Blumeria graminis is a fungus that belongs to the biggest division of fungi, named ascomycota. It has several subspecies and each has a specific host such as, *B. graminis* f.sp. *hordei* infecting barley, *B. graminis* f.sp. *tritici* infecting wheat and *B. graminis* f. sp *secalis* infecting rye (Inuma et al., 2007; Wyand and Brown, 2003). This pathogen is an economically important problem since it causes the yield and quality losses up to 20 % (Czembor, 2002). This pathogen is an obligate biotrophic parasite; therefore, it can not survive without its host (Nowara et al., 2010). The pathogen does not need the water presence in order to infect the host since wet leaf surfaces inhibit the its development. However, it needs cool and humid environments for spore germination (Sooväli and Bender, 2006). Thus, the disease is widespread in crowded plantings where air circulation is not sufficient.

Blumeria graminis f.sp. *hordei* produces cylindrical-shaped conidia in chains that are spread by blow of wind. These conidia fall on a barley leaf surface to infect

(Carver at al., 1996). If a host has *Mlo* (*Mildew Locus O*) gene, the pathogen can invade most efficiently (Büschges et al., 1997). *Mlo* gene is mutation-induced recessive alleles and produces plant specific integral membrane protein, whose biochemical role is still unknown (Devoto et al., 1999). *Mlo* has a negative regulator function on cell death; thus, mutations at this locus, causing loss of function, result in resistance to powdery mildew disease *via* the cell-death response mechanism and the accumulation of a callose rich barrier at infection site (Wolter et al., 1993).

The asexual life cycle of *Blumeria graminis* proceeds in a highly synchronous way (Both et al., 2005). When wind-dispersed conidia of the pathogen reach to a leaf surface, the process of infection begins. After infection, an extracellular matrix is instantly produced. The function of the extracellular matrix is thought that it helps fungus to bind the leaf surface and to get signal cues of surface (Carver et al., 1999; Wright et al., 2002). An hour post inoculation (hpi), a short primary germ tube occurs from a conidium. It provides the perception of the surface nature where the conidia proliferate (Kinane et al., 2000; Nielsen et al., 2000). After a short time, appressorium germ tube, also called secondary germ tube, appears. Then, appressorium germ tube forms the appressorium, obviously noticeable at 8 hpi. This swollen-hooked structure adheres to the surface. At 15 hpi, a penetration peg occurs from appressorium. This hyphal strand destroys the epidermal cell wall by turgor pressure and enzymatic activity (Francis et al., 1996; Pryce-Jones et al., 1999). The penetration peg does not invade the plasmalemma; therefore, a haustorium, forming from penetration peg, develops in between the inner membrane and the cell wall, called periplasmatic space. The function of the haustorium is to feed *Blumeria graminis* by providing nutrients from epidermis cells of the host plant. It allows the pathogen to pullulate rapidly on the leaf surface. The pathogen colonies can be observed by naked eye at approximately 3 days post inoculation (dpi). Then, the colonies start to generate numerous conidiospores. At about 5 dpi, conidiospores produce conidia, asexual and non-motile spores. These can spread by wind to infect the other host plants

(Both et al., 2005). The whole life cycle of the pathogen development is briefly described in Figure 1.4.

Figure 1.3 refers to a conidium having primary germ tube, appressorium and penetration peg. This image was taken 24 hpi and visualization was achieved by the tryphan blue staining under light microscopy.

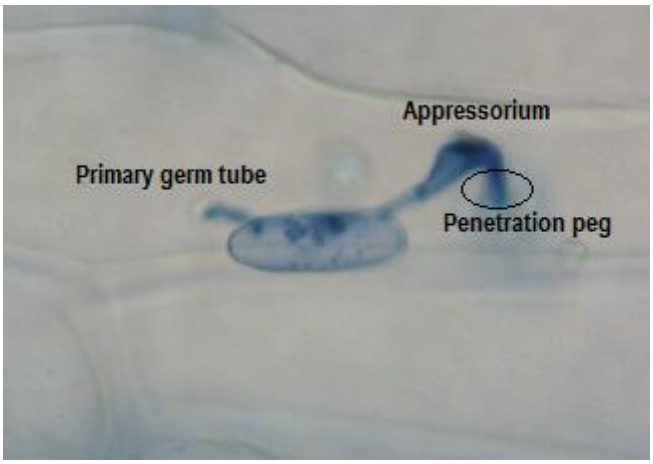

Figure 1.3 A conidium at 24 hpi, consisting of primary germ tube, appressorium and penetration peg (taken by Yağmur Aksoy).

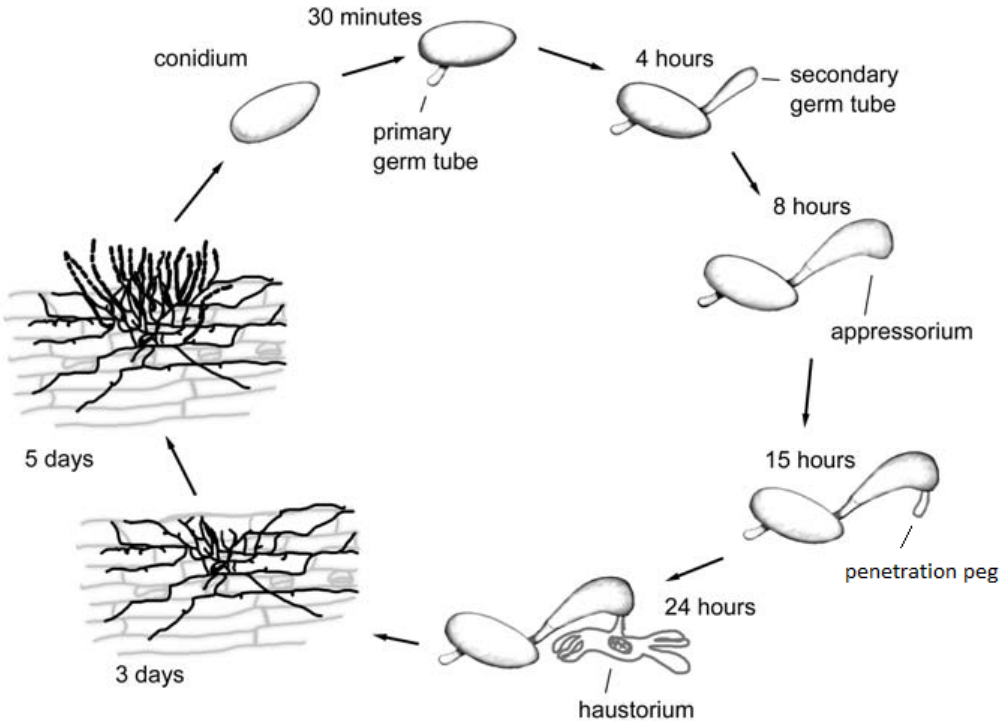

Figure 1.4 Asexual life cycle of *Blumeria graminis* f.sp. *hordei* (Both et al., 2005).

1.2 Plant defense mechanisms

Plants mean rich-nutrient source for various organisms such as bacteria, fungi, insects and protists. The defense mechanism of plants are different from that of mammals in terms of having no immune cells, capable of mobile to infection site and lack of circulatory system to discern the pathogens (Spoel and Dong, 2012). However, in order to prevent pathogen invasions, they have two effective mechanisms that are basal defense, also called innate immunity, and resistance gene (R-gene) mediated defense. Besides of these differences, the molecular level of defense mechanisms of plants and mammals has several similarities. The studies have posed that the components of signal transduction and receptors, playing a role in plant defense mechanism, have considerable similarities with the mammalian innate immunity pathway (Cohn et al., 2001).

The previous studies revealed that there are two branches of the plant immune system as mentioned above. One of them, called basal resistance, uses trans-membrane pattern recognition receptors (PRRs). This reciprocates microbial / pathogen associated molecular patterns (MAMPs/PAMPs) (Zipfel and Felix, 2005). The second one, gene-for-gene resistance, moves to exceedingly inside the plant cell. It uses the products of the polymorphic nucleotide-binding and leucine-rich repeat (NB-LRR) proteins. The NB-LRR protein is encoded by R-genes (Dangl and Jones, 2001). NB-LLR disease mechanism is efficient against pathogens that are obligate-biotrophs, growing on living tissues, and hemi-biotrophs (Glazebrook, 2005).

1.2.1 Basal resistance

Several factors affects the plants in nature; however, plants can handle most of them *via* their basal defense or non-host resistance mechanism (Hückelhoven, 2005). In pathogens especially rust and mildew species, one common property is the lack of haustorium formation on non-host plants, which is namely pre-haustorial or penetration resistance (Niks, 1987; Heath, 2002; Collins et al., 2007; Hardham et al., 2007). This type of defense results in the formation of cell wall strengthening, termed cell wall appositions or papillae (O'Connell and Panstruga, 2006; Hückelhoven, 2007). Generally, pre-haustorial non-host resistance is supported by hypersensitive post penetration resistance (Lipka et al., 2005). In non-host plants, the period of cell death is not essential as in host species; thus, the result of gene-for-gene interaction is also not essential (Christopher-Kozjan and Heath, 2003). Recent studies have represented that non-host resistance shows strong similarities and relation with the basal defense to adapted pathogens in plants (Niks and Marcel, 2009).

Basal defense is generally activated by molecules, called microbial/pathogen associated molecular patterns (MAMPs/PAMPs). PAMPs are small molecular

motifs and highly conserved within a class of pathogens, recognized by the innate immune system cells (Niks and Marcel, 2009).

The process of resistant plant-pathogen interaction consists of several stages. In early stage, basal resistance is induced when plant identifies the highly conserved molecules (PAMPs) of the pathogen. This defense mechanism is termed as 'PAMP-triggered immunity' (PTI). There are several types of MAMPs. Bacterial lipopolysaccharide (LPS), bacterial flagellin, nucleic acid variants generally involved in viruses (dsRNA), fungal chitin, Pep-13 can be given as examples for MAMPs distinguished by plants. In spite of obtaining weak immunity, pattern recognition receptors (PRRs) prevent spread of pathogen (Dangl and Jones, 2006; Bent et al., 2007). In second stage, so as to handle basal resistance, pathogen induces some host-specific modifications. Therefore, basal resistance is not enough to obstruct pathogen attacks in later stages of infection (Dangl and Jones, 2006; Bent et al., 2007).

1.2.2 Gene-for-gene resistance mechanism

Most pathogens are tremendously specialized and result in disease on a narrow host range. These pathogens are able to overcome the preformed defense mechanisms. Thus, plants must inhibit the disease improvement by specially identifying the invaders and ceasing their growth. This specific defense mechanism is called gene-for-gene resistance (Holt et al., 2000). This kind of resistance is accomplished *via* the interaction of a receptor derived from plant and a pathogen molecule, corresponding with the receptor. This pathogen molecule is termed as 'elicitor'.

In the beginning, elicitor term was used for molecules that are able to stimulate the products of phytoalexin, however, it is now frequently used for compounds that can induce all types of plant defense mechanisms (Ebel and Cosio, 1994; Hahn, 1996; Nüernberger, 1999). There is not any general chemical structure for

elicitors, since they compose of different types of compounds such as glycoproteins, peptides, lipids, oligosaccharides and proteins (Angelova et al., 2006). Elicitors can be categorized as general elicitors and race-specific elicitors. General elicitors are capable of triggering host and non-host defense mechanisms while race-mechanism resulting in resistance only specific host cultivars. 'Race specific elicitors' are also called 'effectors' having the same function with elicitors but consisting of only protein molecules.

Effectors that are produced by avirulence (*Avr*) gene, coming from a particular race of a pathogen, lead to resistance only in a host plant having the corresponding R-gene. In the absence of either R-gene or *Avr* gene, the disease occurs (Hammond-Kosack and Jones, 1997; Tyler, 2002).

R-genes generally located either on the plasma membrane or in the cytosol (McDowell and Dangl, 2000). They are basically grouped in five classes; intracellular protein kinases, receptor like protein kinases including extracellular leucine rich repeat (LRR) domain, intracellular LRR proteins having nucleotide binding site (NBS) and a coiled-coil (CC) motif (NBS-LRR-CC), intracellular NBS-LRR proteins with a region similar to the TIR (NBS-LRR-TIR) and LRR proteins producing membrane bound extracellular proteins (Ellis et al., 2000). Among of these classes, NB-LRRs are the major type of R-genes.

The protein encoded by NB-LRR resistance gene contains a nucleotide binding site (NBS) and a leucine-rich repeat (LRR). The NB-LRR proteins are necessary for the effector-triggered immunity (ETI) (Song et al., 1995; Dardick and Ronald, 2006).

A specific R-gene and its correponding *Avr* gene interacts with each other physically by initiating a complex signalling network. This network includes large ion fluxes (Ca^{2+}, H^{+}), jasmonate, salicylic acid, reactive oxygen intermediates, and ethylene (Delaney et al., 1994; Reymond and Farmer, 1998; Knoester et al., 1998; Ananieva and Ananiev, 1999). All these molecules induce the defense mechanisms. Additionally, the R-genes produce pathogenesis-related

(PR) proteins, that are chitinases, glucanases, enzymes associated with the phytoalexin biosynthesis, the oxidative stress protection, tissue repair and others, all of which are related to the defence system (Odjakova and Hadjiivanova, 2001).

In early stage of the R-*Avr* interaction, there are changes in the permeability of plasma membrane, which are calcium (Ca^{2+}) / proton influx and potassium / chloride efflux (McDowell and Dangl, 2000). Afterwards, the flux of ions triggers the induction of extracellular production of reactive oxygen intermediates (ROI); superoxide (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl free radical (OH•) (Somssich and Hahlbrock, 1998). All these changes promote the hypersensitive response (HR), which is localized cell and tissue death at the infection region to prevent pathogen growth by limiting further nutrient sources (Van Loon, 1997; Hammond-Kosack and Jones, 1997; Holt et al., 2000). In addition to these changes, during hypersensitive response (HR), in order to inhibit the further pathogen attack, secondary signal molecules such as nitric oxide, phytoalexins, salicylic acid and pathogenesis-related proteins (PR) are rapidly released by the plant (Cohn et al., 2001).

In case the host plant does not possess the R-gene, the effector molecules are not able to be identified by the host; therefore, the *Avr* gene becomes *virulence* gene and disease occurs.

1.3 The overview of gene silencing

Gene silencing is a common term, used for defining the 'switching off ' a gene by a mechanism. In other words, this process interferes the gene expression by a way, other than genetic modification. Generally, gene silencing happens when RNA is not capable of producing a protein during translation (He et al., 2011).

Gene silencing process is achieved at two different levels; transcriptional and post-transcriptional (Wang and Waterhouse, 2002). In transcriptional gene

silencing (TGS), histone modifications occur and they form an heterochromatin environment around a gene. These changes make the gene unreachable for RNA polymerase, transcription factors, etc. (Vaucheret et al., 2001; He et al., 2011). For instance, DNA methylation causes the undesired structural changes on the promoter site by binding the chromatin remodeling proteins to the promoter. By this way, it inhibits the essential transcription factors and other proteins binding to a gene promoter. (He et al., 2011). On the other hand, in post-transcriptional gene silencing (PTGS), mRNA of a specific gene is exterminated or blocked to inhibit translation, and so protein production (Yu and Kumar, 2003). PTGS is accomplished by RNA interference (RNAi). It is an efficient method to protect host against the infections of viruses.

Scientists use the gene silencing systems for many purposes such as to understand how organisms work and how diseases lead to damage. By this way, they improve new treatments. Moreover, gene silencing is used to determine the functions of genes in plants (Yu and Kumar, 2003).

1.3.1 Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing (PTGS) can be defined as an RNA mediated systemic silencing mechanism. This mechanism is identified as ‘quelling’ in fungi (Romano and Macino, 1992) and as ‘RNA interference (RNAi)’ in animals (Fire et al., 1998). In plants, RNA silencing works as a component of the defense system against viruses and it is also part of the regulation of endogenous gene expression (Yu and Kumar, 2003; Voinnet, 2002). RNA silencing does not influence a gene locus transcription. However, it degrades the specific sequence of target mRNAs (Fagard and Vaucheret, 2000). The common property of RNA silencing is to be induced by the presence of double-stranded RNA (dsRNA), further split into small RNAs to be functional in a number of gene silencing processes (Waterhouse et al., 1998; Eckardt, 2002).

Foreign nucleic acid, in particular dsRNA, is a major seditious of the host response to viral infection. When a plant virus infects a host cell, it induces the RNA based defence mechanism to prevent the proliferation of the viral genome (Ratcliff et al., 1999). In early stage of infection, the viral single-stranded RNA (ssRNA) in the infected cell would not be target for the formation of small interference RNA-RNase complex since the rate of this replication intermediate is not enough to be targeted. In the later stages, the accumulation of the viral RNA increases and dsRNA rate becomes more abundant *via* the action of RNA dependent RNA polymerase enzyme (RdRp) (Voinnet, 2001; Kusaba, 2004). Then, dsRNA interacts with Dicer enzyme, cutting the foreign dsRNA into 20-25 nucleotides in length that can be endogenous and exogenous (Bernstein et al., 2001; Hammond et al., 2000). Afterwards, a ribonucleoprotein complex, called the RNA-induced silencing complex (RISC) associates with the small interference RNA (siRNA). By this way, the double-stranded siRNA is untied. This single-stranded siRNA is used as a template to recognize the complementary mRNA. After finding the target mRNA, the single-stranded siRNA binds to it and then, activates the RNase to cleave the target mRNA (Denli and Hannon, 2003). At the end of this process, the translation of the target mRNA is prevented so that it cannot synthesize the protein (Tang et al., 2010). The RNA silencing mechanism is overviewed in Figure 1.5.

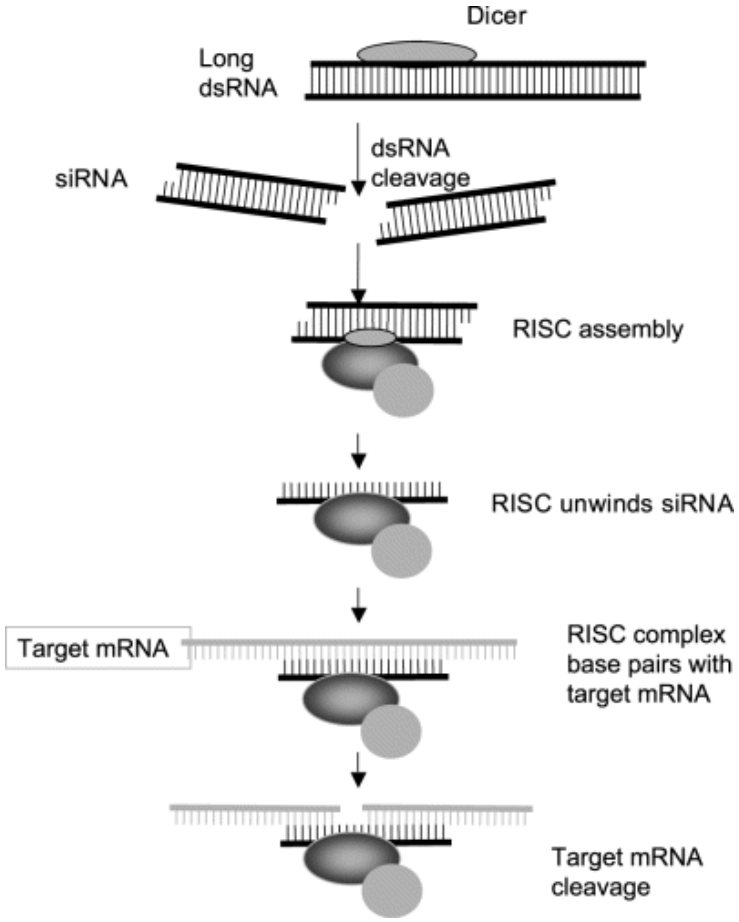

Figure 1.5 RNA interference mechanism in plants (Karpala, 2005).

The RNAi process is managed by either siRNA or miRNA. Although the functions of both are the same, there are subtle differences. siRNA is thought exogenous dsRNA entering through viral vectors or taken up by cells, whereas miRNA is endogenous ssRNA which is non–coding, presenting in the introns of larger RNA molecules (Martinez et al. 2002; Llave et al. 2002a; Reinhart et al. 2002). Moreover, unlike siRNA, miRNAs do not represent perfect compatibility to their target mRNAs (Aukerman and Sakai, 2003). Nevertheless in plants, the compatibility rate between miRNA and its target mRNA is more than that in animal (Palatnik et al., 2003).

1.4 Virus induce gene silencing (VIGS)

The virus induced gene silencing (VIGS) is one of the most utilizable method for gene silencing studies. The 'virus induced gene silencing' term first used by A. van Kammen in 1997 in order to define the recovery phenomenon from virus infection; however the term has since been performed almost only for the technique containing recombinant viruses to suppress the regulation of endogenous genes (Van Kammen, 1997; Baulcombe, 1999; Ruiz et al., 1998).

Virus induced gene silencing (VIGS) is a widely used as a powerful tool in plant functional-genomic studies. With this method, it is possible to target and down-regulate the most of endogenous gene mRNAs in a sequence-specific manner to understand their functions (Lu et al., 2003; Bruun-Rasmussen et al., 2007; Pogue et al., 2002). For instance, the function of *Rar1* in disease resistance was first determined in barley (Shirasu et al., 1999) and later, the similar study was performed to represent that *Rar1* has a same function in tobacco (Liu et al., 2002a). Likely, VIGS has been applied in *Nicotiana benthamiana* to detect the function of *SGT1* gene in gene-for-gene mediated resistance mechanism against bacteria and viruses (Liu et al., 2002b; Peart et al., 2002).

VIGS technology is depended on post transcriptional gene silencing (PTGS) mechanism (Section 1.3.1). In plants, VIGS includes cloning a specific short sequence, belonging to a gene, into a viral vector. This short DNA sequence should not be less than 23 bp; nevertheless, in general, a 23-nucleotide long fragment is not efficient to begin silencing (Ekengren et al., 2003; Thomas et al., 2001). After infection, in a few weeks natural defense mechanisms of the plant suppress the replication of virus by degrading the specific endogenous mRNA, targeted to silence.

Over the past 8 years, many viral vectors have been engineered for VIGS application in many plant species. Tobacco mosaic virus (TMV) is the earliest of these vectors (Kumagai et al., 1995). Recombinant virus transcripts involving a sequence from phytoene desaturase (*PDS*) gene were generated *in vitro* and in

order to silence this gene, *Nicotiana benthamiana* plants were inoculated with them. In early studies, VIGS was commonly applied in the *N. benthamiana* species. Since this wild tobacco species is highly susceptible to virus infection and so it provides effective silencing (Burch-Smith et al., 2004). In addition, potato virus X (PVX) is also used as a vector for *N. benthamiana* species. In spite of being more stable than TMV, PVX has a more restricted host range (Ruiz et al., 1998). Barley stripe mosaic virus (BSMV) can be also used as a viral vector to silence the genes involving in powdery mildew resistance in barley and leaf rust resistance in wheat (Holzberg et al., 2002; Scofield et al. 2005; Hein et al. 2005; 2007; Tai et al. 2007; Shen et al., 2007).

Previously, barley stripe mosaic virus was engineered for effective down-regulation of Phytoene desaturase (*PDS*) gene, which was the first study of gene silencing in monocotyledonous (Burch-Smith et al., 2004). After *PDS* silencing, photobleaching was obviously observed in the plant. Because of producing clear phenotype, *PDS* silencing has been used as a control in VIGS studies (Figure 1.6) (Brigneti et al. 2004; Constantin et al. 2004; Kumagai et al. 1995; Ratcliff et al. 2001, Held et al., 2007). Then, this method was applied on other genes present in wheat (Scofield et al. 2005). Other than these, there are a few more viruses used as a vector in the VIGS technique, which is summarized in Table 1.1.

Table 1.1 The summary of properties of VIGS viral vectors (Burch-Smith et al., 2004).

Vector	Species[a]	Viral symptoms	Efficiency[b]	Adopted for large-scale studies	Reference
TMV	*N. benthamiana*	Severe	Fair; better with antisense Improved with inclusion of direct inverted repeats	Yes	Kumagai *et al.* (1995), Lacomme *et al.* (2003), Fitzmaurice *et al.* (2002)
PVX	*N. benthamiana*	Moderate	Fair; not persistent	Yes	Ruiz *et al.* (1998), Lu *et al.* (2003)
TGMV	*N. benthamiana*	Variable	Fair; varies with gene silenced, size of insert	No	Kjemtrup *et al.* (1998), Peele *et al.* (2001)
TRV	*N. benthamiana*	Mild	Good; persistent and consistent between experiments	Yes, available for GATEWAY cloning	Ratcliff *et al.* (2001), Liu *et al.* (2002b)
	Tomato	Mild	Variable; depends on inoculation technique	Yes, available for GATEWAY cloning	Liu *et al.* (2002a)
SVISS	Tobacco	Mild	Good; persistent and consistent between experiments	No	Gossele *et al.* (2002)
BSMV	Barley	Moderate	Fair; depends on target sequence; not persistent Improved with use of direct inverted repeats of targeting sequence	Yes	Holzberg *et al.* (2002), Lacomme *et al.* (2003), Fitzmaurice *et al.* (2002)
CbLCV	*Arabidopsis*	Moderate in older plants	Fair; effects variable with gene silenced and vector design	No	Turnage *et al.* (2002)

Figure 1.6 The result of the silencing of phytoene desaturase (*PDS*) gene in barley (Helda et al., 2008).

In several respects, VIGS technique is a significantly useful tool for plant functional genomic studies. This method has a rapid experimental process and it does not need full length cDNA sequences. Therefore, complete gene sequence information is not necessary to start experiments. Additionally, because of using viral construct, the influence of transient silencing can be observed with the naked eye, but it is not always possible. Furthermore, VIGS technique is a particularly useful tool for polyploidy plants, such as wheat. Since gene silencing depends on the homology between targeted DNA fragment and its complementary; thus, any genes having at least 85 % sequence identity are likely to be knocked down (Cakir et al., 2010). In other words, it is possible to silence most members of a family *via* a short-DNA sequence from the most conserved region of a gene family. If with in the gene family, a specific member is to be studied, the most diversed region of that gene is targeted, such 3' UTR.

Besides of all these advantages, there are a few limitations affecting the VIGS efficiency. In general, VIGS does not lead to uniform silencing of the gene all-around an infected plant; thus, the efficiency of VIGS method cannot reach to 100 %. In addition, choosing the unsuitable vector also reduces the efficiency of the method. The insert size is another factor that influences the effectivity. Longer than 1500 bp and less than 23 bp insert lengths significantly decrease the stability of vector (Liu and Page, 2008). Moreover reduced and restricted plasmodesmatal activity in a plant causes weak virus induced gene silencing application (Gould and Kramer, 2007). However the efficiency of VIGS technique can be increased by properly setting environmental conditions, using the most suitable virus to infect the plant and determining the correct insert length. Additionally, the susceptibility of the plant against viruses should also be evaluated before applying VIGS (Velásquez et al., 2009).

The genes can be identified *via* VIGS technique whether they have a role in plant defense system against pathogens and whether they are responsible for the endurance to extreme environmental situations or climate shifts (Eybishtz et al., 2010; Voxeur et al., 2011, Lu, 2003). In order to understand the function of a

gene, firstly, specific DNA fragment from a gene to be silenced is cloned into a most suitable viral vector for a plant. Then, the plant is infected with this virus and the phenotypic changes are observed and evaluated. At last, silencing level of the gene is determined *via* quantitative real-time polymerase chain reaction (qRT-PCR).

1.4.1 BSMV mediated virus induced gene silencing

Barley stripe mosaic virus (BSMV) is a rod-shaped positive-sense ssRNA *Hordeivirus*, and consists of three genome components that are α, β, and γ as shown in Figure 1.7 (a) (Palomar et al., 1977). The RNAα and RNAγ are crucial for the replication of the viral genome (Jackson et al., 1989; Solovyev et al., 1996) while RNAβ is essential for cell-to-cell movement (Lawrence and Jackson, 2001).

RNAβ consists of four parts (βa, βb, βc, βd). βa fragment is responsible for encoding a coat-protein, (CP). It is thought that CP serves as a silencing suppressor (Holzberg et al., 2002); therefore in BSMV mediated VIGS studies, βa fragment is removed from RNAβ (β.Δβa) to increase virulence efficiency (Donald et al., 1994, Holzberg et al., 2002).

RNAγ generates a protein, called RNA-dependent RNA polymerase (RdRp). RNAγ composes of γa and γb parts. Cysteine-rich γb fragment has RNA-binding activity *in vitro*. Therefore, it may affect gene expression of the hordeivirus (Donald et al., 1994; Agranovsky et al., 1992; Petty et al., 1990). Because of having a particular influence on viral virulence, long distance movement, symptom severity and RNA silencing suppression, a short-DNA sequence from a target gene is cloned into γb fragment (Lawrance and Jackson, 2001; Donald et al., 1994).

The cloning of targeted gene into γb fragment can be conducted in either sense or antisense direction (Holzberg et al., 2002; Hein et al., 2005). The *in vitro*

transcripts from recombinant γ and modified BSMV components (α, β.Δβa, and γ) are directly applied onto the host leaves in order to silence the target gene (Holzberg et al., 2002; Pacak et al., 2010). The period of silencing changes between 14 and 21 days post inoculation (Cakir et al., 2010; Bruun-Rasmussen et al., 2007). The most common symptoms of BSMV are the yellow stripes and dots appearing on the leaves. Then, these yellow stripes and dots may turn brown color. Additionally, leaf curling and growth interference occur after infection as shown is Figure 1.8 (Tufan et al., 2011).

BSMV can infect several monocot species, such as barley, wheat, oats and maize, which are agriculturally significant (McKinney et al., 1965; Holzberg et al., 2002; Pacak et al., 2010). Because of this property, BSMV is efficiently used as a vector in VIGS technique to understand the functions of endogenous genes present in monocotyledonous plants. Especially, it is successfully performed in gene-function studies that involve in powdery mildew R-genes in barley (Hein et al., 2005).

In our lab, BSMV mediated VIGS method has been successfully applied on monocot species. For instance, by this way, F-box proteins were identified as positive regulators of resistance against powdery mildew disease in *Hordeum vulgare* (Dagdas et al., 2009). Additionally, BSMV mediated VIGS method was applied on *Brachypodium distachyon* to constitute a new model organism for gene function studies. Since it has smaller genome and smaller plant size, compared to barley and wheat, which are difficult to manipulate agriculturally (Demircan and Akkaya, 2010). Furthermore, in the thesis study of Turgay Ünver, it is demonstrated that RAD6 gene is a negative regulator of plant disease resistance against *Blumeria graminis* in wheat and barley (Unver, 2008).

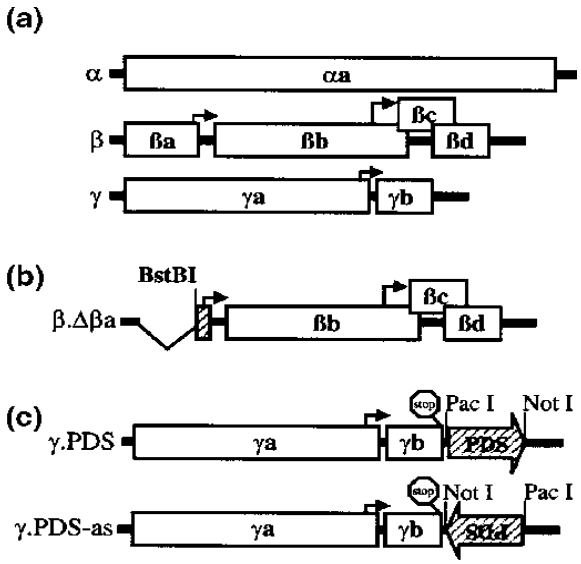

Figure 1.7 BSMV genomic RNAs. a) α, β, γ genomes of BSMV. b) β.Δβa, having deletion in βa fragment. c) Genomic orientations of pγ.bPDS4S and pγ.bPDS4As (Holzberg et al., 2002).

Figure 1.8 Barley: healthy seedlings on the left and seedlings infected with BSMV on the right.

http://www.oregon.gov/ODA/CID/PLANT_HEALTH/pages /barley_stripe_mosaic_virus.aspx

1.5 *RAD23* gene

RAD23 is a ubiquitin (Ub) receptor and has a role in DNA repair mechanism, also called nucleotide excision repair (NER) (Dantuma et al., 2009). This protein is significant factor to recognize DNA lesions and to target ubiquitylated proteins for proteasomal degradation in many eukaryotes from yeast to plants (Elsasser et al., 2005; Vierstra, 2003).

RAD23 and analogous proteins were first explored in yeast mutagenesis experiments. When studied in *Arabidopsis thaliana*, it was discovered that RAD23 proteins are multifunctional (Vierstra, 2003). These functions are binding to DNA during the replication process, inducing the targeting of the Ubiquitin/26S Proteasome system (Vierstra, 2003) and initiating the UV-triggered DNA damage repair (Madura, 1990).

RAD23 consists of two domains binding ubiquitylated proteins to occur a link between the proteasome (26S) and ubiquitylated substrates (Wilkinson et al., 2001). In spite of serving as a Ub receptor to trigger proteasomal degradation, the exact RAD23 activation in nucleotide excision repair mechanism and the possible connection between ubiquitin and proteasome system is still less obvious (Dantuma et al., 2009).

In the previous thesis study performed in our laboratory, the *RAD6* gene, which is the member of RAD family, was functionally characterized *via* VIGS in barley and wheat. After powdery mildew inoculation, pathogen growth was not observed in *RAD6* silenced susceptible barley leaf samples, suggesting that *RAD6* gene function as a negative regulator of disease resistance. Thus, it is likely that *RAD23* belonging to RAD protein family similarly functions. No homology observed among the EST data of the *HvRAD6* and *HvRAD23* or *OsRAD23* is an interesting outcome but full sequence of *HvRAD23* is needed for clarification to claim they have different DNA sequences.

1.6 Aim of the study

Powdery mildew is the serious fungal disease that results in serious quality and yield loss, especially in barley. In terms of reducing the effects of this disease, resistance-related molecular mechanisms are very significant and they can provide understanding for developing strategies against the pathogens.

Previously, *RAD23* gene product was identified as an induced factor in compatible interaction between *Blumeria graminis* f.sp. *hordei* and barley in proteomics studies, performed by Akkaya Lab MSc. Degree student, Neşe Özgazi (Proteome Analysis of *Blumeria graminis* f. sp. *hordei* Inoculated Barley) and Assist. Prof. Dr. Aslıhan Günel. Pallas-01 and Pallas-03 (barley cultivars), after 10 days growth (until the second leaf appears) were inoculated using powdery mildew race of *Bulmeria graminis* f. sp. *hordei*, *Bgh*103, with incompatible and compatible interactions, respectively. The triple biological samples of inoculated seedlings were collected at varying time points of post inoculations (12, 24, and 48 hpi) and crude protein extracts were separated on triple replicates of 2D-PAGE. The differentially expressed proteins were analyzed by nanoLC-MS/MS. Interestingly, RAD23 was only present in virulent race inoculation with 4 fold induction at 12 hpi, on the other hand, it did not appear in the resistant plant. Thus, *RAD23* gene may play a negative regulatory role in plant disease. Thus, in this thesis study, it was aimed to test if the reducing of *RAD23* gene expression also reduce the powdery mildew disease formation and the level of formed hyphae in order to confirm its function as a negative regulator in resistance. For this, virus induced gene silencing (VIGS) method was planned to be performed. The difference of *Blumeria graminis* growth levels between the control (BSMV:00) and silencing groups were aimed to be evaluated based on quantitative and qualitative analyses and the silencing level of the gene was aimed to be detected *via* qRT-PCR.

CHAPTER 2

MATERIAL AND METHODS

2.1 Plant and pathogen

Barley (*Hordeum vulgare* ssp. *vulgare*) differential line Pallas-03 seeds were amplified by Assist. Prof. Dr. Mehmet Aybeke, Trakya University and used as plant material for the RNA isolation and VIGS experiments. The Pallas-03 is well characterized differential line produced by James Kolmer and provided by ICARDA.

Fungal pathogen *Blumeria graminis* f.sp. *hordei*, *Bgh*103 and *Bgh*95, were kindly obtained from Prof. Dr. Mogens Strovring Hovmoller of Aarhus University, Demark (2008), and used as pathogen materials to inoculate leaves in order to assess *HvRAD23* gene silencing level by trypan blue staining. The biologic materials used in this study and the interactions between them are briefly presented in Table 2.1.

Table 2.1 The interaction between the Pallas-03 line and pathogen race.

Barley line	Pathogen race	Interaction between plant and pathogen
Pallas-03	*Bgh*103/virulent	Compatible interaction / Susceptible plant response
	*Bgh*95/avirulent	Incompatible interaction/ Resistant plant response

2.2 BSMV vectors

BSMV vectors pα, pγ:00, pβ, pβ.Δβa, pγ.bPDS4S (sense direction) and pγ.bPDS4AS (anti-sense direction) (Figure 1.7) were obtained from Large Scale Biology Corporation, USA (2003) and pSL039B-1 was obtained from Steven R. Scofield, USA (2003). In this study, pα, pγ:00, pβ.Δβa were used and pSL039B-1 was utilized to silence *RAD23* gene in *Hordeum vulgare* line Pallas-03. The sequences of BSMV vectors and the plasmids are shown in Appendix A.

2.3 Maintenance of Powdery mildew

Bülbül 89 is a cultivar of barley and is susceptible to all the known *Blumeria graminis* f.sp. *hordei* races; thus, Bülbül 89 was used as plant material for maintenance of *Bgh*103 and *Bgh*95.

Bülbül 89 seeds were planted on soil into 650 cm^3 pot and grown for 10 days in a growth chamber, Sanyo Versatile Environmental Test Chamber (Model MLR-351H), (conditions; at 18 ^oC light-period for 16 hr and dark-period for 8 hr). After 10 days, first leaves were cut and approximately 5 cm long pieces were put onto water-agar plates as Figure 2.1 and 2.2 (Agar plates were prepared as follows: 7 g agar (LabM, Lot#: Q34567/129) was dissolved in 450 mL of ddH_2O and the mixture was sterilized in autoclave. After cooling to 50^oC, 50 mL of Benzimidazole (Aldrich, Lot#: 23968 222) (1 g/L, in 0.1 % DMSO) was added.). Then, they were inoculated with pathogens by blowing. The leaves were incubated in a growth chamber at the same conditions as above. This process was repeated every 10-12 days to keep pathogens alive.

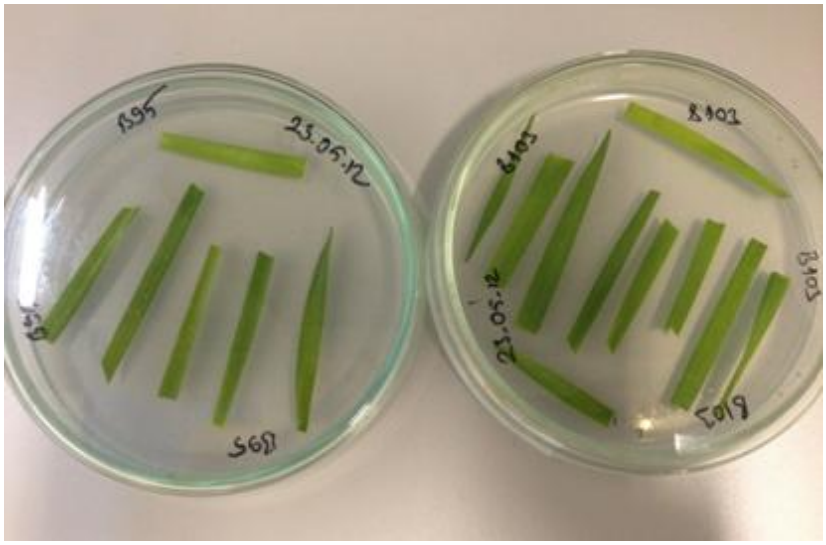

Figure 2.1 Leaf segments just after inoculation with *Blumeria graminis* f.sp. *hordei*, *Bgh*95 and *Bgh*103 isolates.

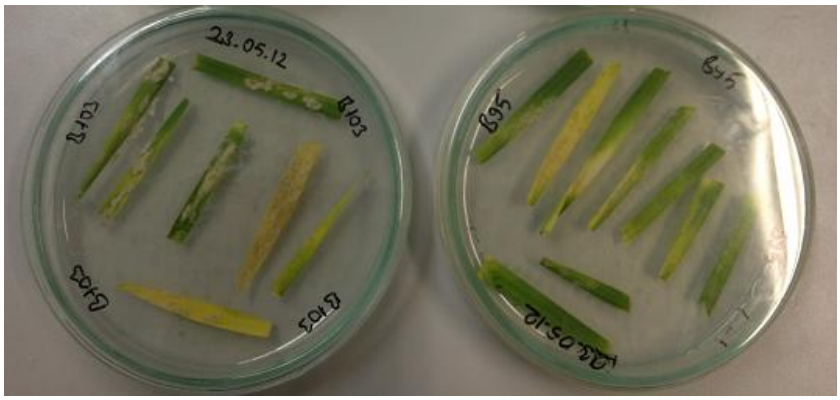

Figure 2.2 Leaf segments after 10-days post *Bgh*103 and *Bgh*95 inoculation (10dpi).

2.4 Cloning of *HvRAD23* gene fragment into pSL039B-1 vector

2.4.1 Synthesis of *HvRAD23* gene

The following *HvRAD23* gene fragment of 193 bp lenght shown in grey in Figure 2.3 was synthesized with additional *Pac*I and *Not*I sites and cloned into pBlueScript (pBSK) vector by Sentegen Biotech, Turkey. *HvRAD23*/*Pac*I&*Not*I presented in Figure 2.4 was used to insert into pSL039B-1 to silence the targeted gene.

ggtcctcccctccctcgcgagtccgaagtcaaggcgaaaaccctcgaggcgaagcgaga
gtcgcagatagggataaccgcgagagtcgagtgagtgggttggaagggaagccgagatg
aagctcaacgtgaagaccctcaagggcacaagcttcgagatcgaggcgacccccgagtc
ctcggtgggtgaggtcaagagaatcatcgagagtgctcaggggcagaatgtgtaccctg
cggatcagctgatgatcatatatcaaggaaaaattctcaaggatgataccactctggat
gctaacaaggttgcagagaacagtttccttgttataatgctgtctaagcctaaggcgtc
atctagtggcgcttcttctgcttcaaaggcacctgttagtcagtctcaacctgctaccc
cagttgctgctgctactccacctgtccctgttgcctcagctgcaagatcaccaccttca
caagcacctgttgctgcatctgaaccggcgcctcccagtgcacagccttcagctgtttc
tgatactccagctgctgcagtaactgcatcaggcgatgctgatgtatacagtcaggctg
catcaaaccttgtctctggcggcattctagaacagacagtccaacaaattcttgacatg
ggtggtggcacctgggaacgtgatatggttgtccgtgctttacgtgctgcgtataacaa
ccctgagagggctattgactacctgtattctggaatacctgaatccgtagaggccccac
ctgtggctcgagcacctgctcctgctcaacaggcacaaaacctgcaggctccatcccag
gctcaggctgcacctctaccggcagtgcagccatctggtggtgtctctgctgggcctaa
tgcgaatcctctaaacctttcccacagggtgttccaagtggtggggccaatgctggtg
ctggtgtgggtgccggtgccggtgcccttgatgcattgcgacagcttccacagttccaa
gcactgcttgcattggtccaggctaatccccaaatcctacagccaatgcttcaagagct
ggggaaacaaaatccacagatcctgcggttgattcaggaaaatcaggctgagtttctcc
gcctggtaaatgaaacacctgagagtggtgctggcggaaatatactaggcgcactggca
gctcaaatgccacaggcagttcaagttactccagaagaacgggaggccatccagcggct
tgagtcaatggggttcaatcgtgagcttgtgttggaggtcttctttgcatgcaacaggg
atgaagaactggctgccaactatcttttggatcatggccacgagtttgaggaacaacaa
tagatgttagattggtgttaccgtcgaaagacagagtcagtgacagaagcagtacagtt
tggtgtcgttcttatatcttgctgctgaatatcactactacttgccattagtgagaagc
tgctgactttcaatttcttgtatgctggaaatccttttaagtacatattaatatcata
tctgtgtgtgcagctggtc

Figure 2.3 *Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein (AK367564.1) complete coding sequence (cds). The sequence in grey is target region for silencing.

TTAATTAAatgttagattggtgttaccgtcgaaagacagagtcagtgacagaagcagt
acagtttggtgtcgttcttatatcttgctgctgaatatcactactacttgccattagt
gagaagctgctgactttcaatttcttgtatgctggaaatccttttaagtacatatta
atatcatatctgtgtgtgcagctggtcGCGGCCGC

Figure 2.4 *HvRAD23/Pac*I&*Not*I-BSMV silencing target. The grey parts show *Pac*I and *Not*I sites, respectively.

2.4.2 Transformation of *E. coli* DH5α with pBSK-*HvRAD23*

The pBSK-*HvRAD23* gene was transformed into *E. coli* cells to prepare stock from a single colony. Prior to transformation, fresh competent cells were prepared from *E. coli* DH5α cells.

E. coli competent cell preparation

Competent cells are used to propagate the plasmids *via* bacterial transformation. The following procedure was applied (Fredrick et al., 1994).

A single colony of *E. coli* (DH5α) was inoculated into 3 mL LB medium (0.01 g/mL tripton, 0.005 g/mL yeast extract, 0.0032 mL/mL NaOH (0.5 N), 0.01 g/mL NaCl; dissolved in 500 mL distilled water *via* autoclave.) and incubated at 37 ^{o}C, 250 rpm for overnight. 1 mL from the overnight-grown cells was inoculated into 100 mL sterile LB medium and incubated at 37 ^{o}C, 250 rpm until A_{600nm} reached to 0.375 absorbance. This step lasted approximately 2 hr and A_{600nm} was measured as 0,380 absorbance. Then, the culture was aliquoted into two 50 mL falcon tubes and they were kept on ice for 10 min. *E. coli* cells were centrifuged for 5 min at 5000 rpm, 4 ^{o}C and supernatant was discarded. 10 mL ice-cold and filter-sterilized 0.1 M $CaCl_2$ was added into each falcon tube to resuspend each pellet by gently shaking and inverting. After resuspension, the falcon tubes were leaved on ice for 10 min. Cells were centrifuged for 5 min at 5000 rpm, 4 ^{o}C and

supernatant was poured off. Then, 10 mL $CaCl_2$ was added into each tube and the pellets were resuspended by the same way and incubated on ice for 30 min. The cells were centrifuged again for 5 min at 5000 rpm, 4 ^oC and supernatant was removed. Each pellet was resuspended into 2 mL of ice-cold 0.1 M $CaCl_2$ and kept on ice for 5 min. Resuspended cells were dispensed into 0.5 mL PCR tubes (100 µL cell/tube) and 1 volume of filter-sterilized 50 % glycerol was added to 1 volume of the cells. After mixing gently, PCR tubes were immediately transfered to -80 ^oC.

Prior to transformation, competent cells in 0.5 mL PCR tube was put on ice and kept until cells dissolved. Then, pBSK-*HvRAD23* was transformed into *E. coli* competent cells according to following procedure.

After merging 1 µL of pBSK-*HvRAD23* with 100 µL competent cell in a sterile 1.5 mL ependorf, the mixture was incubated on ice for 20 min. Then, heat-shock was performed for 45 sec at 42 ^oC in the water bath. Afterwards, the transformation product was immediately put onto ice without shaking and held on for least 5 min. Then, 300 µL of LB medium was added to the transformation product and the mixture was incubated at 37 ^oC for 1 hr at 150 rpm. After incubation, 10 µL of the product in LB medium was spreaded on LB-agar plate having 100 µg/mL ampicillin (LB-agar plates were prepared as follows; 0.01 g/mL tripton, 0.005 g/mL yeast extract, 0.0032 mL/mL NaOH (0.5 N), 0.01 g/mL NaCl, 0.013 g/mL agar were dissolved in 500 mL distilled water and sterilized in autoclave. After cooling to 50 ^oC, ampicillin (100 µg/mL) was added). Then, the plate was incubated at 37 ^oC for overnight (16-18 hr).

A single colony was randomly selected from plate. The single colony and pγ.PDS4AS were separately inoculated into LB mediums having 100 µg/mL ampicillin and incubated at 37 ^oC, 250 rpm for overnight in order to isolate plasmids.

2.4.3 Plasmid isolations

QIAprep® Spin Miniprep Kit (Cat No: 27104, Lot# 139313379) was used for the plasmid isolation experiments. The manufacturer's protocol was applied with minor changes.

The *E. coli* Dh5α cells grown in 4 mL LB medium having 100 μg/mL ampicillin at 37 ^{o}C, 250 rpm for overnight were harvested by centrifugation (CLP Model 3410 Microcentrifuge) for 1 min at 13,000 rpm at room temperature. Supernatant was poured off and 250 μL of Buffer P1 was added into each tube in order to undergo resuspend pelleted bacterial cells by vortexing. Then, 250 μL of Buffer P2 was added. At this step vortex was not used since this would lead to sharing of genomic DNA. Therefore, samples were mixed by gently inverting until the solutions homogeneously turned blue and incubated at room teperature for approximately 5 min. The solutions turned blue as Buffer P2 included LyseBlue reagent. Afterwards, 350 μL of N3 Buffer was added and mixed immediately by softly inverting until the solution turned a homogeneous colorless suspension. After mixing, samples were centrifuged by using microcentrifuge for 10 min at 13,000 rpm, room temperature. The upper phase of the samples was transferred into QIAprep spin columns in a provided 1.5 mL collection tubes by pipetting and the spin columns were centrifuged for 1 min at 13,000 rpm, room temperature. The flow-through into collection tubes was discarded and the spin columns were washed by adding 750 μL of Buffer PE, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded again and the spin columns were additionally centrifuged for 1 min in order to remove residual wash buffer. After completely discarding residual wash buffer, the contents of the spin columns were tranferred into new sterile 1.5 mL microcentrifuge tubes and the DNA plasmids were eluted by adding 35 μL of Buffer EB (elution buffer, 10mM TrisCl, pH 8.5). After adding elution buffer, the spin columns were kept for approximately 5 min to allow the absorption of elution buffer from the membrane and then, they were centrifuged for 5 min at 13,000 rpm, room temperature. Finally, spin columns were removed from the collection tubes and the concentrations of eluted DNAs

were measured by NanoDrop (ND-1000 spectrophotometer). The samples were stored at -20 ^{o}C.

2.4.4 Excision and release of *HvRAD23* insert from pBSK-*HvRAD23*

Preparation of pγ vector and *HvRAD23* insert for cloning

pBSK-*HvRAD23* and pSL039B-1 vector, containing *PDS*4as gene fragment (185 bp), as in Figure 3.6, were double digested with the restiriction enzymes, *Pac*I and *Not*I-HF (high fidelity).

1U *Pac*I (NEB, Lot# R0547L), 2U *Not*I-HF (NEB, Lot# R3189L, 10X BSA (NEB, Lot# 1005), 1X Buffer4 (NEB, Lot# 799), purified templates and ddH_2O up to 10 µL were combined into 0.2 mL sterile PCR tube for double digestion as in Table 2.2.

Table 2.2 The combination of restriction enzyme double digestion master mix.

Components	Per reaction (µL)
Buffer 4 (10X)	1
BSA (100X)	0.1
*Not*I–HF (10 U/µL)	0.25
*Pac*I (20 U/µL)	0.5
Plasmid	8.15
TOTAL	10

Digestion mixture was incubated at 37 ^{o}C for 3 hr and the reaction was inactivated at 65 ^{o}C for 20 min.

Products were observed on 1.5 % agarose gel in 1X TAE buffer (AppliChem, Lot# 7B010350) in the presence of 0.5 µg/mL EtBr in order to excise of *HvRAD23* fragment and pγ linearized vector using a clean, sharp scalpel.

Gel extraction of *HvRAD23* fragment and pγ linearized vector for cloning

The excised DNA bands were purified by using QIAquick® Gel Extraction Kit (QIAGEN, Lot# 139312248). The manufacturer's procedure was applied.

Before starting, the weight of the gel slice into 1.5 mL ependorf was measured and 3 volumes Buffer QG was added to 1 volume of band. Water bath was heated to 50 ^{o}C and the sample was incubated into water bath until the gel slice completely dissolved. This lasted approximately 10 min. After the gel slice completely dissolved, 1 volume of 100 % isopropanol was added to 1 volume of band and mixed by inverting the ependorf several times. Then, the mixture was transferred to a QIAquick spin column in a provided 2 mL collection tube to bind DNA and the spin column was centrifuged for 1 min at 13,000 rpm by using microcentrifuge. Flow-through was removed and the spin column was fixed in the same collection tube. Afterwards, 0.5 mL of Buffer QG was added to spin column to remove all traces of agarose and the sample was centrifuged for 1 min at 13,000 rpm and flow-through was poured off. In order to wash the spin column, 0.75 mL of Buffer PE was added and it was centrifuged for 1 min at 13,000 rpm. However, before centrifugation, the spin column was incubated at room temperature for 5 min to reduce salt concentration in elute which inhibits the ligation. After centrifugation, the flow-through was discarded and the spin column in the same collection tube was centrifuged for an additional 1 min at 13,000 rpm. Later, the spin column was placed into a new sterile 1.5 mL ependorf and Buffer EB (elution buffer, 10 mM Tris-Cl, pH 8.5) of 20 µL was added to the center of the membrane to elute DNA. The spin column into new collection tube was kept at room temperature for approximately 5 min so as to ensure the absorption of elution buffer from the membrane and centrifuged for 2 min at 13,000 rpm.

Finally, spin column was removed from the collection tube and eluted DNA was stored at -20 ^{o}C.

All centrifugations were performed at room temperature and this procedure was separately applied for both excised DNA bands, *HvRAD23* insert and pγ linearized vector.

Eluted DNAs were analyzed on the 1.5 % agarose gel in 1X TAE buffer in the presence of 0.5 μg/mL EtBr so as to certain whether DNAs were extracted from the gel bands.

2.4.5 Ligation of *HvRAD23* gene fragment in pγ (as) vector

HvRAD23 gene fragment was ligated with pγ linearized vector from their *Not*I and *Pac*I sites by combining following reaction components: 16 μL of *HvRAD23* insert, 1 μL of pγ linearized vector, 2 μL 1X T4 DNA ligase buffer (Fermentas, USA) and 1 U T4 DNA ligase (Fermentas, USA) with the final volume of 20 μL.

After briefly spining, ligation mixture was incubated at 22 ^{o}C for 1 hr and reaction was inactivated at 65 ^{o}C for 20 min.

2.4.6 Transformation of *E. coli* competent cells with pγBSMV:*HvRAD23*

10 μL of ligation product was transformed into *E. coli* DH5α cells. Transformation was performed as in Section 2.4.2. At the end of transformation, the mixture was spreaded on LB-agar plates with ampicillin (100 μg/mL) as 100 μL, 150 μL, 200 μL of transformation product per plate. These plates were incubated at 37 ^{o}C for overnight (16-18 hr).

In order to ensure whether colonies contained insert or not, PCR amplification from randomly selected recombinants was conducted. This technique provides convenience in terms of rapidly screen of plasmid inserts directly from *E. coli* colonies.

2.4.7 Verification of the positive colonies

Each randomly selected 14 single colony was separately placed into 0.2 mL PCR tubes including 12 µL ddH_2O. After pipetting, 2 µL of the mixture was taken and put into 1.5 mL eppendorf containing 1 mL LB medium having 100 µg/mL ampicillin. This process was repeated for each single colony. Then, the mixtures were incubated at 37 ^oC, 250 rpm. The rest of the mixture (10 µL) with pre-incubation at 95 ^oC for 10 min was used for polymerase chain reaction. Master mix was prepared by combining following components and equally distributed among all the 16 tubes (Table 2.3). One of 16 samples was negative control (no template, containing just ddH_2O) and one was positive control (including pBSK-*HvRAD23*).

Table 2.3 The combination of PCR master mix.

Components	**Per reaction (µL) (1X)**	**Master-mix (µL) (17X)**
HvRAD23 F 10 pmol /µL (Heliksbiyoteknoloji)	2.00	34.0
HvRAD23 R 10 pmol /µL (Heliksbiyoteknoloji)	2.00	34.0
PCR Buffer (10X) (Gene Mark, Lot# F00322)	2.50	42.5
$MgCl_2$ (25 mM) (Fermentas, Lot# 6300)	1.50	25.5
dNTP (10 mM) (Gene Mark, Lot# G00814)	0.50	8.50
Taq Pol. (5U/uL) (Gene Mark, Lot# F00315)	0.20	3.40
ddH_2O	6.30	107.1
Template (single colony)	10.0	-
TOTAL	25.0	255

The PCR was performed on thermocycler (Eppendorf Mastercycler Gradient) under the following conditions; initial denaturation at 94 ^{o}C for 5 min in 1 cycle, followed by 35 cycles at 94 ^{o}C for 30 sec, 50 ^{o}C for 30 sec and 72 ^{o}C for 45 sec, 72 ^{o}C for 5 min in last cycle.

Primers used for verification of positive colonies corresponds to the beginning and the end of the *HvRAD23* fragment presented in Figure 2.4 (Table 2.4).

Table 2.4 Primer sequences amplifying *HvRAD23* used for single colony PCR test after cloning.

Primer name	**Sequence 5' to 3'**	**Product size**
HvRAD23 R	gaccagctgcacacacagat	193 bp
HvRAD23 F	atgttagattggtgttaccg	

Following single colony PCR, samples were observed on 1.5 % agarose gel in 1X TAE buffer and plasmid isolation was performed from a few positive colonies, as in Section 2.4.3.

After overnight growth in LB medium with amphicilin, 1 volume of filter-sterilized 50 % glycerol was added to 1 volume of each selected positive colonies to store at -80 ^{o}C for possible further studies. Then, isolated plasmids were double digested with restiriction enzymes, *Pac*I and *Not*I-HF (high fidelity) to ensure whether vectors had insert at espected size (Section 2.4.4) and analyzed on 1.5 % agarose gel in 1X TAE buffer. One of the clones having insert was selected and sequencing of this clone was performed by RefGen A.Ş., Turkey.

2.5 BSMV mediated virus induced gene silencing of *HvRAD23* in barley

2.5.1 Preparation of BSMV vectors

BSMV pα, pβ.Δβa, pγ, pγ.PDS4AS and pγBSMV:*HvRAD23* (as) plasmids in DH5α *E. coli* cells were separately inoculated into 4 mL LB medium with 100 µg/mL ampicillin and grown for overnight at 37 ^{o}C, 250 rpm. The plasmids were isolated as in Section 2.4.3.

2.5.2 Linerization of the plasmids

In order to linearize pα, pβ.Δβa, pγ, pγ.bPDS4AS and pγBSMV:*HvRAD23* (as) were digested with restriction enzymes. Plasmids with 4 µg were completed to 43 µL ddH_2O. Then, 2 µL of 10 U restriction enzyme and 5 µL of 1X enzyme buffer were combined with the plasmids in the 0.2 mL PCR tubes with the 50 µL final volume. The reactions were performed as in Table 2.5.

Table 2.5 The restriction enzymes used for linearization of BSMV vectors.

Restriction Enzyme	Plasmid	Enzyme Buffer	Reaction Conditions,
*Mlu*I (Fermentas, Lot#:00053835)	pα and pγ	Buffer R (Fermentas, Lot#:4518)	Incubation at 37 ^{o}C for 4 hr, inactivation at 80 ^{o}C for 20 min
*Bcu*I (*Spe*I) (Fermentas, Lot#:00054530)	β.Δβa	Buffer Tango (Fermentas, Lot#:00086159)	Incubation at 37 ^{o}C for 4 hr, inactivation at 80 ^{o}C for 20 min
*BssH*II (Biolabs, Lot#:028011)	pγBSMV:*HvRAD23* and pγ.bPDS4(as)	NEB Buffer 3 (NEB, Lot#:1105A)	Incubation at 50 ^{o}C for 4 hr, inactivation at 80 ^{o}C for 20 min

1 µL of products were observed in 1.5 % low agarose gel in 1X TAE buffer to confirm linerization. Samples were stored at -20 ^{o}C until *in vitro* transcription.

2.5.3 *In vitro* transcription from linearized vectors

The linearized pα, pβ.Δβa, pγ:00, pγ.bPDS4AS and pγBSMV:*HvRAD23* were *in vitro* transcribed according to the manufacturer's protocol. mMessage mMachine T7 *in vitro* transcription kit (Ambion, Lot#:1009036) was used.

The mixture was prepared by combining the following components; 5 µl of 2X NTP, 1 µl of 1X Buffer, 1µl T7 RNA polymerase enzyme and 3µL linearized plasmid with the 10µL final volume. Then, the samples were incubated at 37 ^{o}C for 2 hr, followed by a hold at 7 ^{o}C.

2.5.4 Inoculation of barley with the mixture of BSMV vectors

The first leaves of 10-days old plants were inoculated with BSMV viral RNAs. BSMV:00 and only FES treated plants (mock) were used as control groups. FES solution was prepared as follows; 20 % (v/v) 10X GP (18.77 g glucose, 26.13 g K_2HPO_4, ddH_2O up to 500 mL and autoclave for 20 min.), 0.01 g/mL sodium pyrophosphate, 0.01 g/mL bentonite, 0.01 g/mL celite were dissolved in ddH_2O and sterilized in autoclave for 20 min (Pogue et al., 1998). FES solution contributes to virus infection by damaging the plant cell walls.

Transcripts of each BSMV vectors were combined in 1:1:1 ratio and they were mixed with 27.5 µL FES solution (Table 2.6).

Table 2.6 The components of BSMV inoculation mixtures.

Components	**Mock**	**BSMV:*HvRAD23***	**BSMV:*PDS* as**	**BSMV:00**
α_{mix}	-	1.5 µL	1.5 µL	1.5 µL
$\beta.\Delta\beta a_{mix}$	-	1.5 µL	1.5 µL	1.5 µL
pγBSMV:*HvRAD23*$_{mix}$	-	1.5 µL	-	-
pγPDS$_{mix}$	-	-	1.5 µL	-
pγ	-	-	-	1.5 µL
FES	32 µL	27.5 µL	27.5 µL	27.5 µL
TOTAL: 32 µL				

After preparing the mixtures, they were immediately applied from bottom to top of first leaves by rubbing. Then, plants were incubated in the growth chamber at the same conditions, described in Section 2.3.

2.5.5. *Blumeria graminis* f.sp. *hordei* inoculation

At 15 days post-inoculation, the second leaves of the plants were collected and cut off 4 pieces. Two of them were stored at -80 ^{o}C in order to determine silencing level of the gene by qRT-PCR. The other two pieces were put onto agar plates, as in Figure 2.5, to inoculate with *Blumeria graminis* f.sp. *hordei* races, *Bgh*103 and *Bgh*95. The powdery mildew inoculation was performed as in Section 2.3. The whole plan of VIGS experiments was described in Figure 2.6.

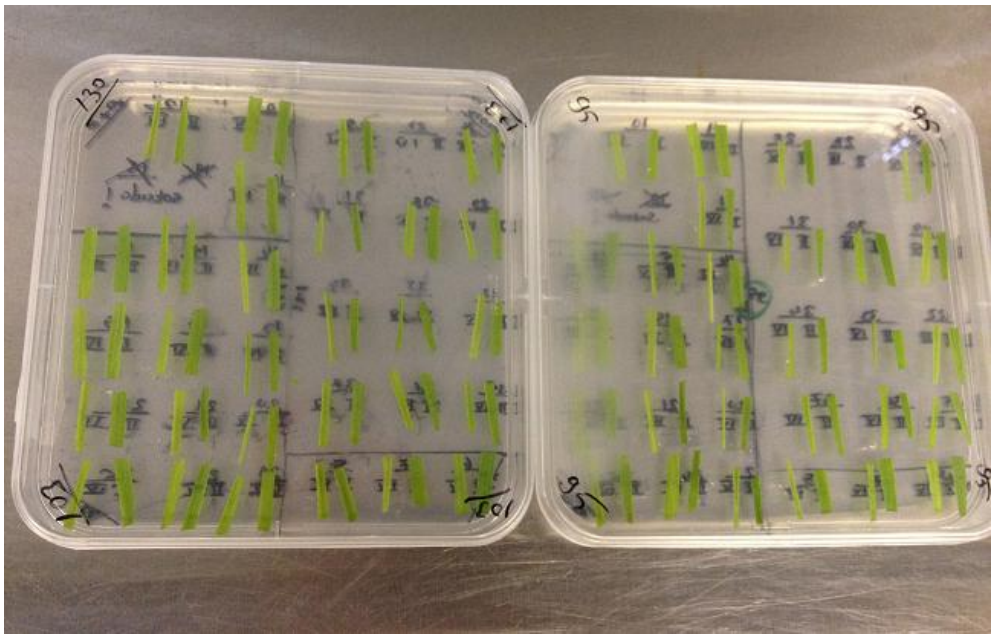

Figure 2.5 The 14-days post silenced leaf pieces ready for *Bgh*103 and *Bgh*95 inoculations.

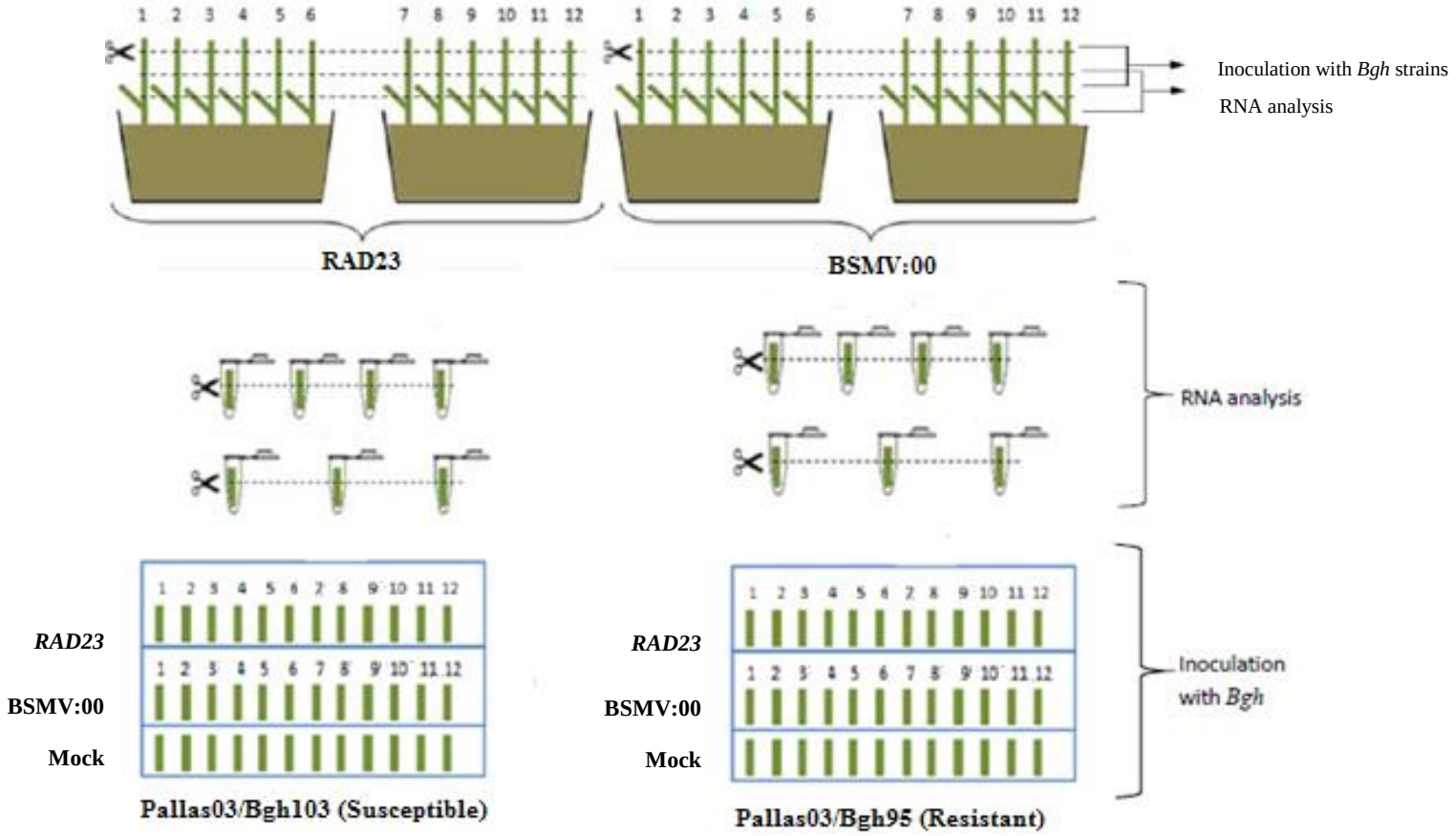

Figure 2.6 The overall plan for VIGS experiments.

2.5.5 Trypan blue staining

The fungal structures can be visualized by tryphan blue staining. This method provides to assess the infection levels of fungal pathogens. Thus, in this way, silenced and control groups were compared after staining to determine *Blumeria graminis* f.sp. *hordei* hyphal development levels. In this study, trypan blue staining was performed as Vogel and Somerville's procedure (2000).

The leaf segments from the plant which were silenced and control treated, 3 and 5 days after inoculation were collected from water-agar plates and put into 95 % ethanol for overnight to bleach green background. Bleaching leaves were stained with tryphan blue solution for 15 min at room temperature (250 µg/mL trypan blue (Applichem; Lot#: 7D008258) in a solution of lactic acid (Fluka; Lot#: BCBC5008): 87 % glycerol: ddH_2O (1:1:1)). Then, samples were transferred to rinsing solution (Lactic acid: 87 % glycerol: ddH_2O (1:1:1)) and kept for approximately 4 min at room temperature. Afterwards, leaf segments were placed on glass slides and the structures was observed under light microscope (Leica, DFC 280). The lengths of all primary hyphae, secondary hyphae and longest hyphae were measured (µm). Then, the average was calculated for all classes of hyphae.

Primary hyphae emerges from a spore while secondary hyphae arises from a primary hyphae (Figure 2.7).

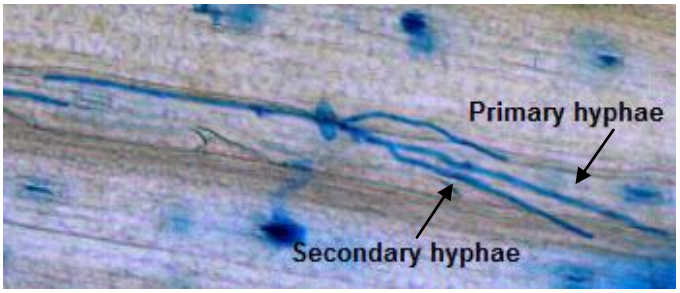

Figure 2.7 Primary and secondary hyphae of a spore at 3 dpi, barley Pallas-03 inoculated with *Bgh*103 (compatible interaction).

2.6 qRT-PCR

2.6.1 Total RNA isolation from the leaves

Total RNA isolation was conducted to determine expression level of *HvRAD23* gene fragment *via* qRT-PCR. RNeasy Plant Mini Kit (QIAGEN, Lot# 136265242) was used for the total RNA isolation. The manufacturer's protocol was carried out.

In order to inactivate ribonucleases (RNases), eppendorf tubes, spoons, mortars and pestles were submerged in 0.1 % diethyl pyrocarbonate (DEPC) in ddH_2O for overnight. Plastic wares were autoclaved to remove any trace of DEPC and then, oven baked at 50 ^oC. Spoons, mortars and pestels wrapped in aluminum foil were directly baked at 200 ^oC for overnight after DEPC treatment.

Before homogenization, mortar and pestel were chilled with liquid nitrogen. Then, the leaf segments stored at -80 ^oC were put into mortar and powdered in liquid nitrogen by using pestel. While homogenizing, polyvinyl-pyrrolidone (PVP) ($2x10^{-5}$ g/µL lyses buffer, Sigma, Lot# 116H0685) was added to increase RNA quality. The powdered leaf segments with polyvinyl-pyrrolidone were transferred to 1.5 mL RNase free eppendorf tube and immediately 450 µL of lysis buffer RLT (containing 10 µL β-Mercaptoethanol per 1 mL buffer RLT) was added. The homogenized lysate was vigorously vortexed. Then, the lysate was transferred to a lilac spin column (supplied by manufacturer) and centrifuged for 2 min at 13,000 rpm. Afterwards, 0.5 volume of 100 % ethanol was added to 1 volume of the lysate and mixed by pipetting. The mix was transferred to an QIAshredder spin column (pink) placed in a 2 mL collection tube and centrifuged for 30 sec at 13,000 rpm. The flow through was discarded. Then, 700 µL of Buffer RW1 was added to the spin column and centrifuged for 30 sec min at 13,000 rpm. Then, 500 µL of Buffer RPE (containing 4 volumes of 96 % ethanol) was added to wash the spin column membrane and centrifuged for 30 sec min at 13,000 rpm. The flow through was discarded and this washing step was performed again. Afterwards, the spin column was placed in a new collection

tube and centrifuged for 2 min at 13,000 rpm to remove any residual carryover of Buffer RPE. Then, the RNeasy spin column was placed into a new 1.5 mL sterile eppendorf tube and 30 µL of RNase free water was directly added to the spin column membrane so as to eluate total RNA. The spin column in 1.5 mL tube was incubated for 5 min at room temperature to allow the absorption of RNase free water from the membrane and then, centrifuged for 1 min at 13,000 rpm. The concentration of eluated total RNA was measured by NanoDrop (ND-1000 spectrophotometer) and the eluated total RNAs were stored at -80 ^{o}C.

For centrifugation, CLP Model 3410 Microcentrifuge was used and all steps were performed at room temperature.

2.6.2 DNase treatment

Prior to first strand cDNA synthesis, total RNAs were treated with DNase (Fermentas, DNase I, RNase-free Lot# 93831) to degrade any DNA contamination. DNase treatment was conducted as suggested by the manufacturer's instructions.

162 ng total RNA was put into a DEPC treated 0.2 mL PCR tube and the volume was completed to 4.75 µL with ddH_2O. Then, 0.55 µL 10X reaction buffer and 0.2 µL enzyme were combined with total RNA and the mixtures were incubated at 37 ^{o}C for 30 minutes. Afterwards, 0.55 µL EDTA (50 mM, Fermentas, Lot# 93072) was put into mixture and incubated at 65 ^{o}C for 10 minutes for enzyme inactivation.

After DNase treatment, in order to ensure the absence of DNA contamination, PCR was performed as in Section 2.4.7. Elongation Factor primer set (Table 2.7) was used for amplification reaction. Then, the products were analyzed on 1.5 % agarose gel.

2.6.3 cDNA synthesis

SuperScript II Reverse Transcriptase (Invitrogen, Lot# 1038858) was used for first strand cDNA synthesis. It was conducted according to the manufacturer's protocol.

DNase treated RNA of 5 µL (134 ng) was combined with 1 µL of 10 µM random hexamer, 1 µL of 10 µM oligo dT primer, 1 µL of 10 mM dNTP and ddH_2O up to 12 µL. The mixture was put into an RNase-free 0.2 mL PCR tube, and incubated at 65 ^oC for 5 min. After that, the reaction mixture was rapidly transferred to ice and incubated for 5 min. After briefly spinning, 4 µL 5X First-Strand Buffer (Invitrogen, Lot# 1005582), 2 µL of 0.1 M DTT (Invitrogen, Lot# 1005609), 1 µL of RiboLock RNase Inhibitor (40u/µL, Fermentas, Lot# 96098) were added into the tube and mixed by pipetting. The mixture was incubated at 25 ^oC for 2 min and 42^oC for 2 min. Then, 1 µL of 200 U SuperScript™ II Reverse Transcriptase (Invitrogen, Lot# 1049412) was added and mixed by pipetting. The final volume of the cDNA synthesis is 20 µL.

The first strand cDNA synthesis reaction was performed under the following conditions; 25 ^oC for 10 min, 42 ^oC for 50 min and 70 ^oC for 15 min for enzyme inactivation. All incubation steps were performed *via* Eppendorf Mastercycler Gradient.

In order to verify cDNA synthesis, PCR was performed as in Section 2.4.7. Elongation Factor primer set was used for amplification reaction as in Section 2.6.2. Then, 1 µL of the amplified products were analyzed on 1.5 % agarose gel to check cDNA synthesis efficiency.

2.6.4 Determination of reference genes

cDNAs synthesized from BSMV:00 (control) and BSMV:*HvRAD23* (silenced) treated leaves were used as templates for the qRT-PCR. Elongation Factor (EF),

Actin, Ubiquitin (Ubi), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin (Table 2.7) were the candidate reference genes, two of which were used to normalize the expression data. The reference gene sequences were shown in Appendix B.

The reactions were performed as triplicates for each cDNAs and non-template controls (NTC). For qRT-PCR analysis, SYBR Green JumpStart Taq Ready Mix (Sigma, Lot# 031M6054) was used and the reaction was conducted according to the manufacturer's protocol.

Master mix sets were separately prepared for each of the cDNAs as in Table 2.8 and then, 9.8 μL of master mix was dispensed to reaction tubes. Each reaction contained 0.84 ng cDNA. The plate setup was described in Table 2.9. After distributing master mix, 0.2 μL of 1 μm primer mix (F&R) was put into each well. Following briefly spinning, the tubes were placed on Stratagene Mx3005P Real-Time PCR System to start reaction. Prior to start, SYBR Green with Dissociation Curve option was chosen as experiment type. Then, the plate sets were chosen and the type of wells was selected as unknown. 'FAM' was selected as collection of the fluorescence data and then, the reaction was carried out starting with a denaturation step at 95 ^{o}C for 10 min, followed by amplification and quantification step at 95 ^{o}C for 30 sec, 55 ^{o}C for 1 min and 72 ^{o}C for 1min for 40 cycles with fluorescence determination once at every 55 ^{o}C. The final cycle was incubation at 95 ^{o}C for 1 min, 55 ^{o}C for 30 sec, heating to 95 ^{o}C with a fluorescence determination to constitute dissociation curve, and at last followed by 95 ^{o}C for 30 sec incubation.

In the analyses of qRT-PCR, GeNorm software was used to calculate the M values of the candidate reference genes to eliminate the genes with the smaller M values which explains the variation of a gene compared to all other genes. The elimination process was repeated until there were only two reference genes, which were the optimum, in otherwords with no fluctuation in expression levels at different experimental conditions. The candidate genes having the highest M

value were not co-regulated with the target gene. It should be lower than 1.5 to be acceptable the most stable gene expression in different conditions.

(GeNorm software: http://www.biogazelle .com/genormplus)

Table 2.7 Primer sequences of candidate reference genes

Name	Accession #	Primer name	5'- 3' Sequence
Elongation Factor	Z50789.1	EF (Fwd)	ATG ATT CCC ACC AAG CCC AT
		EF (Rev)	ACA CCA ACA GCC ACA GTT TGC
Ubiquitin	M60175.1	Ubi (Fwd)	GCC GCA CCC TCG CCG ACT AC
		Ubi-R (Rev)	CGG CGT TGG GGC ACT CCT TC
Actin	AY145451.1	Actin (Fwd)	AAT GGT CAA GGC TGG TTT CGC
		Actin (Rev)	CTG CGC CTC ATC ACC AAC ATA
GAPDH	M36650.1	Hv-GAPDH-cw1 (Fwd)	CGT TCA TCA CCA CCG ACT AC
		Hv-GAPDH-ccw1 (Rev)	CAG CCT TGT CCT TGT CAG TG
α-tubulin	Y08490.1	α-tubulin (Fwd)	AGT GTC CTG TCC ACC CAC TC
		α-tubulin (Rev)	AGC ATG AAG TGG ATC CTT GG

Table 2.8 The composition of the master mix for qRT-PCR

Reaction components	Per reaction	Master mix (17X)
cDNA (1/20 diluted)	2.5 µL	42.5 µL
ddH_2O	2.3 µL	39.1 µL
SYBR mix	5 µL	85 µL
Total	9.8 µL	166.6 µL

Table 2.9 The plate setup for qRT-PCR to determine M value.

	1	2	3	4	5	6
A	00 / Actin	00 / Actin	00 / Actin	R / Actin	R / Actin	R / Actin
B	NTC / Actin	NTC / Actin	NTC / Actin	00 / EF	00 / EF	00 / EF
C	R / EF	R / EF	R / EF	NTC / EF	NTC / EF	NTC / EF
D	00 / α-tubulin	00 / α-tubulin	00 / α-tubulin	R/ α-tubulin	R/ α-tubulin	R/ α-tubulin
E	NTC/ α-tubulin	NTC/ α-tubulin	NTC/ α-tubulin	00 / Ubi	00 / Ubi	00 / Ubi
F	R / Ubi	R / Ubi	R / Ubi	NTC / Ubi	NTC / Ubi	NTC / Ubi
G	00 / GAPDH	00 / GAPDH	00 / GAPDH	R / GAPDH	R / GAPDH	R / GAPDH
H	NTC/ GAPDH	NTC/ GAPDH	NTC/ GAPDH			

00:BSMV:00–cDNA of naked virus treated leaf (C-1), **R**: *HvRAD23*-cDNA of silenced leaf sample (S-5), **NTC:** Non-template control, **Actin**: using Actin primer set, **EF**: using Elongation Factor primer set, **α-tubulin**: using α-tubulin primer set, **Ubi**: using Ubiquitin primer set, **GAPDH**: using Glyceraldehyde 3-phosphate dehydrogenase primer set. (**C:** control, **S:** silenced)

2.6.5 qRT-PCR for transcription level determination of candidate genes

Prior to determination of transcription level of the targeted gene, qRT-PCR was carried out to calculate the amplification efficiency of cDNAs. The cDNA from BSMV:*HvRAD23* inoculated leaf was diluted to 1/8, 1/16 and 1/32 ratios to detect the concentration range for the most efficient amplification. The experiment was performed as in Section 2.6.4. The plate setup was described as in Table 2.10.

Table 2.10 The plate setup for qRT-PCR to detect the amplification efficiency.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1/8 Actin	1/8 Actin	1/8 Actin	1/16 Actin	1/16 Actin	1/16 Actin	1/32 Actin	1/32 Actin	1/32 Actin	NTC Actin	NTC Actin	NTC Actin
B	1/8 EF	1/8 EF	1/8 EF	1/16 EF	1/16 EF	1/16 EF	1/32 EF	1/32 EF	1/32 EF	NTC EF	NTC EF	NTC EF
C	1/8 Ubi	1/8 Ubi	1/8 Ubi	1/16 Ubi	1/16 Ubi	1/16 Ubi	1/32 Ubi	1/32 Ubi	1/32 Ubi	NTC Ubi	NTC Ubi	NTC Ubi
D	1/8 GAPDH	1/8 GAPDH	1/8 GAPDH	1/16 GAPDH	1/16 GAPDH	1/16 GAPDH	1/32 GAPDH	1/32 GAPDH	1/32 GAPDH	NTC GAPDH	NTC GAPDH	NTC GAPDH
E	1/8 α-tub	1/8 α-tub	1/8 α-tub	1/16 α-tub	1/16 α-tub	1/16 α-tub	1/32 α-tub	1/32 α-tub	1/32 α-tub	NTC α-tub	NTC α-tub	NTC α-tub
F	1/8 RAD23	1/8 RAD23	1/8 RAD23	1/16 RAD23	1/16 RAD23	1/16 RAD23	1/32 RAD23	1/32 RAD23	1/32 RAD23	NTC RAD23	NTC RAD23	NTC RAD23

cDNAs from silenced plant sample (S-5) was diluted to 1/8, 1/16, 1/32 to determine the amplification efficiency for the reference genes.

The coefficient of determination (R^2), describing as the goodness of fit of a regression, is expected to be ≥ 0.98. The higher R^2 indicates the better variance. The calculation of amplification efficiency is shown as below;

$$\text{Efficiency (E)} = 10^{(-1/\text{slope})}$$

After determining qRT-PCR efficiency of the genes, one more qRT-PCR was conducted to detect expression levels of candidate genes. The experiment was performed as in Section 2.6.4 with only one difference. This was the use of

primers for normalization analysis (Elongation Factor (F&R) and Ubi (F&R). Additionally, the gene specific primer set producing 116 bp product size (Fwd: 5'TGAAGGTGGTGATCTTGCAG3' and Rev: 5'CTAGTGGCGCTTCTTCTGCT3') were used to detect the *HvRAD23* silencing level (The amplification region of this primer set was given in Appendix C). cDNAs from BSMV:*HvRAD23* treated leaves at 14-days post silencing (6 samples, silenced group) and from BSMV:00 treated leaves at 14 dpi (3 samples, control group) were used to compare expression levels of the *HvRAD23* gene. In order to calculate the silencing level of the gene, Pfaffl (2001) method was used. This method, also called delta-delta method, enables to use of ΔCp (crossing point) values between silenced and control groups for targeted gene and reference genes. ΔCp value implies the difference between treshold cycle (Ct) values of silenced and control groups. The calculation was performed according to the equation given in below;

$$\text{ratio} = 2^{-[\Delta CP \text{ sample} - \Delta CP \text{ control}]}$$

$$\text{ratio} = 2^{-\Delta\Delta CP}$$

In order to calculate silencing levels, the equation given in below was used;

$$\% \text{ Silencing} = 100 \times 1 - \left(\frac{2^{\Delta Cp} \text{ Gene of interest (control} - \text{sample)}}{2^{\Delta Cp} \text{ Reference gene (control} - \text{sample)}} \right)$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Cloning of *HvRAD23* gene fragment into pSL039B-1 (pγ) vector

In the proteomics study previously conducted in our laboratory in order detect the genes differentially expressing upon powdery mildew inoculation of the resistant and susceptible barley lines, we found *RAD23* of *Oryzae sativa* (BM817007) (Figure 3.1) was one of the spots that was induced in susceptible pathogen inoculation. In order to conduct VIGS experiments, we have searched if a sequence of *RAD23* is present in available sequences of barley. Instead of finding a full gene sequence we have detected an undesignated/un-annotated piece of sequence (AK367564.1) in barley (Figure 3.2). AK367564.1 was corresponding 1553 bp coding region of *RAD23* from *Oryzae sativa* between 17-668 bp with a 96 % homology (E-value: 0.0) (Figure 3.3). The 193 bp fragment of the most conserved region (Figure 2.3) was cloned directly into pγ plasmid of the barley stripe mosaic virus (BSMV) in anti-sense direction. The barley homolog of *RAD23* gene is refered as *HvRAD23* throught of the thesis.

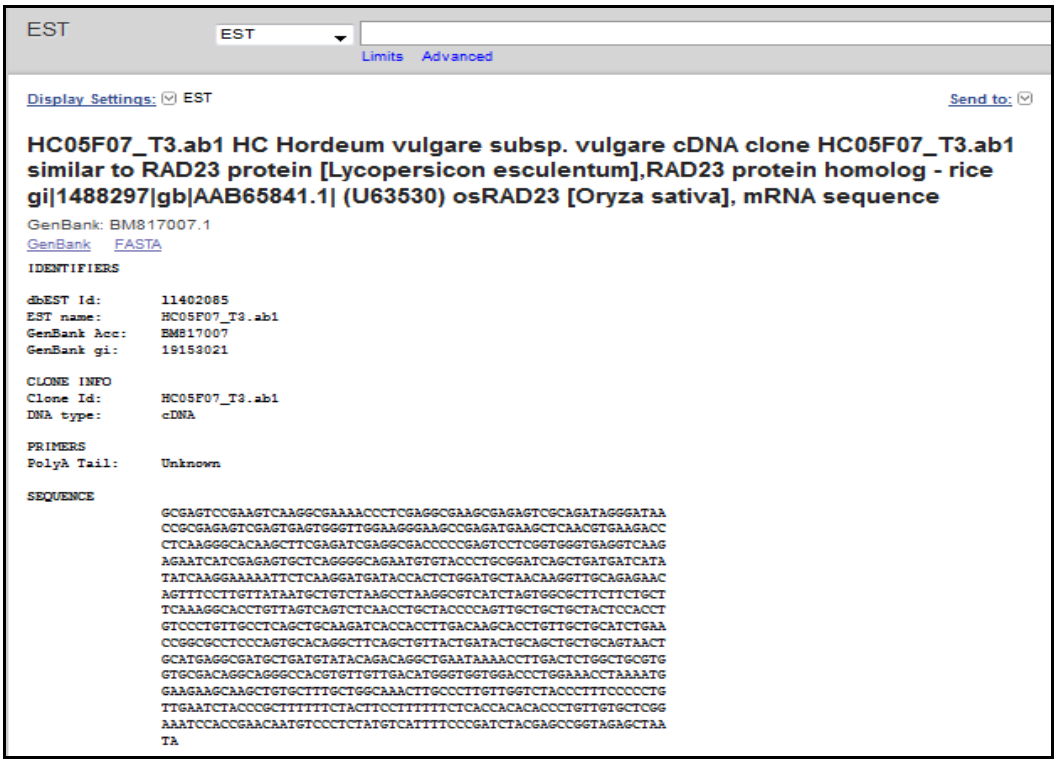

Figure 3.1 Expressed sequence tag (EST) of *RAD23* gene in *Oryzae sativa* homolog to *HvRAD23.*

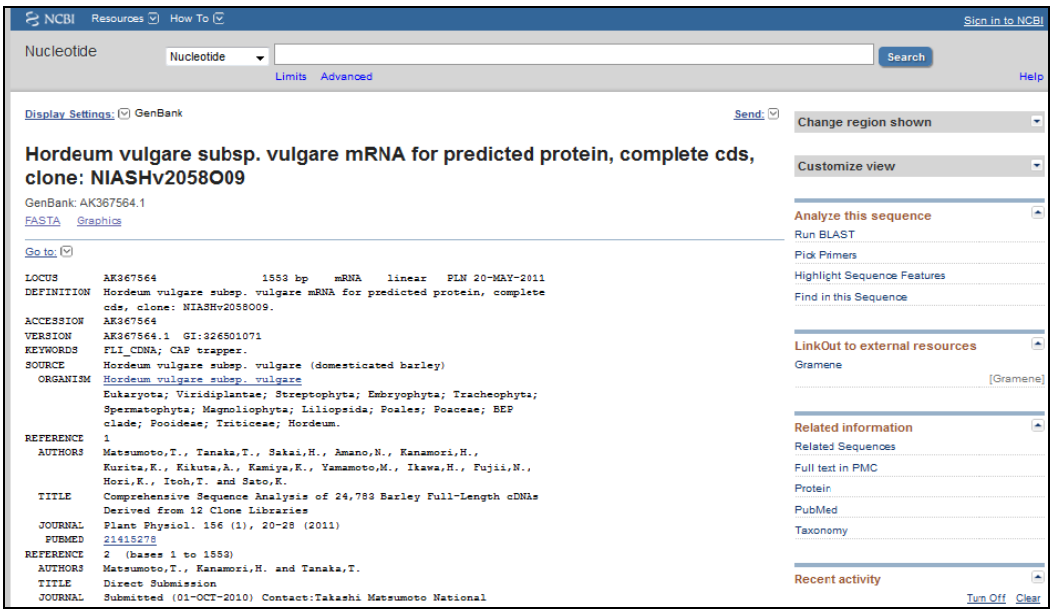

Figure 3.2 Complete coding sequence (cds) of *RAD23* gene in barley.

Figure 3.3 Multiple alignment of *RAD23* from *Oryzae sativa* and EST of *RAD23* gene in barley to show homology. **Query Accession#:** BM817007 **Subject Accession#:** AK367564.1

3.1.1 Plasmid isolation from pBSK-*HvRAD23* and pγ.bPDS4As

The pγ.bPDS4As plasmid, also known as pSL039B-1(containing *HvPDS* (185 bp)) vector, was used to construct pγ.RAD23As by replacing 185 bp bPDS fragment with 193 bp *HvRAD23* insert by restriction enzyme digestion. Before digestion, plasmid isolation from pBSK-*HvRAD23* and pγ.bPDS4As (pSL039B-

1) was performed as in Section 2.4.3. The concentration of the isolated plasmids, pBSK-*HvRAD23* and pγ.bPDS4As (pSL039B-1), were measured on NanoDrop in ng/µL (Figure 3.4).

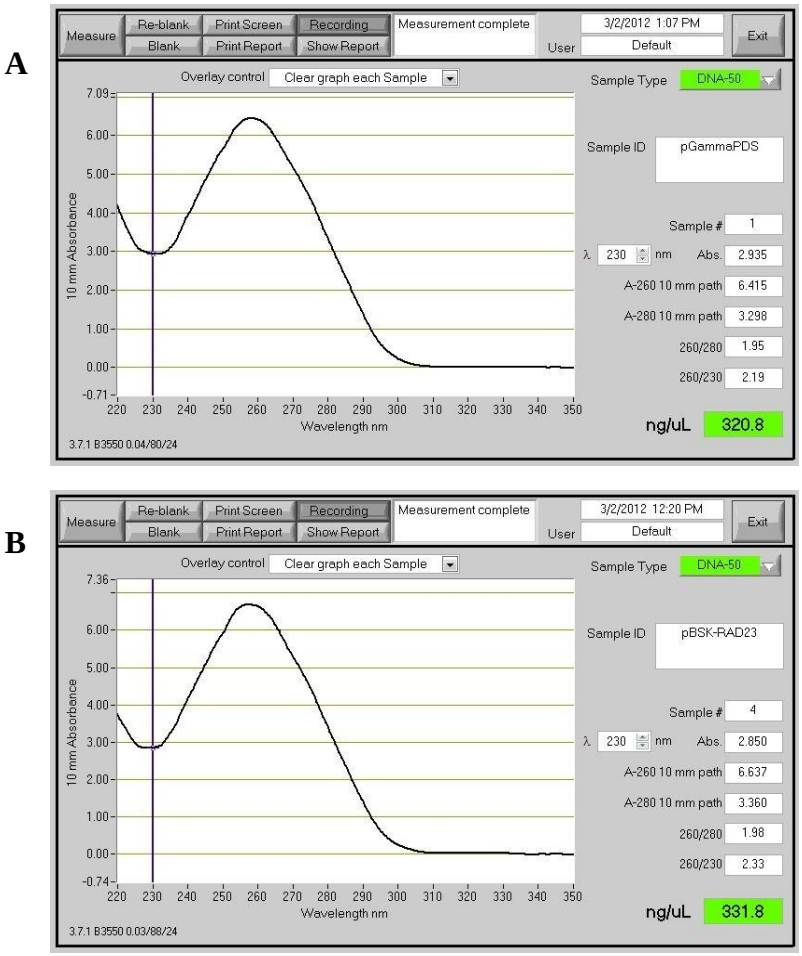

Figure 3.4 Absorbance spectra of plasmids. **A)** pBSK-*HvRAD23* (A_{260}/A_{280}=1.98 A_{260}/A_{230}=2.33) and **B)** pγ.bPDS4As (A_{260}/A_{280}=2.19 A_{260}/A_{230}=1.95).

These ratios A_{260}/A_{280} and A_{260}/A_{230} are very significant to determine the quality of DNA. A_{260}/A_{280} ratio should not be less than 1.8 for DNA to be accepted as pure. Lower A_{260}/A_{280} value means that there is protein contamination while higher value shows that plasmid isolation contains too much RNA. Therefore, the optimum range is between 1.80-1.90 for DNA purity. In addition, A_{260}/A_{230} ratio is also important for DNA purity. This ratio should be about 2.00. If it is

appreciably lower, it means that there is a contamination resulting from polysaccharide, guanidine hydrochloride. All these sources increase the absorbance at 230 nm and results in lowering A_{260}/A_{230} value. Considering these parameters, the plasmid isolations in this thesis were at their optimums.

3.1.2 Preparation of pγ vector and *HvRAD23* insert

First, both plasmids were double digested with *Pac*I and *Not*I restriction enzymes as in Section 2.4.4. Then, the digestion products were separated by electrophoresis on 1.5 % agarose gel to verify the correct sizes and to extract them from the gel. The agarose gel was prepared with TAE buffer rather than TBE since the borate ions in TBE buffer could block the ligase activity.

According to the DNA bands, the fragment in 193 bp length (*HvRAD23*) and ~6 kb pγ vector (pSL039B-1) were at the expected size as shown in Figure 3.5.

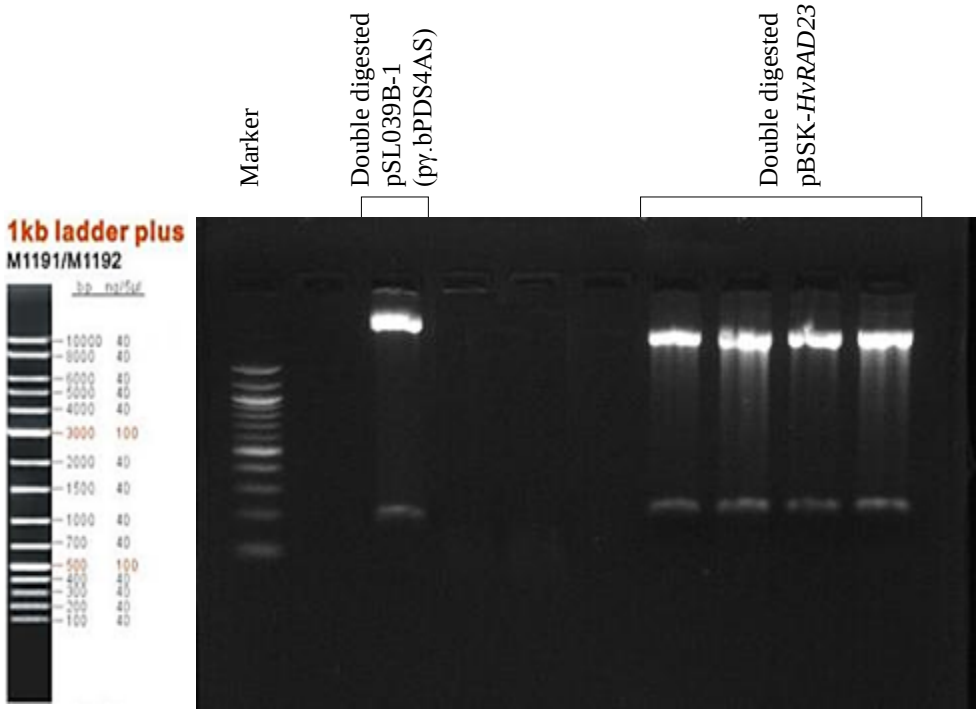

Figure 3.5 Double digestion of pBSK-*HvRAD23* and pγ.bPDS4As. **Marker:** 1 µL of 1 kb DNA ladder (0.5 µg/µl, Fermentas).

After visualization, required DNA bands were excised from the agarose gel. In this step, it was observed that too much UV light exposure significantly decreased the ligation efficiency by causing damage on DNA. Therefore, DNA bands were excised from the gel as fast as possible to prevent the UV damage. Then, DNA bands were purified *via* QIAquick® Gel Extraction Kit as mentioned in Section 2.4.4. Because ligation is a salt-sensitive application, during gel extraction, the DNA-bound membrane was kept in buffer PE (wash buffer) for prolonged time to reduce salt concentration. Additionally, in elution step, it was determined that holding the membrane in elution buffer/ddH_2O for elongated period led to increase the amount of recovered DNA.

After gel extraction, eluted DNAs were analyzed on the 1.5 % agarose gel to ensure whether DNA samples were successfully extracted from the bands (Figure 3.6).

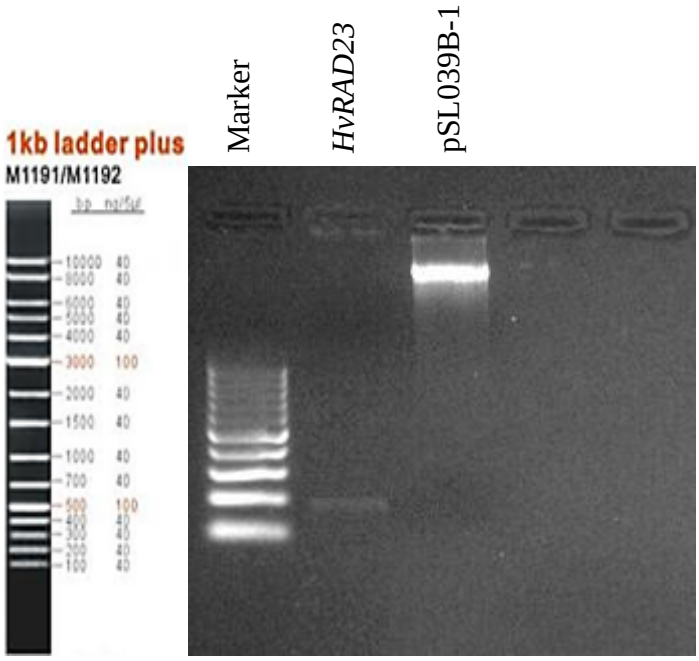

Figure 3.6 Agarose gel of the *HvRAD23* insert (193 bp) relased from pBSK-*HvRAD23* and pSL039B-1 vector. **Marker:** 1 µL of 1 kb DNA ladder (0.5µg/µl, Fermentas).

Following the conformation of the extracted DNA having the expected sized with sufficient amounts, ligation reaction was performed as in Section 2.4.5.

3.1.3 Ligation reaction

There are several crucial factors that affect the ligation efficiency. Molar ratio of insert and vector is very significant to gain high efficiency in cloning. In general, 1:3 molar ratio of Vector:Insert works well. In addition to this, vector should be completely double digested to prevent positive colony artifacts. Furthermore, too much UV exposure of DNA and high salt or EDTA concentration in DNA elution reduce the ligation efficiency as mentioned above. All these factors were taken into consideration during the ligation experiments to increase efficiency.

Following ligation, transformation was conducted as in Section 2.4.6. In order to improve the yield of transformants, heat inactivation of ligase was performed. Moreover, too much ligation product should not be added to competent cells, which decreases the transformation efficiency. Therefore, 10 µL of ligation product was added to 100 µL of competent cells ($OD_{600:}$ 0.380 nm). The candidate colonies of 14 supposedly having *HvRAD23* insert were randomly picked on ampicillin plates. In order to verify the positive colonies, single colony PCR was conducted using specific primers, which only amplify the *HvRAD23* fragment (Section 2.4.7).

After single colony PCR, samples were analyzed on 1.5 % agarose gel *via* electrophoresis. According to the electrophoresis result, it was observed that all contained the insert (Figure 3.7).

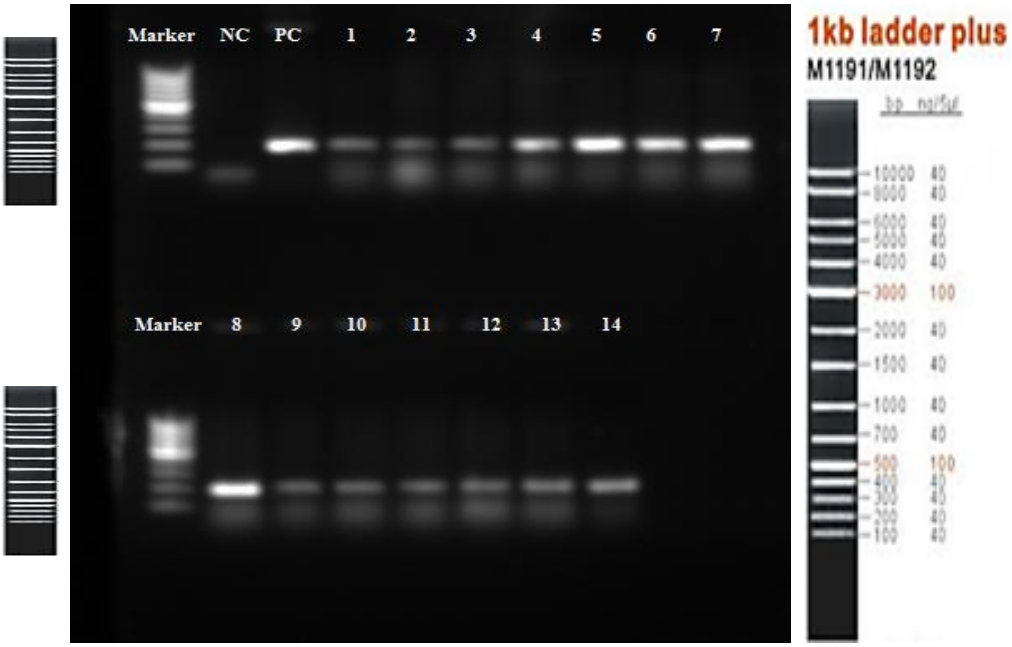

Figure 3.7 Single colony PCR of pγBSMV-*HvRAD23* colonies. **Marker:** 1 µL of 1 kb DNA ladder (0.5 µg/µl, Fermentas). **NC** (Negative control)**:** No template. **PC** (Positive control)**:** pBSK-*HvRAD23* plasmids were used as a template. Lanes 1-14 are the randomly picked colonies.

The five colonies were collected for plasmid isolation to confirm the presence of inserts by releasing with *Not*I-HF and *Pac*I restriction enzyme double digestion as in Section 2.4.4. The digested products were analyzed on 1.5 % agarose gel (Figure 3.6). The released insert fragments from all the plasmids were identical and they possessed the expected sizes. Finally, the plasmid of the **clone number 1** in Figure 3.8 was selected and sequenced by RefGen A.Ş., Turkey. The result of the sequencing is shown in Figure 3.9 and the map of pSL039B-1 vector is presented in Figure 3.10 to indicate the direction of cloning, which must be counter-clockwise, anti-sense.

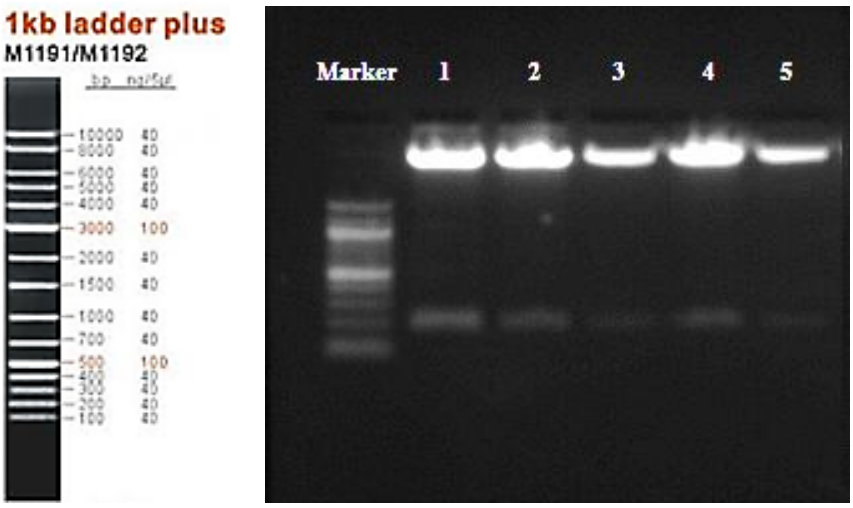

Figure 3.8 Agarose gel (1 %) of double digested pγBSMV-*HvRAD23*. **Marker:** 1 µL of 1 kb DNA ladder (0.5 µg/µL, Fermentas). Lanes 1-5 are the digested plasmids of the randomly selected colonies

>1-M13R

CCCCAGGCTTGATGACGCGCGCGTGGTCTTCCCTTGGGGGACCGAAGCTGAGCTTCGGCTCAGT
ATGCACACTCTTAAAGTGTGACGCAGCTACCGGAAGTTGTAGCTTACATACCAATTCCTAAATT
TTCTCTCCAGAGTCCGTTAAGATTCATGGTTTCCAATTCAGGCATCGTTTTCAAGTTCGATTAT
AGTGGACTTGCAAACACTCCCATCATATGGTTGATGGGCACCATCAGATTTGAATGATCTGATC
AAACATTTTTTTTTTTTTTTAGCTAGCTGATTAATTAAATGTTAGATTGGTGTTACCGTCGAAA
GACAGAGTCAGTGACAGAAGCAGTACAGTTTGGTGTCGTTCTTATATCTTGCTGCTGAATATCA
CTACTACTTGCCATTAGTGAGAAGCTGCTGACTTTCAATTTCTTGTATGCTGGAAATCCTTTTT
AAGTACATATTAATATCATATCTGTGTGTGCAGCTGGTCGCGGCCGCTCAGCTAGCAACGGAAG
AAGAATCATCACATCCAACAGAATCTTCAAAAGAAGAAGCTACGGACTTACGTATTGCGTTAAC
CTCACTTTCAAGCTTAGCCATTTTTACGATATGAGAAAGTTTCAGCTCCTGCATCTTCTTCTGG
AGAAATTCAAGAAGTAACTCCTGTTCAGAACGTTTCAGAAGTGAATCATACAGATCCGCATGCT
TTTGGCCACAGAACCTACTCACGATTGGCAGTTGATCACAAGCCTCTTCCGCAATGGAGCATGG
CATTCCACAACTGTGTCCAACGATTCCATTCAGGGGCGCATTTTTTGCGGTTCCAATAGATACT
TCTTGTAAAGTTCCAATCTCTTATTTCCTTGTTTCAGAATATACATGGCTTTCGCTCACATCTC
TTACCACAGTAAGTACTTGGAGTTAAGGTACCACAACACCCCCAAGAGAAAGTAGCCATCAT

Figure 3.9 The DNA sequence data of clone 1 obtained with M13 reverse primer corresponding to vector sequence. The sequence shown in grey is *HvRAD23* fragment in 193 bp length. Sequences in red refer to restriction enzyme cut-sites, *Pac*I and *Not*I sites, respectively. Sequences in blue represent the *Nde*l and *Hind*III sites, respectively, which were used to check cloning direction (counter-clockwise).

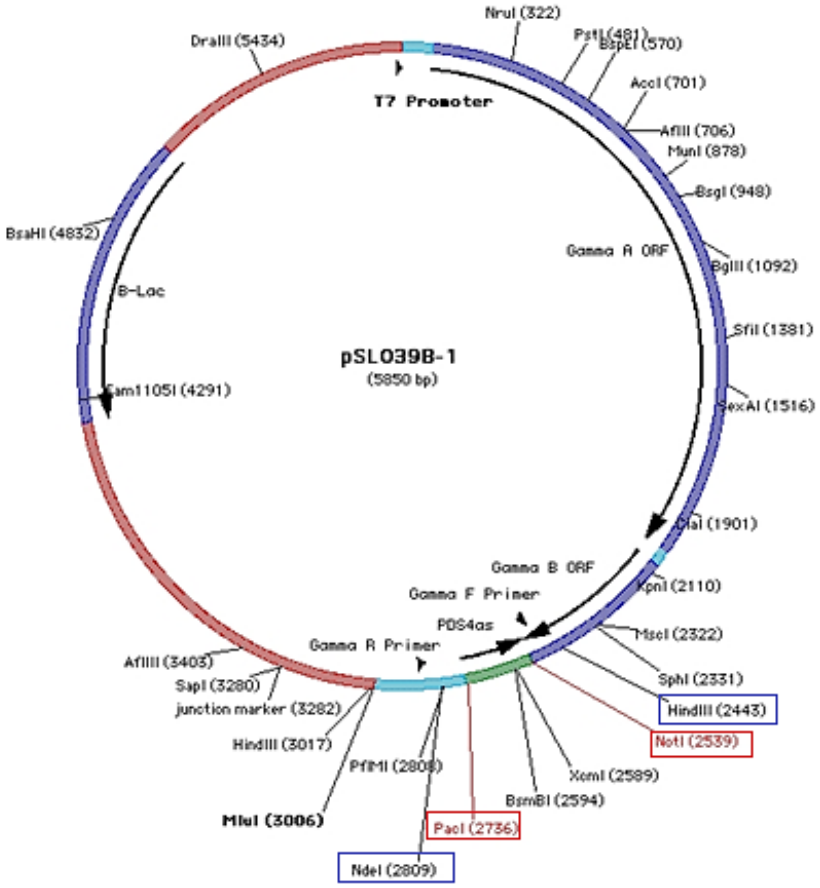

Figure 3.10 The map of pγ vector including a 185 bp fragment of the bPDS gene (pSL039B-1). The sequence of the vector is presented in Appendix A.

3.2 Plasmid isolations and linearization for VIGS

Plasmids of pα, pβΔβa, pγ:00, pγb.PDSAs, and pγBSMV:*RAD23* were performed as in Section 2.4.3. They were linearized using suitable restriction enzymes for *in vitro* transcription as in Section 2.5.3.

According to their $A_{260/280}$ and $A_{260/230}$ ratios (Table 3.1) the quality of plasmids was excellent for further enzymatic reactions.

Table 3.1 Concentrations of isolated BSMV plasmids using NanoDrop.

Plasmid	Concentration	A_{260} / A_{280}	A_{260} / A_{230}
pα	203.7 ng / µL	1.90	2.28
pβΔβa	123.2 ng / µL	1.92	2.24
pγ	476.3 ng / µL	1.90	2.32
pγb.PDSAs	626.5 ng / µL	1.90	2.28
pγBSMV:*RAD23*	407.3 ng / µL	1.89	2.33

These plasmids were linearized at equal amounts (adjusted to 4 µg) (Section 2.5.2) and then, cut and uncut plasmids were electrophoresed on 1 % agarose gel. The cut and uncut plasmids had clear bands without the smear formation as shown in Figure 3.11. Uncut plasmids had more than one band since plasmids could be topologically different; such as open circular, supercoiled circular, and concatamers; which affect the migration of DNA during the electrophoresis.

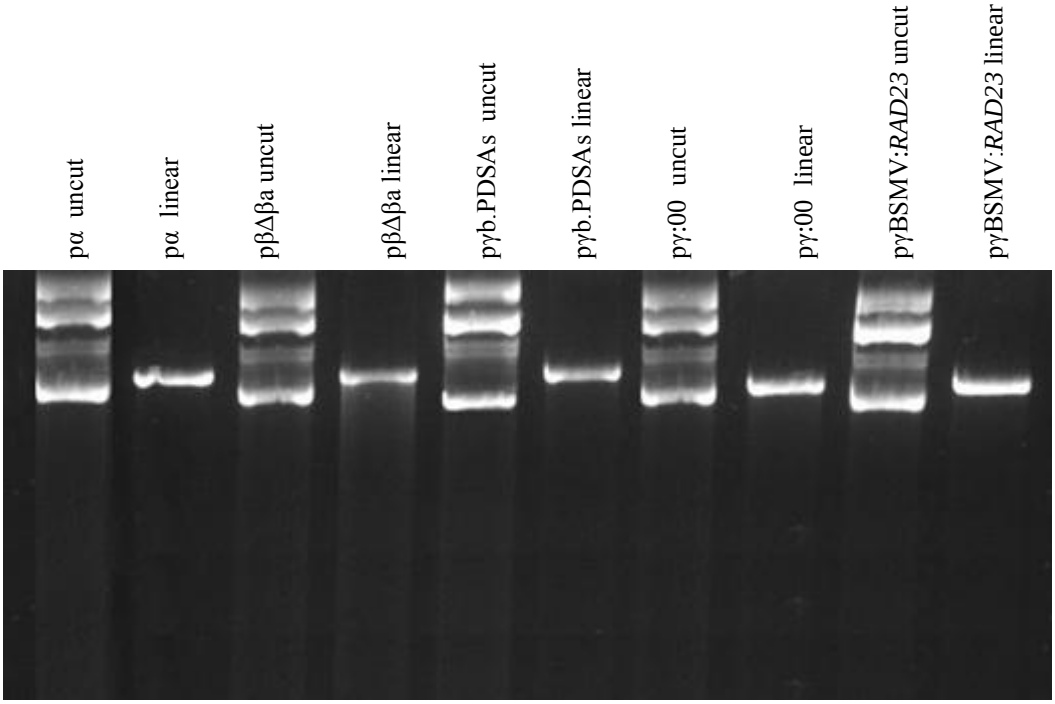

Figure 3.11 The agarose gel picture of cut and uncut pα, pβΔβa, pγb.PDS4As, pγ:00, and pγBSMV:*RAD23*.

3.2.1 Inoculation of BSMV transcripts

In vitro transcription was conducted from linearized plasmids by using mMessage mMachine T7 *in vitro* transcription kit (Section 2.5.3). This kit contains T7 RNA polymerase that transcribes only DNA downstream of a T7 promoter and catalyzes the RNA formation in 5'-3' direction.

Transcript products in 1:1:1 ratio from all the three genomes in FES solution (prepared as in Table 2.6) were immediately applied on barley leaves by rub-on inoculation. The plants were let grow in the growth chamber (Section 2.5.4) prior to samples collections.

BSMV infection generates in yellow stripes, spots and mottled presence on the leaves, like the fungal disease barley stripe (ODA commodity inspection division, 2012). Studies show that the earliest symptoms occur at 5 dpi, and persist until 14 dpi on the leaves (Bruun-Rasmussen et al., 2007). However, in our previous VIGS experiences, we hardly observed viral symptoms (Dagdas, 2009; Unver, 2008). Because of this, although there were no symptoms, the second leaves of the silenced and control plant samples were harvested at 14 dpi (except BSMV:*PDS* treated leaves). A leaf from each group was left on the pots to observe the further viral effects with naked eye. Except the mock, the earliest symptoms were observed at 19 dpi, which were not severe. These symptoms appeared as yellow spots as presented in Figure 3.12 (b, c, d).

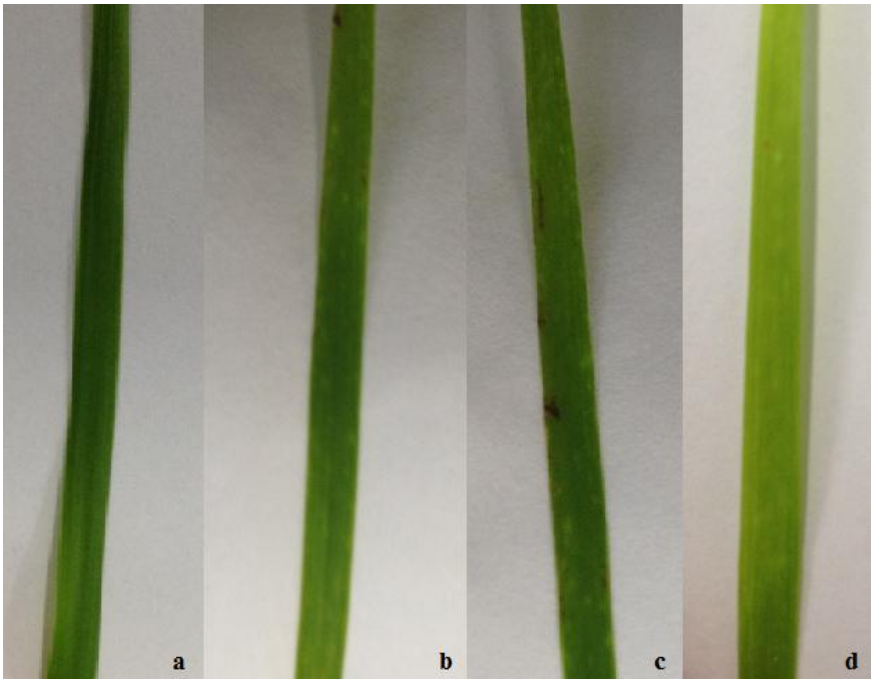

Figure 3.12 Second leaves of the plant seedlings at 19-days post inoculation. **a)** Mock **b)** BSMV:00 treated Pallas-03 **c)** BSMV:*RAD23* treated Pallas-03 **d)** BSMV:*PDS* treated Pallas-03

The silencing of the *PDS* gene is a common positive control because it leads to photobleaching in silenced regions and being rapidly visible phenotype (Kumagai, et al., 1995). In the study of Holzberg, photobleaching was scored as a percentage in barley inoculated with BSMV expressing bPDS4, with and without the βa (coat protein). It was demonstrated that the first photobleaching in BSMV:*PDS* treated barley was observed at 5 dpi with the low percentage and the most severe photobleaching appeared at 14 dpi. This process lasted between 5 and 21 dpi; however, at 21 dpi, leaves were unlikely to have photobleaching (Holzberg et al., 2002).

In our study, although photobleaching was observed at second leaves when *PDS* was silenced, it was not as intense as desired. High ratio whitening was expected between 14 and 17 dpi with respect to Holzberg study. However, the first photobleaching appearance was obvious at 19 dpi indicating a low rate (Figure 3.13, **b**). The following days, this ratio gradually increased and the effects were

observed more obviously but still, the rate of bleaching was not too high compared to the mock sample (**a**). Especially, the bleached patches expanded between 26 dpi (**d**) and 30 dpi (**e**). After 30 dpi, there was not considerable change on the leaves. The BSMV:*PDS* inoculated leaves in a pot are shown in Figure 3.14.

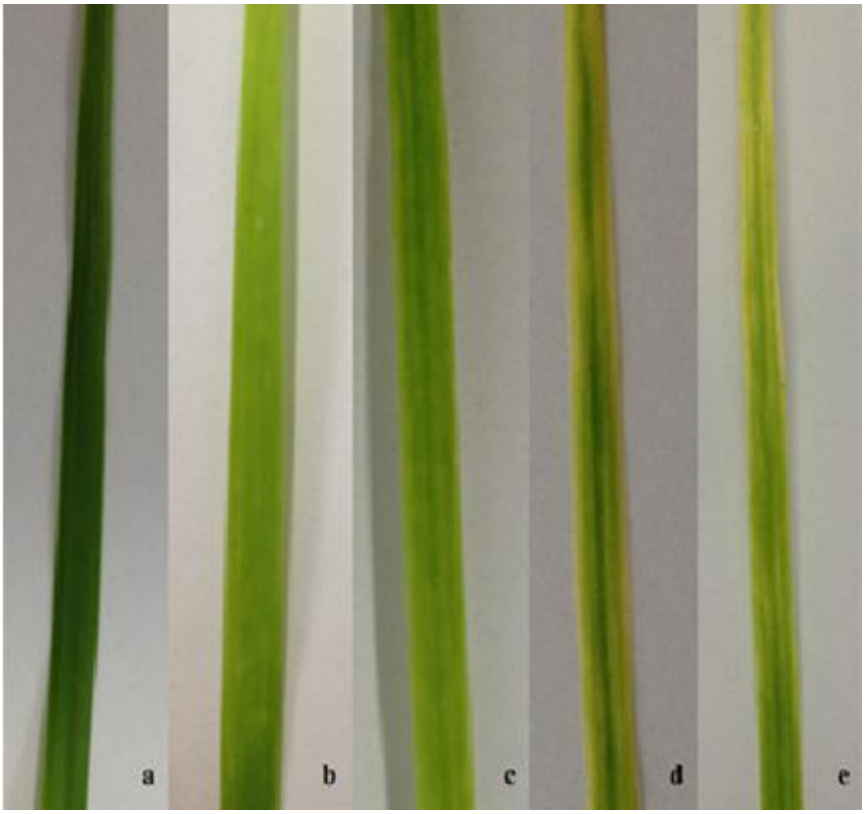

Figure 3.13 Induced photobleaching by BSMV: *PDS* inoculation in barley. **a)** Mock inoculated Pallas-03. The second leaves of BSMV:*PDS* treated plants **b)** at 19 dpi, **c)** at 22 dpi, **d)** 26 dpi and **e)** at 30 dpi.

Figure 3.14 BSMV:*PDS* inoculated Pallas-03 plants at 30 dpi.

Extensive gene silencing is dependent on an vigorous interaction between viral spread and plant growth. Both of them can be affected by environmental conditions (Burch-Smith, 2004). Temperature is one of the most significant factors for efficient virus spread and successful silencing. In the study of Bruun-Rasmussen, VIGS experiment was performed at different temperatures; 16 ^{o}C, 20 ^{o}C, 24 ^{o}C and 28 ^{o}C to detect the extent to which temperature affecting *PDS* silencing in barley. They indicated that there were no appreciable differences in photobleaching between the 20 ^{o}C and 24 ^{o}C. The plants at 16 ^{o}C showed deferred onset and decreased extent of photobleaching while the deceleration in growth and very restricted silencing were observed in plants at 28 ^{o}C. When considering all of these, this study indicates that the optimal silencing with BSMV without coat-protein is conducted at 20 ^{o}C or 24 ^{o}C (Bruun-Rasmussen et

al, 2007). However, in our study, BSMV mediated VIGS was performed at 18 ^{o}C light-period for 16 hr and dark-period for 8 hr and the low temperature conditions may have limited the BSMV spread causing the reduction of *PDS* gene silencing, which decreases the appearance of photobleaching phenotype on the leaves. This situation may have affected *RAD23* gene silencing as well.

Insert stability is the other important factor. DNA insert in a vector can be eliminated during viral infection. Researchers were found that there is a positive relation between insert size and insert loss (Qu et al., 2005). In other words, shorter inserts are more likely to result in long-lasting silencing (Bruun-Rasmussen et al., 2007). Recent studies express that the optimum range of insert size should be ~200 bp to ~350 bp for successful VIGS (Burch-Smith et al., 2004; Burch-Smith et al., 2006; Zhang et al., 2010). Thus, the fragment in 185 bp length for *PDS* silencing and the fragment in 193 bp length for *RAD23* gene silencing were used in our study. Except in the mock inoculation using only FES alone, the necrotic lesions were observed in the all third leaves of BSMV inoculated plants, which was not severe (Figure 3.15). However any bleaching phenotype was not observed in the third leaves of BSMV:*PDS* inoculated plants. This means that viral spread may have lasted until third leaves but there may have been insert loss, which prevents the silencing of the genes.

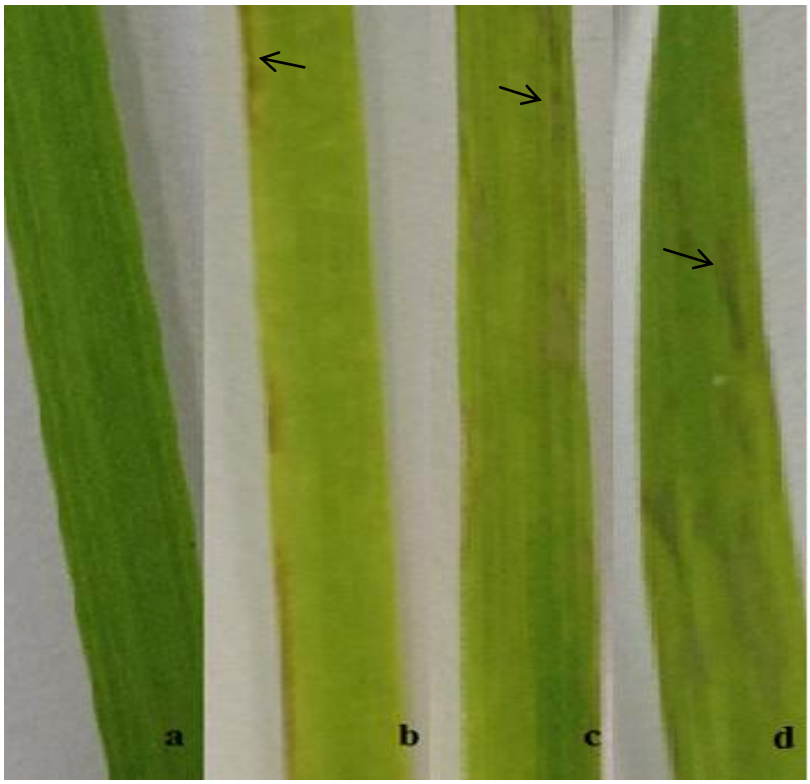

Figure 3.15 The necrotic lesions on the third leaves of the BSMV inoculated plants at 30 dpi. **a)** Mock, inoculated only FES alone. **b)** BSMV:00 treated plant sample. **c)** BSMV:*PDS* treated plant sample. **d)** BSMV:*RAD23* treated plant sample. In picture b, viral symptoms like yellow spots were obvious, as well as necrotic lesions. Arrows indicate the necrotic lesions.

3.3 *Blumeria graminis* f.sp. *hordei* inoculation

After 14-days of BSMV inoculation, the second leaves of plants were collected for total RNA isolation and *Bgh*103/*Bgh*95 inoculation experiments, as explained in Section 2.5.5 and as described in Figure 2.7. Pathogen infection was performed to determine the difference between the hyphae growth ratio of silenced and control groups.

Leaves at 3 dpi and 5 dpi of *Blumeria graminis* (*Bgh*103/*Bgh*95) inoculation were stained with tryphan blue dye to measure the hyphae lengths on light microscope

(Leica DM4000B microscope / DFC 280 camera). However, measurements of hyphae lengths were not performed at 5 dpi due to the long length and high concentration of spores. Thus, hyphae measurements were conducted at 3 dpi.

According to the previous proteomics studies in our laboratory, it is thought that *RAD23* has a critical role in disease formation. Since this protein appeared in *Bgh*103 inoculated Pallas-03 (compatible interaction) with 4 fold induction at 12 hpi while it was absent (not expressed) in *Bgh*103 inoculated Pallas-01 which is resistant against the pathogen. Because of this, after *Bgh*103 inoculation, the reduction of hyphae formation was expected in silenced groups. As expected, the number of growing pathogens in BSMV:*RAD23* inoculated leaves showed decrease compared to control samples as indicated in Figure 3.16. Moreover, there was a little difference in pathogen growth between the only FES treated (Figure 3.16 a), and BSMV:00 treated (Figure 3.16 b1, b2) plants; in the later one, less pathogen growth was observed. This means that inoculation of barley stripe mosaic virus induces plant basal resistance mechanism.

In the previous proteomics studies performed in our laboratory, *RAD23* gene was not expressed in *Bgh*103 inoculated Pallas-01 samples (incompatible interaction) as mentioned above. Because of this, silenced and control leaf samples were inoculated with *Bgh*95 as a control experiment. There was no *Bgh*95 growth on both as expected (Figure 3.17) since Pallas-03 includes *Mla6* and *Mla14* R genes and so it is resistant to *Bgh*95 possessing corresponding avirulent gene, *Mla6*. This result indicates that silencing of *RAD23* gene did not cause change in the plant resistance mechanism against powdery mildew in incompatible interaction.

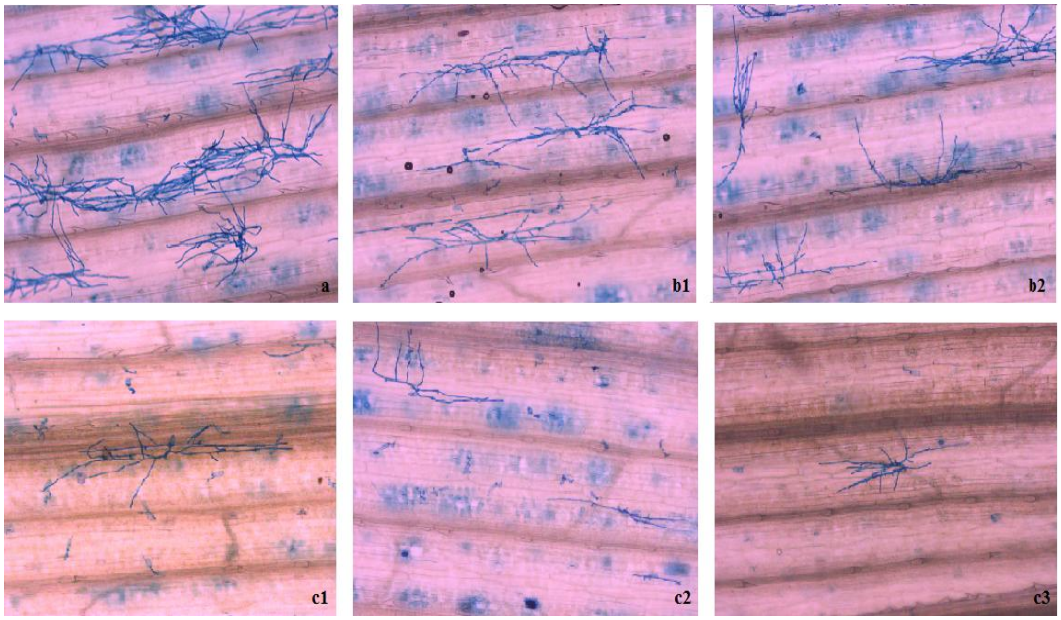

Figure 3.16 The pathogen growth (*Bgh*103) differences at 3 dpi on the control and silenced group leaves. *Blumeria graminis* f.sp. *hordei* (*Bgh*103) inoculation was conducted on 14-days after BSMV inoculation of 10-days old plant (Pallas-03). Pathogen staining was performed using tryphan blue dye and all pictures were taken in 40X magnification by light microscobe. **a)** Mock; only FES treated leaf sample. **b)** BSMV:00 treated leaves (b1, b2). **c)** *HvRAD23* gene silenced: BSMV:*HvRAD23* treated samples (c1, c2, c3).

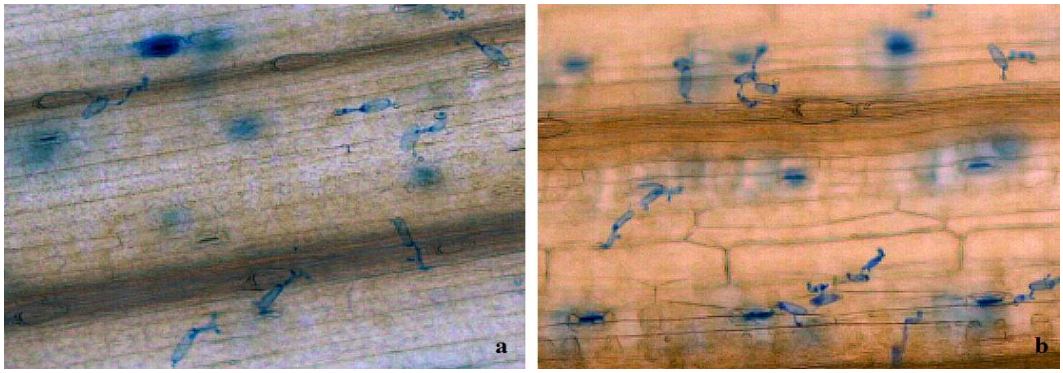

Figure 3.17 Resistance against powdery mildew disease (*Bgh*95/Pallas-03). The pictures were taken in 40X magnification by light microscobe. In resistance interaction, the pathogen is capable of forming just primary germ tube and appresorium without haustoria. **a)** BSMV:00 treated Pallas-03, inoculated with *Bgh*95 (control group). **b)** BSMV:*RAD23* treated Pallas-03, inoculated with *Bgh*95 (silenced group).

All hyphae lengths at 3 dpi were individually measured and were classified according to primary, secondary and longest hyphae of silenced and control groups (Section 2.5.5). The measurements were performed on a total of 284 *Bgh*103 spores and the average of hyphae lengths of each leaf were separately calculated to use for the calculation of the average hyphae lengths of each group as indicated in Figure 3.18. The results indicate 21 % difference in the primary hyphae lengths (Figure 3.18, a), 22 % difference in secondary hyphae lengths (Figure 3.18, b) and 16 % difference in the longest hyphae lengths (Figure 3.18, c) in between the control (BSMV:00) and silenced groups (BSMV:*HvRAD23*).

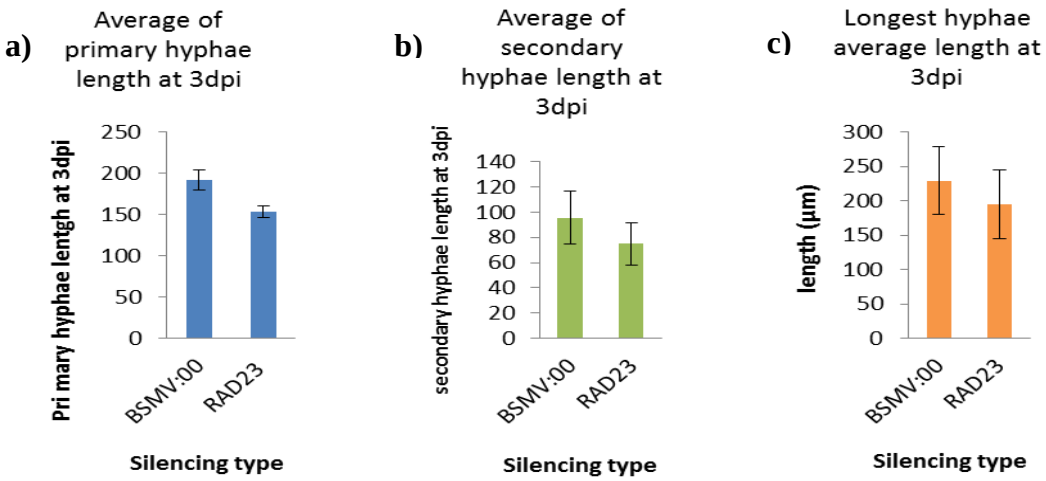

Figure 3.18 Graphic representation of the difference of *Blumeria graminis* growth at 3 dpi. Averages of **a)** the primary hyphae length (n=608, p= $9x10^{-7}$), **b)** the secondary hyphae length (n=484, p= $8x10^{-3}$), **c)** the longest hyphae length (n=247, p= $4x10^{-7}$) of BSMV:00 and BSMV:RAD23 treated plant samples. (**p:** p-value)

The p-value was calculated by performing t-test *via* MS excel in order to show that the calculations are statistically significant (p-value <0.05). For the t-test calculations, the means of the hyphae sizes on individual samples were used. One tail option was selected since the direction can be estimated; increased pathogen growth ratio in silenced group with regard to control group. The type 3 was chosen since the variants are not equal, *HvRAD23* gene silencing may result in several changes in the cells.

A leaf from each group was left on agar plates to observe pathogen growth with naked eye. At 10 dpi, there was no difference between silenced and control groups of Pallas-03 inoculated with *Bgh*95 as shown in Figure 3.19 (c, d). Silencing of Hv*RAD23* gene did not influence the pathogen growth in Pallas-03/*Bgh*95 because of resistance interaction. However after inoculated with

*Bgh*103, in terms of pathogen growth rate, the obvious difference was observed between two groups as indicated in Figure 3.19 (a, b).

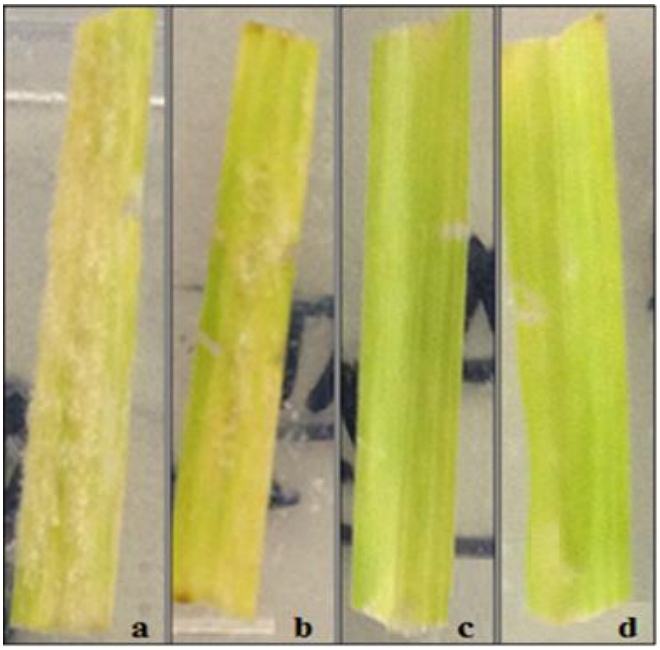

Figure 3.19 The images taken from the leaves at 10 days post pathogen inoculation. **a)** BSMV:00 treated Pallas-03, inoculated with *Bgh*103. **b)** BSMV:*RAD23* treated Pallas-03, inoculated with *Bgh*103. **c)** BSMV:00 treated Pallas-03, inoculated with Bgh95. **d)** BSMV:*RAD23* treated Pallas-03, inoculated with Bgh95.

3.4 qRT-PCR

3.4.1 Total RNA isolation from control and silenced leaf samples

In order to evaluate the silencing level of *HvRAD23* gene, leaf samples (Figure 2.8) were collected at 14-days post BSMV inoculation. Total RNA isolation was conducted from these leaves as in Section 2.6.1 for qRT-PCR analyses. The quality of the total RNAs is significant for further enzymatic reactions. Nucleic

acids give a maximum Absorbance peak at 260 nm, which does not depend on the pH of the solution but some contaminants like proteins in the solution gives a pH-dependent peak at 280 nm. Therefore protein source of contamination decreases the A_{260}/A_{280} ratio and also RNA quality. In order to say 'pure' for RNA, A_{260}/A_{280} ratio should be around 2.00. Additionally, proteins, phenolic compounds and chaotropic salts like guanidinium isothiocyanate sources of contaminants give absorbance peaks at 220-230 nm. All these contaminants can originate from either tissue sample or lysis solutions. The optimum range of A_{260}/A_{230} ratio is between 1.60-2.00 for RNA purity. Lower than 1.60 may lead to inhibition of enzymatic reactions. In our study, we did not have any problem in A_{260}/A_{280} ratio but there was a major problem in A_{260}/A_{230} ratios of isolated total RNAs. The A_{260}/A_{230} ratios were lower than 1.00 and it was observed that holding the spin column membrane in Buffer RPE (wash buffer) for elongated period led to increase A_{260}/A_{230} ratio, resulting from the reduction of the amount of guanidinium isothiocyanate in eluted RNA but still it was not enough to be said pure. Thus, it was thought that this problem was not derived from lyses buffer and it resulted from tissue samples. Phenolic compounds derived from tissue sample might significantly have reduced A_{260}/A_{230} ratio. To prevent this, while homogenizing, polyvinyl-pyrrolidone (PVP) ($2x10^{-5}$ g/μL lyses buffer, Sigma Lot# 116H0685) was added since 'PVP' forms complexes with polyphenolics through hydrogen bonds to remove them from the homogenate (Maliyakal, 1992). In this way, the problem related to low A_{260}/A_{230} ratio was overcome (Table 3.2).

Table 3.2 Concentrations of isolated total RNAs.

Samples	RNA type		Concentration	A_{260} / A_{280}	A_{260} / A_{230}
C-1	BSMV:00	replicate 1	46.5 ng/µL	1.99	1.85
C-2	BSMV:00	replicate 2	72.9 ng/µL	2.11	1.81
C-3	BSMV:00	replicate 3	64.3 ng/µL	2.12	2.00
S-1	BSMV:*HvRAD23*	replicate 1	60.2 ng/µL	2.13	2.29
S-2	BSMV:*HvRAD23*	replicate 2	74.7 ng/µL	2.12	2.20
S-3	BSMV:*HvRAD23*	replicate 3	41.3 ng/µL	2.12	1.92
S-4	BSMV:*HvRAD23*	replicate 4	102.7 ng/µL	2.17	2.07
S-5	BSMV:*HvRAD23*	replicate 5	34.1 ng/µL	2.00	1.79
S-6	BSMV:*HvRAD23*	replicate 6	47.2 ng/µL	2.08	2.22

C: Control samples, inoculated with BSMV:00 **S:** Silenced samples, inoculated with BSMV:*HvRAD23.*

As seen in Table 3.2, the results indicate that the RNA qualities are sufficient. The yields of RNAs are not high but this did not pose a problem during enzymatic reactions.

3.4.2 DNase treatment

The amounts of RNAs were adjusted to 162 ng to DNase treatment, performed in Section 2.6.2. At the end of the DNase treatment, EDTA was used to inactivate to DNase enzyme in order to inhibit the degradation of RNA samples in the further enzymatic reactions. Then, PCR was conducted (Section 2.4.7) so as to ensure whether there was genomic DNA contamination in eluted RNA. Since genomic DNA source of contamination (even if a small amount) may lead to significant differences in qRT-PCR due to the 2^{40} fold amplification of ever signals at the end of qRT-PCR. The amplification products were electrophoresed on 1.5 % agarose gel. According to the electrophoresis result, there is no genomic DNA contamination in RNA samples (Figure 3.20).

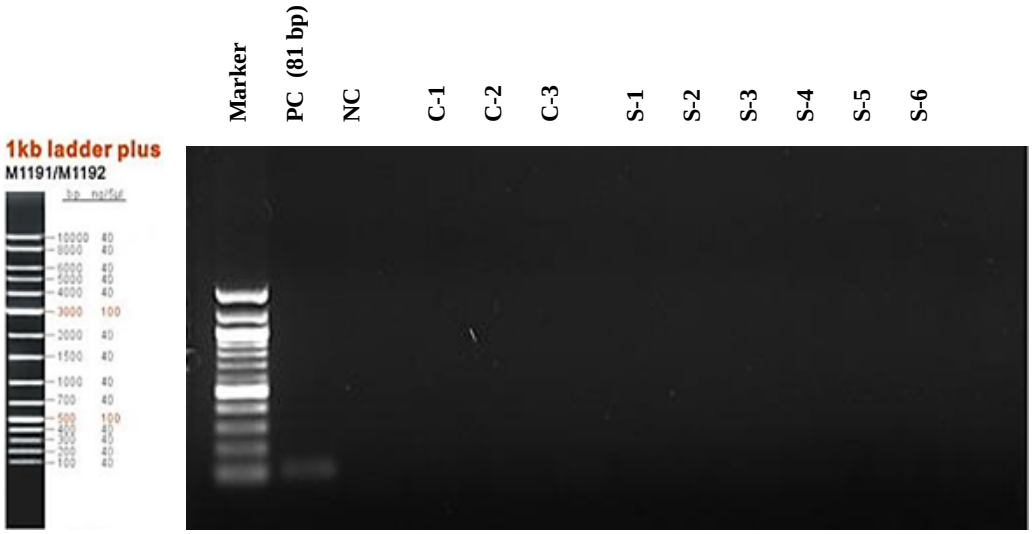

Figure 3.20 The agarose gel picture of DNase treated RNAs from control and silenced leaf samples. **C:** Control **S:** Silenced **PC:** pBSK-*HvRAD23* plasmids were used as a template. **NC:** No template. Elongation Factor primer sets (EF) (Table 2.7) were used for amplification; thus, in PC the band was expected to present at 81 bp.

3.4.3 cDNA synthesis

cDNA synthesis was conducted from DNase treated total RNAs from control and silenced samples in order to perform qRT-PCR analyses. For this reaction, to obtain full length cDNA synthesis, two type primers were used together; oligo (dT) binding poly-A tails of mRNAs and random hexamer annealing to random complementary sites on mRNAs. Pre-incubations are important for binding of the primers to the template and to eliminate the minor structures in the RNA. After cDNA synthesis, PCR was performed to verify it (Section 2.4.7). EF primer set was used for cDNA amplification. Then, samples were analyzed on 1.5 % agarose gel *via* electrophoresis. The electrophoresis results express that cDNAs are sufficiently synthesized from total RNAs (Figure 3.21).

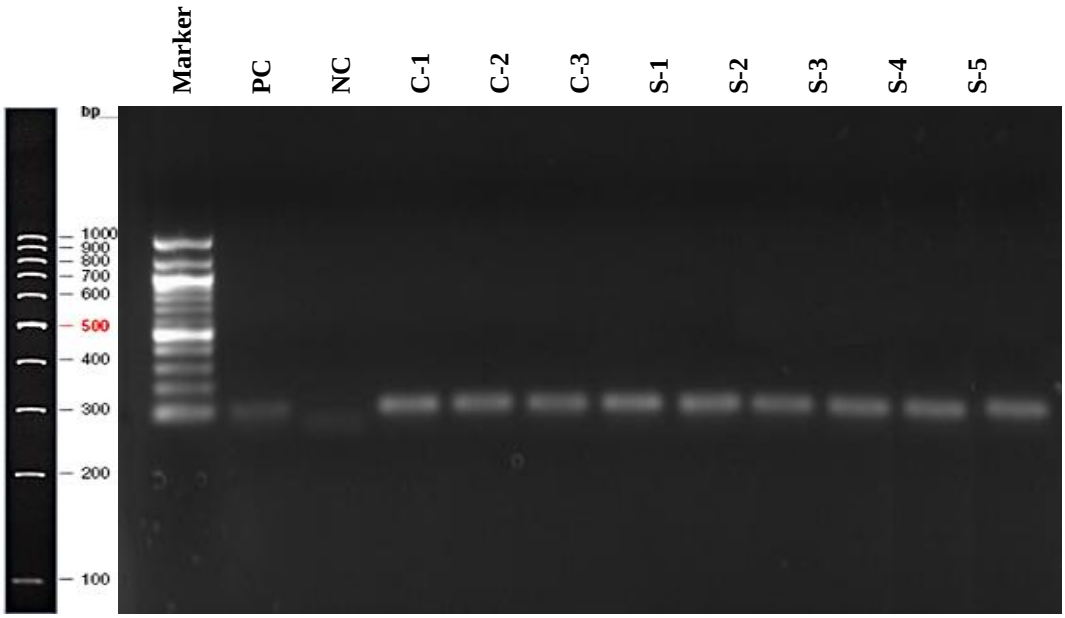

Figure 3.21 The agarose gel picture of PCR results of cDNAs. **C:** Control **S:** Silenced **PC** (Positive control)**:** pBSK-*HvRAD23* plasmids were used as a template. **NC** (Negative control)**:** No template. **M:** 1 µL of 1 kb DNA marker (0.5 µg/µl, Fermentas).

3.4.4 Determination of reference genes

Normalization is very important in qRT-PCR analysis due to the requirement of compensation for inter and intra kinetic variations of real-time PCR, resulting from starting amount difference between the samples, RNA integrity difference, loading variation of cDNA samples or difference in real time effectivity.

In this thesis, for normalization, five candidate reference genes were tested to find out the most reliable two reference genes whose expression levels are not influenced under our experimental conditions. Tested reference genes are Actin, Elongation Factor (EF), Ubiquitin (Ubi), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin, which are the most common reference genes used for normalization in plants as internal standards. Following the qRT-

PCR, computer based statistical method, called geNorm, was applied to determine the most stable two housekeeping genes which are used for the quantification of further qRT-PCR experiments as references. This determination is based on M-values of the genes, which identify a gene variation compared to other candidate reference genes. The reference genes having the smaller M-value than 1.5 show the most stable gene expression while the genes possessing M-value higher than 1.5 M-value express the variable gene expression. In this study, all M-values were lower than 1.5 as indicated in Table 3.3. Following the automatically M-value calculation, geNorm software compares all until finding out the most stable two reference genes. In this study, after determining the average expression stability values of remaining control genes (Figure 3.22), the Elongation Factor and Ubiquitin were chosen as the most reliable reference genes by the geNorm program in order to use in silencing level determination *via* qRT-PCR. The amplification plots and dissociation curves are shown in Figure 3.23. The amplification plots indicates the increases in fluorescence from BSMV:00 and BSMV:*RAD23* samples. The cross-section between an amplification curve and a threshold line is called 'threshold cycle' (Ct). Ct value describes as the number of cycles needed for the fluorescent signal to intersect the threshold. All Ct values of the samples were given in Appendix D.

SYBR green dye can bind to any double-stranded DNA; therefore, to remove this misbinding, a dissociation curve is plotted by continuously reading of the fluorescence (in every 2 ^{o}C changes in temperature) until whole DNA denaturation is achieved. Therefore, the dissociation curve is more than one if there are different DNA fragments. In this study, all products gave a single peak as expected as shown in Figure 3.23, graphs on left.

Table 3.3 M-values of candidate reference genes according to geNorm software.

Reference Gene	M-value
Actin	0.425
EF	0.500
α-tubulin	0.772
Ubi	0.436
GAPDH	0.372

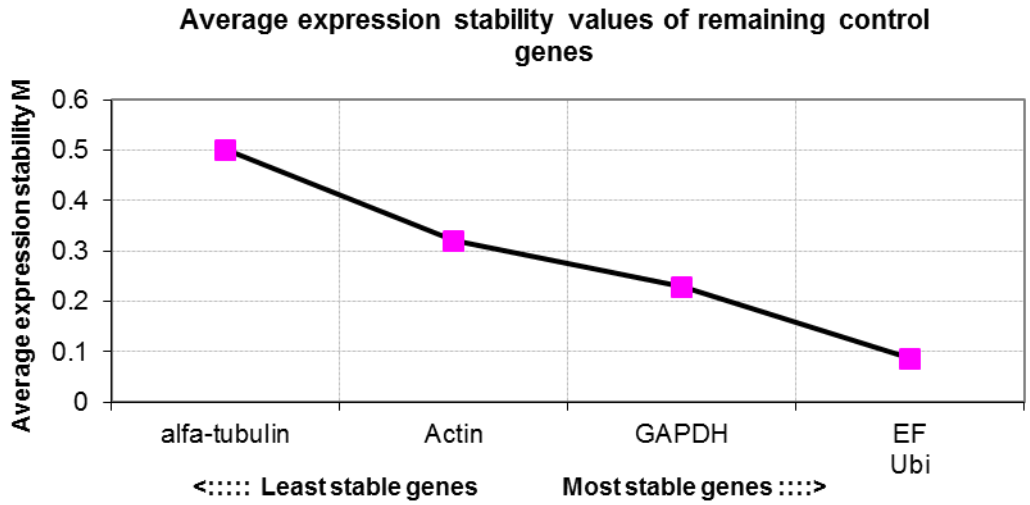

Figure 3.22 Graphic representation of the M-values of reference genes.

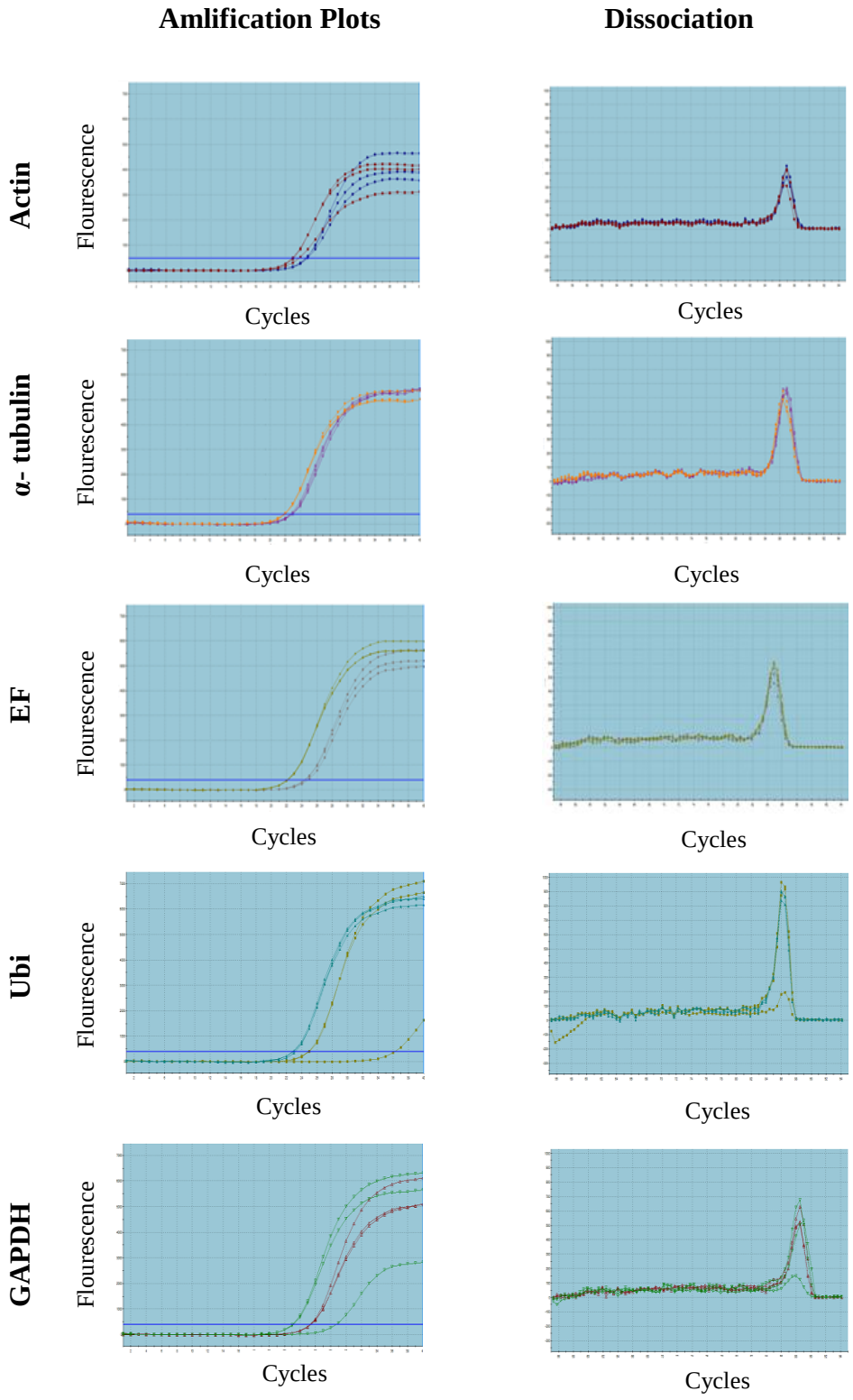

Figure 3.23 Amplification plots (left) and dissociation curves (right) of the reference genes.

3.4.5 Expression level determination of *HvRAD23*

The calculation of amplification efficiency is necessary for the exact quantification since the efficiency of PCR reaction can influence Ct value. Therefore, before expression level detection, qRT-PCR was performed using a dilution series (1/8, 1/16, 1/32) of cDNAs from BSMV:*HvRAD23* inoculated leaf sample to determine the most efficient amplification. The amplification efficiency of the genes was described in figure 3.24.

The coefficient of determination (R^2) should be ≥ 0.98 to the the better variance. For this experiment, R^2 values are 0.9426 for Actin, 0.9942 for Elongation Factor, 0.9972 for Ubi, 0.9933 for GAPDH, 0.9906 for α-tubulin and 0.9752 for *RAD23*.

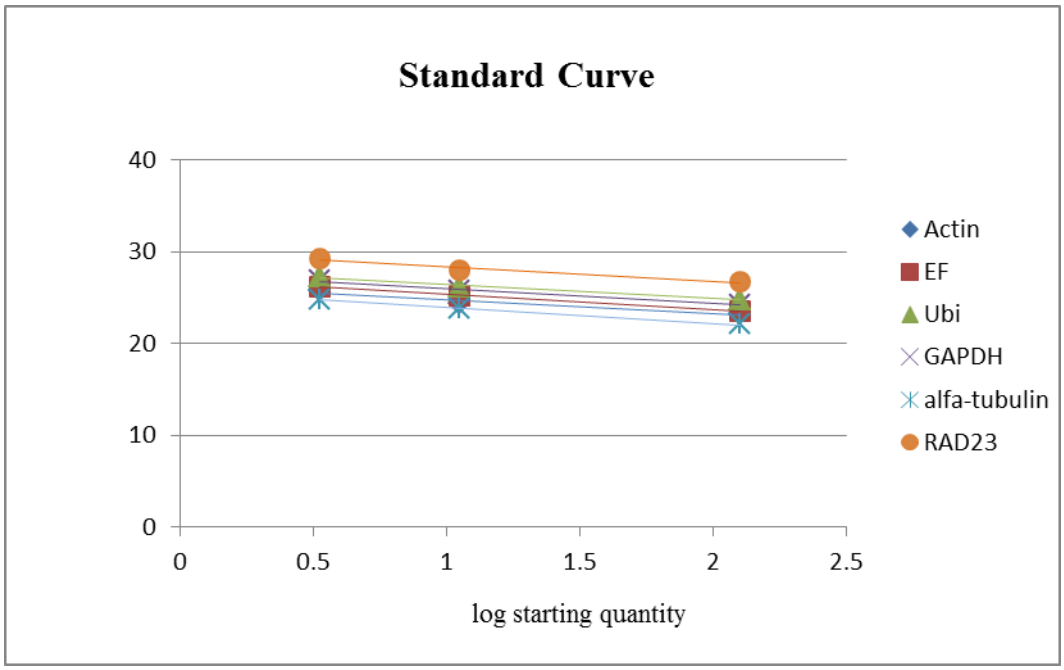

Figure 3.24 The efficiency and linearity of qRT-PCR amplification.

Following the selection of the reference genes and the calculation of the amplification efficiency, one more qRT-PCR was conducted as in Section 2.6.4 so as to determine the silencing level of the targeted gene (*HvRAD23*). Silencing level for each sample was calculated according to Pfaffl (2001) (Table 3.4). For which, the arithmetic mean of the average was calculated to Ct values in C-1, C-2 and C-3 in order to obtain one Ct value. Then, the geometric means of the Ct values obtained by reference genes Elongation Factor and Ubiquitin primer sets computed to obtain one Ct value again. The following calculations were conducted as in Section 2.6.5. The graphic representation of the expression levels of *HvRAD23* gene in each silenced samples is given in Figure 3.25. *HvRAD23* gene silencing was not seen in silenced samples; S-3, S-4 and S-5. In addition, there is no significant difference in *HvRAD23* expression levels between control and the silenced samples.

Table 3.4 Silencing levels of *HvRAD23* gene in silenced plant samples.

Sample	**Silencing level (%)**
S-1	27
S-2	15
S-3	No silencing
S-4	No silencing
S-5	No silencing
S-6	10
Average	**17 %**

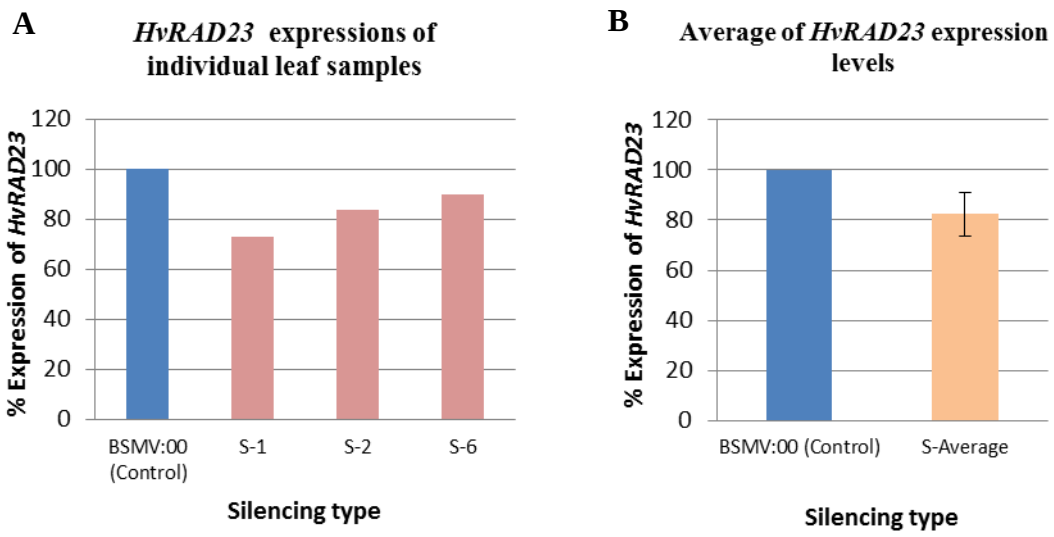

Figure 3.25 Graphic representations of the expression levels of *HvRAD23* gene in silenced and control samples. BSMV:00 treated samples were used as a control to compare the *HvRAD23* gene silencing levels in silenced plant samples. **A)** *HvRAD23* gene expression level in each leaf sample. **B)** The average of *HvRAD23* gene expression levels of silenced samples. **S:** *HvRAD23* silenced sample.

CHAPTER 5

CONCLUSION

Barley (*Hordeum vulgare* ssp. *vulgare*) is one of the most important members of the grass family used for food and feed, also in beer making industry. Due to it signification in consumption the functional genomics and gene function studies capture a quite attention. Powdery mildew disease of crops is a serious fungal disease affecting a wide range of plants and leads to yield loss, especially in barley. In this thesis, the functional characterization of *HvRAD23* gene in plant disease resistance against powdery mildew was studied. For this purpose, the functional genomics application, VIGS, was performed.

In the previous proteomics studies, conducted by Neşe Özgazi and Assist. Prof. Dr. Aslıhan Günel, the *RAD23* gene product was only observed in compatible interaction between the pathogen and barley (*Bgh*103/Pallas-03) which means that *RAD23* gene may possess a negative regulatory role in plant defense mechanism. To confirm the hypothesis, BSMV mediated virus induced gene silencing (VIGS) was applied on the barley plants. Following VIGS, the pathogen growth rate evaluation was performed on the BSMV treated leaf samples and then the silencing level of *HvRAD23* gene was determined *via* qRT-PCR.

The *HvRAD23* silenced Pallas-03 seedlings were analyzed upon virulent (*Bgh103*) and avirulent races (*Bgh95*) of powdery mildews. In the silenced plants with the susceptible pathogen race treatment (*Bgh*103), very low level of hyphal formations was observed. This was also verified with the measurements of the

hyphae of each spore, which indicates the 21 % difference in primary hyphae lengths, 22 % difference in secondary hyphae lengths and 16 % difference in the longest hyphae lengths between BSMV:00 (control) and BSMV:*RAD23* (silenced) treated groups. However, in qRT-PCR analysis, it was observed that among six samples, the *HvRAD23* silencing was observed in only three silenced leaf samples and unfortunately, the average of silencing levels was not high (17 %). Indeed, the silenced leaf samples which showed low pathogen growth after *Bgh*103 inoculation were lost in RNA isolation experiments due to the problem mentioned in Section 3.4.1. The silencing efficiency in our experiment was low; nevertheless, the difference in the development of the hyphae formation on the compatible pathogen inoculated silenced and control plants were statistically significant and the level of silencing were correlated well. This preliminary result supports the *RAD23* acting as a negative regulator of disease resistance mechanism in plants.

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APPENDIX A

BSMV GENOME SEQUENCES

BSMV α genome (Accession # : U35767.1)

GTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCACATAATCGTAATCGATTCTTCTTGA
TCTCTAAACAACACTTTCCCGTTAGCATGGCTAGCGATGAGATTGTCCGCAATCTGATCTCCCGT
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GACAGAATACCTCTTAGATATGGTTCCAATGAAAGAGACTTTTGTTTATGACATTGGTGGTAACT
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BSMV β genome (Scofield, 2007)

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCT
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BSMV γ genome (Scofield, 2007)

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Sequence of γ.bPDS4As (pSL39B-1) (Scofield, 2007)

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PLASMID SEQUENCES

pα plasmid (Scofield, 2007)

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pβ plasmid (Scofield, 2007)

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pSL39B-1 plasmid (Scofield, 2007)

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AAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAT
CCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTC
CACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGCCCAC
TACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAAC
CCTAAAGGGATGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAGGAAGGG
AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCAC
CACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGCTGCGCAACTG
TTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTG
CAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAACGACGGCCAGTG
AATTAATACGACTCACTATA

APPENDIX B

SEQUENCES of CANDIDATE REFERENCE GENES

Actin (Accession # : AY145451.1)

GAATTCCATTCTGTAGGAAATGGCTGACGGTGAGGACATCCAGCCCCTTGTCTGCGACAATGGAA
CCGGAATGGTCAAGGCTGGTTTCGCTGGAGATGATGCGCCAAGGGCTGTTTTCCCTAGCATAGTT
GGTCGCCCTCGGCACACTGGTGTCATGGTAGGGATGGGGCAGAAGGATGCTTATGTTGGTGATGA
GGCGCAGTCCAAGAGAGGTATCCTCACGCTCAAGTACCCCATCGAGCACGGTATCGTAAGCAACT
GGGATGACATGGAGAAAATCTGGCATCACACTTTCTACAATGAGCTCCGTGTGGCACCCGAGGAG
CACCCTGTGTTGCTCACTGAGGCCCCTTTGAACCCAAAAGCCAACAGAGAGAAGATGACCCAGAT
TATGTTTGAGACTTTCAATGTTCCTGCCATGTACGTCGCTATTCAGGCCGTGCTTTCCCTCTATG
CAAGTGGTCGTACTACTGGTATCGTTCTCGACTCTGGTGATGGTGTCAGCCACACTGTGCCCATT
TATGAAGGATACGCGCTTCCCCATGCCATTCTTCGTTTGGATCTCGCTGGTCGGGATCTCACGGA
CTCCCTTATGAAGATCCTCACCGAGAGAGGTTACTCCTTCACAACCTCAGCTGAGCGGGAAATTG
TAAGGGACATCAAGGAGAAGCTTGCGTACGTTGCCCTTGATTATGAACAGGAGCTGGAGACTGCC
AAGAACAGCTCCTCAGTTGAGAAGAGCTACGAGCTTCCTGATGGTCAGGTGATCACGATTGGCGC
AGAGAGGTTCAGGTGCCCTGAGGTCCTCTTCCAGCCATCCATGATCGGCATGGAGTCTTCTGGAA
TCCACGAGACGACCTACAACTCCATCATGAAGTGTGACGTGGATATCAGGAAGGACTTGTATGGA
AACATCGTGCTCAGTGGTGGCACAACTATGTTCCCAGGTATCGCTGACCGTATGAGCAAGGAGAT
CACCGCCCTTGCTCCGAGCAGCATGAAGATCAAGGTCGTCGCTCCACCTGAGAGGAAGTACAGTG
TCTGGATCGGAGGGTCCATCCTAGCCTCACTCAGCACTTTCCAACAGATGTGGATATCCAAGGAT
GAGTACGACGAGTCTGGCCCAGCGATCGTCCACAGGAAGTGCTTCTGATCTCCACGAGCGCTCCA
CTGCTGTTATCATCTAGTCTTCGGTTATGTTTGGTTCATTCTTCTAGAAATGTATTGCGTATTTG
CAAGCTATGTTTTTTTCCAGACGTGACGTGGGTACTCTTGGGATACGCCACCTATATACGTGGCG
GCTCCATGGTGCAAGTGCAAGTACACTATCTATGTTTGTGCATTGTCAGTGTGTTTGTGGGATCA
GTTGTCAAACTTGGGTTGGCTTGATTTGTTGTTGGGAATTGTCTGTAAAGGAATTC

GAPDH (Accession # : M36650.1)

GTCAACGACCCGTTCATCACCACCGACTACATGACCTACATGTTCAAGTATGACACTGTCCACGG
ACAGTGGAAGCACCATGAAGTTAAGGTGAAGGACTCCAAGACCCTTCTCTTCGGTGAGAAGGAGG
TTGCTGTGTTTGGTTGCAGAAACCCCGAGGAGATTCCATGGGCCGCTGCTGGTGCTGAGTACGTT
GTGGAGTCCACCGGTGTTTTCACTGACAAGGACAAGGCTGCAGCTCACATTAAGGGTGGTGCCAA
GAAGGTCATCATTTCTGCTCCCAGCAAGGACGCTCCCATGTTTGTCTGTGGTGTCAACGAGAAGG
AATACAAGTCAGACATCGACATTGTCTCCAATGCTAGCTGCACCACCAACTGTCCTGCTCCTCTT
GCTAAGGTTATCAATGACAGGTTTGGCATTGTTGAGGGTTTGATGACCACTGTCCATGCCATGAC
TGCTACCCAGAAGACTGTTGATGGTCCTTCAAGCAAGGACTGGAGAGGTGGAAGGGCTGCTAGCT
TCAACATCATTCCAAGCAGCACTGGTGCTGCAAAGGCCGTTGGCAAGGTGCTCCCAGAACTTAAC
GGAAAGTTGACTGGAATGGCCTTCCGTGTTCCCACTGTTGATGTTTCTGTTGTTGATCTGACTGT
TAGACTTGCCAAGCCAGCCACCTATGAGCAGATTAAGGCTGCTATCAAGGAGGAGTCTGAGGGAA
ACCTCAAGGGCATTTTGGGTTATGTCGATGAGGACCTTGTTTCCACTGACTTCCAGGGTGACAGC
AGGTCCAGCATCTTTGATGCCAAGGCCGGGATTGCTCTGAACGACAACTTTGTCAAGCTTGTCTC
ATGGTACGACAACGAGTGGGGATACAGCACCCGTGTGGTCGACCTCATCCGCCACATGCACAGCA
CCAAGTAAATGAGCCAAAGCATGAAGATACAGGGAGTGTGGTTTGCCCCAGAGAAGAGAAGAGTG
TACAACCTCTTCCGAGAATAAATTTTTGTATGGAATTATGGCAACTAAAAAAAACCTTTTATTGG
ATGATCCTGATGGTTGGTTGAGCTTAGCGGCTCACATTTTGGTGGTATTATGTACTTGCTTGAAC
TAAATCATGAGTTATTTTCACTCATCGTCATGGGTT

Elongation Factor (Accession # : Z50789.1)

CGGCCTCGCTTTGCGACCCCTCCGTTCGTTCTTCTTCTTCGAGTTTGTTATCAGCCATGGGTAAG
GAGAAGACTCACATCAACATCGTGGTCATTGGCCACGTCGACTCTGGCAAGTCGACCACCACTGG
CCACTTGATCTACAAGCTTGGTGGCATTGACAAGCGTGTGATCGAGAGGTTCGAGAAGGAAGCCG
CTGAGATGAACAAGAGGTCATTCAAGTACGCGTGGGTGCTTGACAAGCTCAAGGCTGAGCGTGAG
AGAGGTATCACCATCGATATTGCCCTCTGGAAGTTCGAGACCACCAAGTACTACTGCACCGTCAT
TGATGCCCCTGGTCACCGTGACTTCATCAAGAACATGATCACGGGTACCTCCCAGGCTGACTGTG
CTGTTCTCATCATTGACTCCACCACTGGTGGTTTTGAGGCTGGTATCTCCAAGGATGGCCAGACA
CGCGAGCACGCTCTCCTTGCTTTCACTCTTGGAGTGAAGCAGATGATCTGCTGCTGCAACAAGAT
GGACGCCACCACTCCCAAGTACTCGAAGGCACGTTATGAAGAAATTGTTAAGGAGGTCTCTTCCT
ACCTGAAGAAGGTCGGCTACAACCCTGACAAGGTTCCCTTCGTCCCCATCTCTGGGTTTGAGGGT
GACAACATGATTGAGAGGTCCACCAACCTTGACTGGTACAAGGGCCCAACCCTGCTTGAGGCGCT
TGACCAGATCAACGAGCCCAAGAGGCCCTCAGACAAGCCCCTCCGTCTTCCCCTCCAGGACGTTT
ACAAGATTGGTGGCATTGGAACTGTGCCTGTTGGCCGTGTTGAGACTGGTGTCATCAAGCCTGGT
ATGGTTGTGACCTTTGGTCCCACTGGTCTGACAACTGAGGTCAAGTCCGTTGAGATGCACCATGA

GTCTCTCCTGGAGGCGCTTCCCGGTGACAACGTCGGCTTCAACGTCAAGAACGTTGCTGTGAAGG
ATCTGAAGCGTGGGTTTGTTGCATCCAACTCCAAGGATGACCCTGCCAAGGAGGCAGCCAACTTC
ACCTCCCAGGTCATCATCATGAACCACCCTGGTCAGATTGGCAACGGCTACGCTCCAGTGCTGGA
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GTAAGGAGCTGGAGGCCCTGCCCAAGTTCCTCAAGAATGGTGATGCTGGTATAGTGAAGATGATT
CCCACCAAGCCCATGGTTGTGGAGACCTTTGCCACTTACCCTCCTCTTGGTCGTTTTGCTGTCCG
TGACATGAGGCAAACTGTGGCTGTTGGTGTCATCAAGGGCGTGGAGAAGAAGGACCCCACCGGAG
CCAAGGTGACCAAGGCTGCCATCAAGAAGAAATAGAGGCGTTTACCTGAATCCATCTATGTCTTG
CTTTTGAGATCTCTCTAGTAGTTTAGTAAGTTTTGCTACTGTTAGCGCCGTGTGATATGCGCCCT
GTTGATGTGTCATTTGAGTACTGTCGTGTTATTTCGGCTTTCGGGAAAACATGGATGCTTCTGAA
GACTAATCATTTTATCATCTGTCTGCTTTCGAAAAAAAAAAAAAAAAAA

Ubiquitin (Accession # : <u>M60175.1</u>)

GAATTCATGAGCATGTATACATCTCACTAACCTGTTAACTTCGATGCTACCTCGGAAAAAGAAAC
GAAGCGATGCTGTGACAGTCGTAACCAATGTTTTTTTTTTTTTGCGAAAGTAACCAATTTTCGA
ATCTGTAGTCTTCCTAGTTTATGTATATCTTATTAATTGCGAGGCCAATTCTTTTGCTGGACAAA
ATGTAGAACTTACACATTTACTCTTATATAAAAATAATTTCCACCACTAAATTGAATTTACATAT
TTCTAGGGCTAAATGAATGGAATTTACACGTTTCTTGACTGGAACGAGTTCCTCAGGAGGCATGG
GCCCGAAGCAAAATCCAGCCCAGCCCAAAATAGCGTCCACGAAAGCCAGCCGTCCGATCTGGAAC
CCCTAGATCCAACAGCTAATATGTCCCGCCTACTTGTACTTATAAGAAGGCAGACCCTTTCTGCA
CCCTAGCCTCCGTTCCATCCCGCCGCCCCCGCCCAAACCCCAGCGCGCCGCCGCCACCGTCGCCG
CCGCAGCGCGCGGAGGCGACCACCCCACCGCCGCCAAGATGCAGATCTTCGTGAAGACCCTGACG
GGCAAGACCATCACGCTGGAGGTGGAGTCGTCGGACACCATCGACAACGTCAAGGCCAAGATCCA
GGACAAGGAGGGCATCCCGCCGGACCAGCAGCGCCTCATCTTCGCCGGCAAGCAGCTCGAGGACG
GCCGCACCCTCGCCGACTACAACATCCAGAAGGAGTCCACCCTCCACCTGGTGCTCCGACTCCGC
GGTGGCGCCAAGAAGCGCAAGAAGAAGACGTACACCAAGCCCAAGAAGCAAAAGCACAAGCACAA
GAAGGTGAAGCTCGCCGTCCTCCAGTTCTACAAGGTCGACGACGCCACCGGCAAGGTAACCAGGC
TCAGGAAGGAGTGCCCCAACGCCGACTGCGGTGCCGGGACCTTCATGGCCAACCACTTCGACCGC
CACTACTGCGGCAAGTGCGGACTCACCTATGTCTACAACCAGAAGGCTTAGAACTGGCCTGTGTT
TGCTCTGCTCTTTTACCTATCGCGAATAGAACTCATTTATGTGTCCAGTTTGTCTTTGAAAACTG
AAACCTTGGAGTAATATGTTGTGTTTCCTGGATATTTGATCGCCTATTGCTGAGATGTGATGCGA
GCTTTAAGTTTTGTTCCGTATGCTATGCTATCATTGCTCTGATTGATGCCATCATGGGATATATC
TTGTTGATACAAGTGTTCTTGAGCTTAACCCTTTATTGCAGGTGTTAGTAGCCAATAGCTATGCA
TGGCTTGATATGTGGATGAATATTTTGCTACTGCTGGTTTATGATATGTTGATTGTTGATGTGTG
TGATCTTTGTCACTTGCAATTATAATTCAGTTCATCCCAAGTGATTGCGTGGTTGGTTTGTTTTA
GATACATGTGTTATTTCATCCCTAGATGTGTCTGAAACTGTGGCTGTAGACAGTTGCAGTGTGAT

GATTGCTAGTGTGGAGCATATTGGTGCCCTGTGGTTTATCCACCATTCACCATTGCCTACCCTGT
GGTGCTAAATTTGGTTGGGTCTATCATACCATATGTGACATGTCTATTGGTTTACAGTTGTAGAT
GTAGTTGAGTTTTGCTGTTCTCTTTTCATTTTGTTCTTGGGTTGTGATAAAGTCATTTCACATAC
GCCGATGATATGTAATTTACAACTAATACATAAGATGGCTCAAATGCATTGTATGTGAATGCAAA
CTTGTCACAAATACTACGAGAATTATCTAAACTGCAACATCACTTCATGCATTTGAATTC

α-tubulin (Accession # : <u>Y08490.1</u>)

GGAAAGGCGTCTTCGTACTCGCCTCTCTCCGCGCACACGAGCTCTCGCCCCCTTCCTCCAACCCA
TCTCGCCAGCGGCGCAGCCCAACCACCCGCCACAATGAGGGAGTGCATCTCGATCCACATCGGCC
AGGCCGGCATCCAGGTCGGAAACGCGTGCTGGGAGCTCTACTGCCTCGAGCATGGCATTCAGCCT
GATGGTCAGATGCCCGGTGACAAGACCGTTGGGGGAGGTGATGATGCTTTCAACACCTTCTTCAG
CGAGACTGGTGCTGGGAAGCACGTCCCCCGTGCAGTCTTTGTTGATCTTGAGCCTACTGTGATTG
ATGAGGTGAGGACTGGTGCTTACCGCCAGCTCTTCCACCCTGAGCAGCTTATCAGTGGCAAGGAG
GATGCAGCCAACAACTTCGCCCGTGGTCATTACACCATTGGCAAGGAGATTGTTGATCTGTGCCT
TGACCGTATCAGGAAGCTGTCCGACAACTGCACTGGTCTCCAGGGCTTCCTTGTCTTCAATGCTG
TTGGAGGTGGAACTGGCTCTGGCCTTGGTTCTCTTCTCCTAGAGCGTCTCTCTGTTGACTATGGA
AAGAAGTCCAAGCTTGGGTTCACAGTGTACCCATCTCCCCAGGTGTCCACCTCTGTTGTTGAGCC
ATACAACAGTGTCCTGTCCACCCACTCCCTCCTTGAGCACACCGATGTCTCTATCCTGCTTGACA
ATGAGGCCATCTATGACATCTGCCGCCGCTCCCTTGACATTGAGCGCCCAACATACACCAACCTC
AACAGGCTTGTTTCTCAGGTCATATCATCACTGACTGCTTCCCTGAGGTTTGACGGTGCTCTGAA
TGTTGATGTGAATGAGTTCCAAACCAACCTGGTGCCCTACCCAAGGATCCACTTCATGCTTTCCT
CCTATGCCCCAGTGATATCAGCAGAGAAGGCTTACCATGAGCAGCTGTCTGTTGCCGAGATCACC
AACAGTGCATTCGAGCCTTCCTCCATGATGGCCAAGTGTGACCCCCGCCATGGCAAGTACATGGC
CTGCTGTCTCATGTACCGTGGGGATGTCGTGCCCAAGGACGTCAACGCTGCTGTGGCCACCATCA
AGACCAAGCGCACTATCCAGTTTGTTGACTGGTGCCCCACTGGCTTCAAGTGCGGTATCAACTAC
CAGCCACCTGGTGTCGTCCCAGGGGGCGACCTTGCCAAGGTCCAGAGGGCTGTGTGCATGATCTC
CAACTCCACCAGTGTTGTTGAGGTCTTCTCCCGCATCGACCACAAGTTTGACCTGATGTACGCCA
AGCGTGCCTTTGTCCACTGGTATGTGGGTGAGGGTATGGAGGAGGGAGAGTTCTCTGAGGCCCGT
GAGGACCTTGCTGCCCTGGAGAAGGACTATGAAGAAGTTGGTGCTGAGTTCGACGATGGTGAGGA
TGGTGACGAGGGTGATGAGTACTAGAGCCTGCCTCCTGGTGCTTTCCCAAGGCGTACTACTGCTA
TCCTATGATCTGCCCAAGCGGCTTTATCTGTTATCTGTCTGTTTGAATGTTTGCTTTGTGGTGTT
TGTTTTACAACCTGTTGTGTTGTAAGAACATATTGGTCTGTCTGAACCTAATGTTATGTGCATGC
GGTTATCTATGCCTTGGCCTCTGTCTCCATTTTCCTGTTTCAATATGTGTTACTATCTGGTTAAA
AAAAAAAAAAAAAAAAA

APPENDIX C

THE AMPLIFICATION REGION

ggtcctcccctccctcgcgagtccgaagtcaaggcgaaaaccctcgaggcgaagcgaga
tcgcagatagggataaccgcgagagtcgagtgagtgggttggaagggaagccgagatga
agctcaacgtgaagaccctcaagggcacaagcttcgagatcgaggcgacccccgagtcc
tcggtgggtgaggtcaagagaatcatcgagagtgctcaggggcagaatgtgtaccctgc
ggatcagctgatgatcatatatcaaggaaaaattctcaaggatgataccactctggatg
ctaacaaggttgcagagaacagtttccttgttataatgctgtctaagcctaaggcgtca
tctagtggcgcttcttctgcttcaaaggcacctgttagtcagtctcaacctgctacccc
agttgctgctgctactccacctgtccctgttgcctcagctgcaagatcaccaccttcac
aagcacctgttgctgcatctgaaccggcgcctcccagtgcacagccttcagctgtttct
gatactccagctgctgcagtaactgcatcaggcgatgctgatgtatacagtcaggctgc
atcaaaccttgtctctggcggcattctagaacagacagtccaacaaattcttgacatgg
gtggtggcacctgggaacgtgatatggttgtccgtgctttacgtgctgcgtataacaac
cctgagagggctattgactacctgtattctggaatacctgaatccgtagaggccccacc
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ctcaggctgcacctctaccggcagtgcagccatctggtggtgtctctgctgggcctaat
gcgaatcctctaaacctttcccacagggtgttccaagtggtggggccaatgctggtgc
tggtgtgggtgccggtgccggtgcccttgatgcattgcgacagcttccacagttccaag
cactgcttgcattggtccaggctaatccccaaatcctacagccaatgcttcaagagctg
gggaaacaaaatccacagatcctgcggttgattcaggaaaatcaggctgagtttctccg
cctggtaaatgaaacacctgagagtggtgctggcggaaatatactaggcgcactggcag
ctcaaatgccacaggcagttcaagttactccagaagaacgggaggccatccagcggctt
gagtcaatggggttcaatcgtgagcttgtgttggaggtcttctttgcatgcaacaggga
tgaagaactggctgccaactatcttttggatcatggccacgagtttgaggaacaacaat
agatgttagattggtgttaccgtcgaaagacagagtcagtgacagaagcagtacagttt
ggtgtcgttcttatatcttgctgctgaatatcactactacttgccattagtgagaagct
gctgactttcaatttcttgtatgctggaaatccttttaagtacatattaatatcatat
ctgtgtgtgcagctggtc

Hordeum vulgare subsp. *vulgare* mRNA for predicted protein, complete coding sequence (cds), **Accession#:** AK367564.1 . The sequence in red is the silencing region. Highlighted sequences indicates the specific primers to determine silencing level of *HvRAD23* gene. (Fwd 5'TGAAGGTGGTGATCTTGCAG3' and Rev:5' CTAGTGGCGCTTCTTCTGCT 3'). These primers were designed from the out of the silencing region.

APPENDIX D

Ct VALUES of REFERENCE GENES

Well Name	Replicate	Threshold (dR)	Ct (dR)
00/ Actin	1	393.559	24.47
00/ Actin	1	393.559	24.64
00/ Actin	1	393.559	24.49
R/ Actin	2	393.559	23.44
R/ Actin	2	393.559	22.63
R/ Actin	2	393.559	22.75
NC/ Actin	3	393.559	No Ct
NC/ Actin	3	393.559	No Ct
NC/Actin	3	393.559	No Ct
00/EF	4	393.559	24.44
00/EF	4	393.559	24.49
00/EF	4	393.559	24.86
R/EF	5	393.559	22.09
R/EF	5	393.559	22.21
R/EF	5	393.559	22.19
NC/EF	6	393.559	34.31
NC/EF	6	393.559	32.75
NC/EF	6	393.559	32.16
00/alta tub	7	393.559	22.77
00/alta tub	7	393.559	22.97
00/alta tub	7	393.559	22.97
R/alfa tub	8	393.559	21.9
R/alfa tub	8	393.559	21.94
R/alfa tub	8	393.559	21.96
NC/alfa-tub	9	393.559	36.75
NC/alfa-tub	9	393.559	38.03
NC/alfa-tub	9	393.559	No Ct
00/Ubi	10	393.559	25.34
00/Ubi	10	393.559	25.4
00/Ubi	10	393.559	25.47

R/Ubi	11	393.559	23.16
R/Ubi	11	393.559	28.73
R/Ubi	11	393.559	23.03
NC/Ubi	12	393.559	35.71
NC/Ubi	12	393.559	No Ct
NC/Ubi	12	393.559	39.52
00- GAPDH	13	393.559	36.28
00- GAPDH	13	393.559	24.93
00- GAPDH	13	393.559	25.02
R/GAPDH	14	393.559	22.91
R/GAPDH	14	393.559	22.96
R/GAPDH	14	393.559	23.24
NC/GAPDH	15	393.559	No Ct
NC/GAPDH	15	393.559	No Ct
NC/GAPDH	15	393.559	No Ct

Ct values in red showed high difference compared to other replicates so they did not participate in the calculation. **00:** BSMV:00, **R:** BSMV:*RAD23* treated plant samples (cDNAs)

Replicate	Well Name	Threshold (dR)	Ct (dR)
1	00/Actin	393.559	24.53
2	R/ Actin	393.559	22.88
3	NC/Actin	393.559	No Ct
4	00/EF	393.559	24.59
5	R/EF	393.559	22.16
6	NC/EF	393.559	32.87
7	00/alta tub	393.559	22.9
8	R/alfa tub	393.559	21.94
9	NC/alfa-tub	393.559	38.03
10	00/Ubi	393.559	25.4
11	R/Ubi	393.559	23.71
12	NC/Ubi	393.559	37.64
13	00/GAPDH	393.559	25.6
14	R/GAPDH	393.559	23.04
15	NC/GAPDH	393.559	No Ct

The average Ct values of the replicates of individual samples.