ELUCIDATION OF THE ROLE OF *GCN2* GENE IN RESPONSE TO POWDERY MILDEW INFECTION

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ABSTRACT

ELUCIDATION OF THE ROLE OF *GCN2* GENE IN RESPONSE TO POWDERY MILDEW INFECTION

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Plant immune system is entirely based on the immunities of the individual cells in which systemic signals originate from the infection sites. Powdery mildew disease is one of the agents causing these infection sites, resulting in significant yield losses, if disease develops. Understanding the molecular basis of plant-pathogen interactions is the new trend for fighting against plant pathogens, since classical methods used in selection of resistant plants are becoming less and less efficient nowadays. Thus, finding out the genes which are responsible in plant's resistance is becoming very important.

In this thesis, effect of 'General Control Nondepressible-2' (GCN2) homolog protein in barley defense mechanism was aimed to be studied. The GCN2 of yeast was

previously identified in our laboratory as an interacting protein when the yeast cDNA library was screened with a putative yellow rust R gene (Yr10) fragment. There are reports available in the literature for the function of GCN2 protein, which makes it a good candidate for a role in disease resistance. Thus, the barley homologue of *GCN2* might have a role in the R protein mediated early disease response of which may be proceeding *via* Programmed Cell Death (PCD). In order to observe such function of *HvGCN2* in barley, silencing of its expression *via* Virus Induced Gene Silencing (VIGS) was investigated. Therefore, the *GCN2* homologue was found to function as dampening the severity of the disease.

The silencing with triple technical replicates was observed in 5 of the 6 samples, at an average of 43.2% by qRT-PCR analysis. The pathogen growth levels at different time points were analyzed under light microscope on the silenced and the control samples by measuring the primary and secondary hyphae lengths. The total of 24 seedlings and 292 individual spores were analyzed, and then the level of disease formation was quantitated with 603 primary hyphae and 106 secondary hyphae measurements. Up to 25% hyphae growth rate differences between the control and silenced groups were observed with a probability value less than 0.05 on t-test.

Keywords: Barley (*Hordeum vulgare*), powdery mildew (*Blumeria graminis* f. sp. *hordei*), qRT-PCR, 'General Control Nondepressible-2' (*GCN2*), 'Virus induced gene silencing' (VIGS).

ÖZ

ARPADA KÜF HASTALIĞINA KARŞI *GCN2* GEN ROLÜNÜN AÇIKLANMASI

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Bitki savunma mekanizması tamamıyla bitki hücrelerinin bireysel savunma mekanizmaları ile enfeksiyon bölgelerinden yollanan sistemik sinyaller sonucunda oluşur. Küf hastalığı bu enfeksiyona neden olan hastalıklardan biri olup, hastalığın gelişmesi durumunda önemli miktarlarda verim kaybına yol açmaktadır. Hastalığa dirençli bitki seçiliminde kullanılan klasik yöntemlerin her geçen gün etkisini yitirmesi dolayısıyla, bitki-patojen etkileşiminin moleküler yapısının anlaşılması günümüzde önem kazanmıştır. Bu nedenle, bitkinin savunma mekanizmasına yardımcı olan genlerin bulunması büyük önem arz etmektedir.

Bu tezde, arpadaki 'General Control Nondepressible-2' (GCN2) homolog proteininin bitki savunma mekanizmasındaki etkisinin gözlemlenmesi amaçlanmıştır. cDNA kütüphanesi taranması sonucunda, mayanın GCN2 proteininin sarı pas R geni (Yr10)

parçası ile etkileşimde olduğu gözlemlenmiştir. Literatürde, GCN2 proteinini bitki hastalıklarına karşı savunma ile ilişkilendirebilecek bir çok rapor mevcuttur. Sonuç olarak, GCN2 proteininin arpa homoloğu, R protein aracılığıyla olan ve programlanmış hücre ölümü şeklinde görülen erken hastalık tepkisinde bir rol sahibi olabilir. Arpadaki *HvGCN2* geninin böyle bir rolü olup olmadığını saptamak için VIGS yöntemi ile ekspresyonunun susturulması incelenmiştir. Sonuç olarak, *GCN2* homoloğunun hastalık etkisini azalttığı gözlemlenmiştir.

Üç teknik tekrar sonucunda 6 örneğin 5'inde %43.2 ortalama susturma olduğu qRT-PCR kullanılarak gözlemlenmiştir. Susturulmuş ve control bitkilerinde farklı zaman dilimlerinde patojen büyüme hızları ışık mikroskobu ile birincil ve ikincil hiflere bakılarak incelenmiştir. Toplamda 24 bitki ve 292 spor analiz edilmiş, hastalık oluşum hızı 603 birincil hif ve 106 ikincil hif uzunluk ölçümleri sonrasında belirlenmiştir. Kontrol ve susturulmuş gruplar arasında %25'e varan hif büyüme hızı farkı gözlemlenmiş ve t-test sonucunda istatistiksel olarak $p<0.05$ düzeyinde anlamlı olduğu saptanmıştır.

Anathar Kelimeler: Arpa (*Hordeum vulgare*), küf hastalığı (*Blumeria graminis* f. sp. *hordei*), qRT-PCR, 'General Control Nondepressible-2' (*GCN2*), 'Virüs-indüklenmesi sonucu gen susturulması' (VIGS).

To my family and the love of my life…

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LIST OF ABBREVIATIONS

ACC:	1-Aminocyclopropane-1-Carboxylic Acid
Avr:	Avirulence gene
APA:	Abscissic acid
PCD:	Programmed cell death
PEG:	Polyethylene glycol
Bgh:	*Blumeria graminis* f.sp. *hordei*
BSMV:	Barley Stripe Mosaic Virus
cDNA :	Complementary DNA
Cp:	Crossing point
Ct:	Treshold cycle
dpi:	Days post-inoculation
dsRNA:	Double-stranded RNA
Edr1:	Enhanced Disease Resistance 1
EF:	Elongation Factor
FAO:	Food and Agriculture Organization
GAPDH:	Glyceraldehyde 3-Phosphate Dehydrogenase
GCN2:	General Control Non-Depresible-2 protein

gDNA:	Genomic DNA
hp:	Hairpin
HR:	Hypersensitive Response
LB:	Liquid Broth
miRNA:	Micro RNA
Mlo:	Powdery mildew resistance gene / Mildew Locus O
mRNA:	Messenger RNA
NTC:	Non-template Control
PAMP:	Pathogen Associated Molecular Patterns
PCD:	Programmed Cell Death
PDS:	Phytoene desaturase
PRR:	Pattern-Recognition Receptor
PTGS:	Post-transcriptional Gene Silencing
qRT-PCR:	Quantitative Real-Time Polymerase Chain Reaction
RdRP:	RNA dependent RNA Polymerase
R-gene:	Resistance gene
RISC:	RNA Induced Silencing Complex
RNAi:	RNA interference
ROS:	Reactive Oxygen Species

SAR:	Systemic Acquired Resistance
siRNA:	Small interfering RNA
u:	Unit
Ubi:	Ubiquitin
VIGS:	Virus Induced Gene Silencing
vir:	Virulence gene

CHAPTER I

INTRODUCTION

1.1. Powdery Mildew of Barley

1.1.1 Barley

Barley, a member of the grass family, (*Hordeum vulgare*) is categorized according to its spike pattern (shattered and non-shattered) and row number (two row or six row). *Hordeum vulgare* has four subspecies; *Hordeum vulgare subsp. spontaneum*, also called *Hordeum spontaneum*, which is a wild barley, *Hordeum vulgare var. distichum*, (two-rowed barley), *Hordeum vulgare var. hexastichon*, (six-rowed barley). Even though barley is considered as a lower rank staple than wheat, it is very adaptable to different climates and can endure harsher environmental conditions such as high salinity, drought, and poor soil (Zohary et al., 2001). According to Food and Agriculture Organization (FAO) (2010), Turkey is the 6th leading barley producer in the World (Figure 1.1). Barley plays an important role in meeting nutritional requirements of humans and animals, also widely used in beer industry (Dean et al., 2012).

Viral and bacterial infections affect barley production, such as *Blumeria graminis* causing powdery mildew disease of barley, *Xnathomonas campestris* that causes bacterial blight and Barley Mild Mosaic Bymovirus that causes necrosis in the tissue of host (Dean et al., 2012).

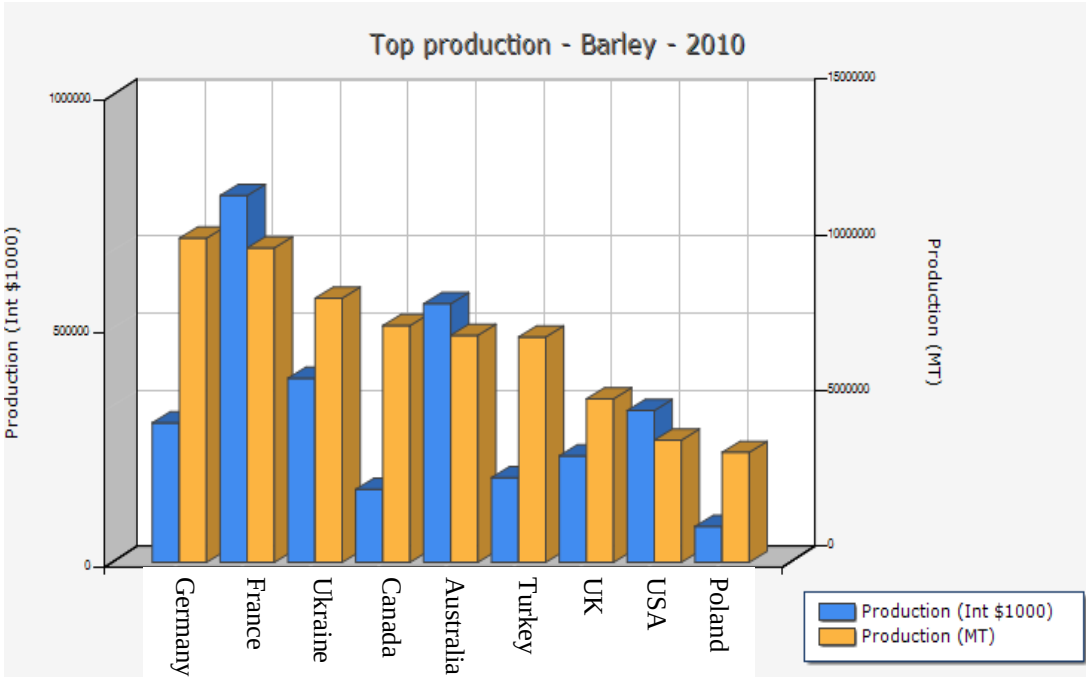


Figure 1.1 World top barley production in 2010 according to FAO. Int $1000: Thousand international dollars, MT: Metric tonne (1000 kg). Image is directly taken from http://faostat.fao.org/DesktopModules/Faostat/Images/T20/ChartPic_omz47wtht75qigzv77p2.png?ca97f000-bce4-4b7f-8ccd-178bdb743b4d

1.1.2 *Blumeria graminis*

Blumeria graminis, also called powdery mildew is obligate parasite causing disease on different varieties of monocotyledonous and dicotyledonous plants worldwide (Ing, 1990). This fungus prefers relatively cool and humid environments to infect and develop (Sooväli and Bender, 2006). The reproduction is through conidia that locates itself onto the host and matures in seven to ten days (Braun et al., 2002). High

specificity in host selection occurs in members of the *Blumeria* family because they affect the *Poaceae* family grasses (Eichmann et al., 2008). Eight different taxonomic groups of *Blumeria graminis* that can infect different members of grass family were identified so far (Braun et al., 2002). *Blumeria graminis* f. sp. *hordei* is the subspecies that can infect *Hordeum vulgare* (Wyand and Brown, 2003). Other than *Blumeria graminis* f. sp. *hordei*, there are other subgroups that can infect oat (f.sp. *avenae*), rye (f.sp. *secalis*), poa (*poae*), ryegrass (*lolii*) and bromus (*bromi*) (Inuma et al., 2007).

Blumeria graminis must be kept under control in the process of agriculture, since it decreases the yield and economical profit (Dean et al., 2012). Currently, fungicides are being used to reduce the infectious capacity of *Blumeria graminis* but the fungus quickly becomes resistant to these chemicals, thus further actions should be taken in order to protect the plants from this pathogen (Glawe, 2008).

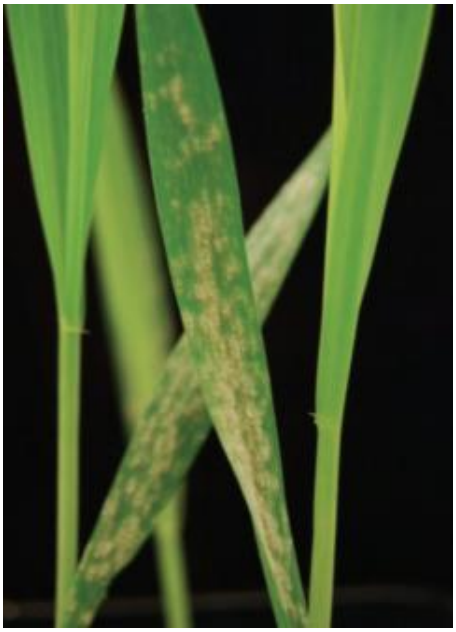


Figure 1.2 *Blumeria graminis* f. sp. *hordei* infection on a barley leaf. Image is directly taken from Dean et al., 2012.

1.1.3 Powdery mildew disease

Powdery mildew is the disease caused by the species of the order *Erysiphales* on many plants. *Blumeria graminis* f. sp. *hordei (Bgh)* infects barley (Wyand and Brown, 2003). In the early years of studies on this pathogen, isolate specific major resistance phenotypic loci were found (Jørgensen, 1994). Later, other different areas in host-pathogen interactions such as compatibility establishment and fungal pathogenesis were studied extensively (Hückelhoven, 2005; O'Connell, 2006). In addition, studying the interaction based on single cell analyses is possible due to cell-autonomous character of plant defense against the powdery mildew infection (Shirasu et al., 1999; Panstruga, 2004). Recently, the genome of *Blumeria graminis* f. sp. *hordei* was sequenced and found to be larger than 120 Mb (Spanu et al., 2010) which will serve producing better strategies against this pathogen.

The asexual life cycle of powdery mildew begins with falling of the spore onto the leaf surface. Several proteins functioning in carbohydrate, lipid and protein metabolism were found to be activated after the pathogen falls onto the leaf, proving that the pathogen is preparing for the catabolism of storage molecules as well as protein synthesis and folding (Noir et al., 2009; Bindschedler et al., 2009). After the spore germination, appressoria development occurs in which the pathogen tries to penetrate the cell wall (Figure 1.3-c). Adhesion of the fungus to the surface of the leaf is thought to be caused by the release of epicuticular alcanes and very long-chain aldehydes (Feng et al., 2009). Another possible reason is that such changes would induce morphogenesis of the fungal structures (Hansjakob et al., 2010).

After the landing of the spore onto leaf, the most important step begins, invading the first cell of epidermis. In order to enter the host cell, the barley must have plant *Mildew Locus O* (*Mlo*) genes (Büschges et al., 1997). In addition, other hosts such as *Arabidopsis thaliana* (Consonni et al., 2006), tomato (Bai et al., 2008), and pea (Humphry, 2011) are also invaded by the pathogens in the presence of *Mlo* genes,

which means there is a common mechanism for the pathogenicity. The gene products of *Mlo* genes are plant-specific integral membrane proteins, however, their biochemical role is still unknown (Devoto et al., 1999). *Mlo* gene products have C-terminal cytoplasmic calmodulin binding domain which enables calcium-dependent calmodulin binding both *in vitro* (Kim et al., 2002) and *in vivo* (Bhat et al., 2005). It was shown that in the sites of fungal penetration at 12-24 hours, when the switch from surface to invasive growth occurs, calmodulin binding to barley *Mlo* increases (Bhat et al., 2005).

After invading the host cell, next step is the formation of haustoria inside the plant cell (Figure 1.3-d). Because they are covered by a separate extrahaustorial membrane, haustoria stay isolated from the host cell. Molecular composition of the extrahaustorial membrane is different from that of conventional host plasmolemma (Koh et al., 2005; Micali et al., 2011).

In the later stages of powdery mildew infection, host cell death suppression occurs. In *Arabidopsis thaliana*, Enhanced Disease Resistance 1 (Edr1) protein is a kinase required for the susceptibility against powdery mildew, whose loss-of-function results in cell death (Frye and Innes, 1998). Increase in expression level of Edr1 is observed after powdery mildew infection, yet Edr1 is not a specific gene for powdery mildew (Christiansen et al., 2011).

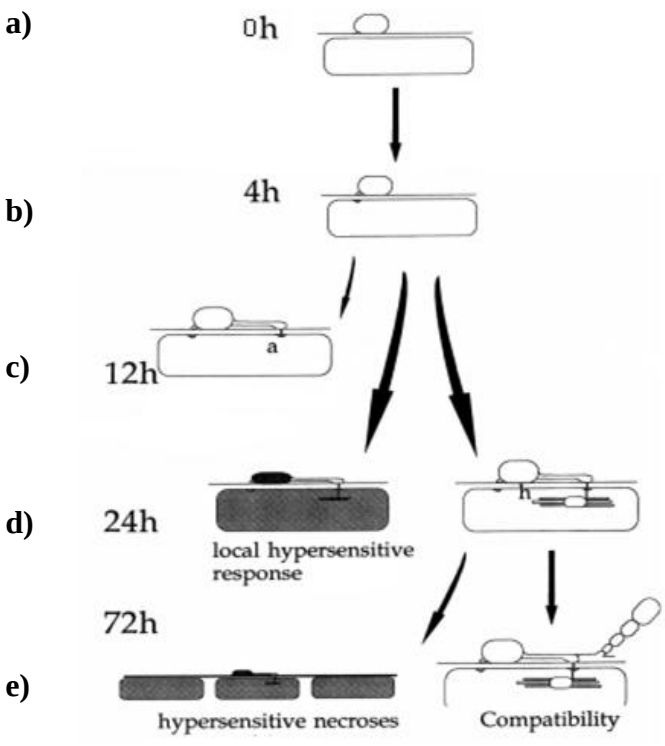


Figure 1.3 Powdery mildew-barley interactions. **a)** The fungus lands onto the cell surface. **b)** After 4 hours, the powdery mildew induces a thickening inside a cell wall named papilla. **c)** After 12 hours, the appressorial germ tube tries to penetrate the cell, marked as a. **d)** After 24 hours, hypersensitive response causes cell death in resistant hosts. In susceptible hosts, haustoria formation occurs. **e)** After 72 hours, extensive multi-cell hypersensitive necroses occur in resistant hosts. In susceptible hosts, the pathogen continues to develop. Image is directly taken from Collinge et al., 1993.

1.2 Disease resistance mechanism in plants

Disease resistance mechanisms in plants are crucial for the protection of host plant from pests, infectious fungi, bacteria, and virus (Brunner et al., 2012). Plant defense mechanisms are far simple than that of mammals because they are devoid of mobile immune cells and they have no circulatory system in order to detect the pathogens (Spoel and Dong, 2012). Instead of that, plant cells use innate immunity of each cell and they use systemic signals which originate from the infection sites (Ausubel, 2005). When the pathogen first gets into contact with the plant, they produce Pathogen Associated Molecular Patterns (PAMP) (Dubery et al., 2012). These PAMPs are recognized by host derived Pattern-Recognition Receptors (PRRs) (Spoel and Dong, 2012). In order to counter, the pathogens that could adapt use effector molecules which suppress plant defense mechanisms (Jones and Dangl, 2006). Through co-evolution with the pathogens, the plants developed intracellular immune receptors in order to counter the effector molecules (Spoel and Dong, 2012). Therefore, effector-triggered immunity is achieved (Figure 1.4). It was hypothesized that direct contact is not obligatory for the activation of Resistance genes (R-genes) (Dangl and Jones, 2001). Avirulence (*Avr*) gene of the pathogen enables it to cause avirulence on the plant in case the host has the corresponding R-gene (Dangl, 1994). It was thought that *Avr* genes encode effector molecules while the host R-gene produce receptors for these elicitors, and after the contact of the receptor and the elicitor, a cascade of events leading to hypersensitive response (HR) occurs (Keen, 1990). Since the cells don't have any circulating system, they have to keep high levels of R-gene products (Dangl and Jones, 2001; Holub, 2001). In case the host does not have the R-gene, the effector molecules cannot be recognized by the host, and the *Avr* gene becomes *virulence* (*vir*) gene.

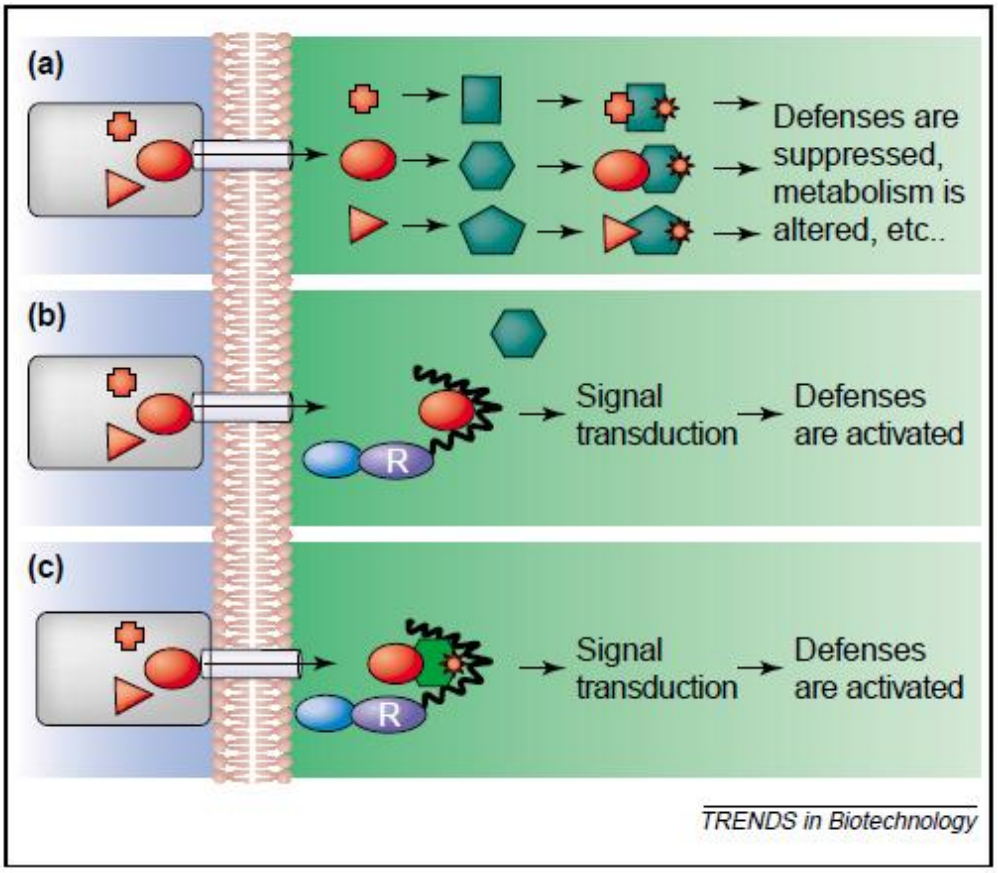


Figure 1.4 Gene for gene resistance model. In order for resistance to occur, both the avirulence gene of the pathogen and the R-gene of the plant must be present. **a)** This picture represents the situation in which the plant has no R-gene. When the pathogen elicitor molecules enter the host cell, plant can't recognize them, and the defenses are not activated or only weakly activated. **b)** If the plant has the R-gene, it may directly bind to the pathogen elicitor molecules which lead signal transduction events. As a result, plant defenses are activated. **c)** This picture represents a typical guard hypothesis. In this case, R protein of the plant senses the presence of a modified host protein (guardee, indicated with a red star), in some cases as a complex with the pathogen elicitor molecules. Image is directly taken from McDowell and Woffenden, 2003.

Although HR is one of the main ways that the plants use to defend themselves against pathogens, there are also other ways such as reinforcement of the tissue or antibiotic production at the site of infection (Hammond-Kosack and Jones, 1996). At the end, these local mechanisms lead to systemic acquired resistance (SAR), which enables the plant to fight against many pathogens (Dong, 2001; Métraux, 2001). SAR needs the use of significant amount of cellular resources as well as genetic programming and metabolic changes (Somssich and Hahlbrock, 1998). Generally, the plants containing the R-gene have complete immunity against one or a few strains of the pathogens, because of which they have been of great interest for years (Pink, 2002). Even though they are extremely effective against certain strains of pathogens, R-genes can easily be overcome by the evolution of the pathogens (Pink, 2002). In order to avoid such a problem, use and deployment of multiple of R-genes into an individual line has become common and effective strategy to fight against the pathogens (Pink, 2002). This methodology is known as gene pyramiding, which exploits the collection of multiple genes of interest into one genotype (Ye and Smith, 2008). The advantage of this technique is that the plant becomes resistant against broad spectrum of diseases (Kim et al., 2012). Also pyramiding of R genes can demonstrate increase in resistance level in the plant that is already resistant to the pathogen of interest (Tan et al., 2010). But this technique requires much time and source; also it is an exhausting process (Ye and Smith, 2008). In addition, the genes of interest should be well characterized or else the pyramiding might not be successful (Ye and Smith, 2008). Besides, this method provides too much selection pressure on the pathogen, forcing it to evolve against the R-genes. Another method can be the use of several plant lines having different R-genes and sowing in the same field, which reduces the selection pressure of the *Avr* on the pathogens (Mundt, 2002). This methodology is known as mixed culture and its advantage lies in the easiness of the technique, since the earth that the plant was sowed acts as a "gene pool" for those plants and enhancing the resistance of those plants by gene uptake (Hoffman et al., 1994). However, the downside of mixed culture is the stability of the taken DNA, since the plant can lose the taken resistance

in a certain amount of time (Hoffman et al., 1994). Maybe the most effective strategy is to use durable resistant genes. Durable resistance of a plant can be defined by its widespread use for a long duration (Johnson, 1983). This characteristic of durable resistance makes it valuable in plant disease resistance, however the necessity for large area and long duration to define a resistance as durable makes it hard to find one (Johnson, 1984). Barley Rpg1 gene is among them (Horvath et al., 2003; Brueggeman et al., 2002). On the other hand, the studies on non-host resistance genes can provide more efficient methods to fortify plant defense against pathogens (Heath, 2000). Non-host resistance means that all genotypes of a plant species are resistant to all strains of a particular pathogen (McDowell and Woffenden, 2003). When successfully isolated and cloned, non-host genes can provide resistance to another plant even they are not closely related (Whalen et al., 1988). The downside of this application is that such non-host genes do not function properly in every plant (Whalen et al., 1988).

There are several sources available in case one desires to look for powdery mildew or other pathogen resistance on wheat and barley. For wheat, one can take advantage of International Wheat Genome Sequence Consortium (http://www.wheatgenome.org/) and especially "Grain Genes: A Database for *Triticeae* and *Avena*" (http://wheat.pw.usda.gov/GG2/index.shtml). Also, Marker Assisted Selection in Wheat (http://maswheat.ucdavis.edu/) can be a valuable source. As for the barley, Barley DB (http://www.shigen.nig.ac.jp/barley/) would be a good start for browsing pathogen resistance genes in barley. On the other hand, Barley Genome Database (http://150.46.168.145/gbrowse_new/index.html) can be used as well as "Grain Genes: A Database for *Triticeae* and *Avena*" (http://wheat.pw.usda.gov/GG2/index.shtml), which also serves for barley pathogen resistance genes.

Up to date, nearly 32 loci have been determined to act on powdery mildew disease on wheat; among which the most important locus is Pm locus (Huang & Röder, 2004). In wheat, *OsGLP* gene on chromosome 8 is responsible of broad spectrum resistance against infections (Manosalva et al., 2009). For barley, Mlo locus cluster holds more

than 30 alleles that are known to act on powdery mildew disease resistance (Huang & Röder, 2004). Mlo locus shows broad spectrum of resistance against infections, mostly to fungal infections (Büschges et al., 1997). During the determination of resistance genes, P21 is used from *Dasypyrum villosum* (Qi et al., 2011). *Dasypyrum villosum* is related to wheat and a member of grass family (Qi et al., 2011).

To introduce resistance loci from alien sources to our host, introgression can be used. Introgression is the application that specific resistance or another gene is introduced into the plant of interest by crossing with another plant that bears the target gene and backcrossing with its parental plants until the gene is flowed into the plant of interest (Jakobson et al., 2012). In order to introduce the resistance loci to the host, one can cross our plant with another plant that is known to have the desired resistance gene (Kindworth et al., 2012). After several crosses and backcrosses that would be designed according to the gene of interest specifically, it can be confirmed that resistance gene is translocated to the plant by several markers and it has the resistance capacity against a particular pathogen (Kindworth et al., 2012). On the other hand, translocation is a process where a part of a genome is carried to another part of the genome, and is integrated to this location (Miyao et al, 2003). Different than introgression, gene translocation happens in the genome of the host itself, not as the result of crossings between plants (Yu et al., 2012). Instead, it can be induced via external factors such as radiation (Yu et al., 2012).

1.3 Gene silencing

Gene silencing is a process in which a gene expression is interfered in a way other than a genetic modification (He et al., 2011). Endogeneous silencing mechanism must have been evolved for defending the host organism against the infecting pathogens, parasites and pests (He et al., 2011). Gene silencing process can take place during transcription and post transcription (Wang and Waterhouse, 2002). In post

transcriptional gene silencing, the target mRNA of a corresponding gene is destroyed in order to prevent its translation (Yu and Kumar, 2003). This process is carried out by RNA interference (RNAi) mechanism, and it is an effective method to defend host against viral infections. In transcriptional gene silencing, mRNA of a corresponding gene is prevented to be transcribed, mostly due to DNA methylation that prevents the necessary transcription factors and other proteins from binding to the promoter of the gene to be silenced (He et al., 2011). Methylation can also lead to the binding of chromatin remodeling proteins to the promoter, causing undesired structural changes on the promoter region by which it leads to silencing, too (He et al., 2011). Gene silencing systems in plants are used to identify the functions of genes and obtaining desired phenotypes in commercial plants (Yu and Kumar, 2003).

1.3.1 RNAi mediated gene silencing

RNAi mechanism, mostly found in eukaryotes to date, silences the target genes with the help of RNA induced silencing complex (RISC), Dicer enzyme, small interfering RNA (siRNA) and micro RNA (miRNA) activity (Yu and Kumar, 2003). RNAi mediated gene silencing is an effective defense system against virus infections and undesired transposon activity (Kusaba, 2004). In the mechanism that siRNA takes place, RNAi mechanism reacts to short double stranded RNA molecules which can be endogenous and exogenous (Hammond-Kosack and Parker, 2003). These short double-stranded RNA (dsRNA) molecules first activate Dicer enzyme, which cuts the dsRNA into smaller fragments of 20 to 25 base pairs (Hannon, 2002). This cleavage process leaves the fragments with two hanging nucleotides at their 3' ends (Voinnet et al., 2000). At this state, these fragments are separated from each other, forming single stranded short RNA fragments (Hammond-Kosack and Parker, 2003). These fragments are defined as siRNA, which are then processed by RISC complexes to be coupled with their target mRNA, preventing their translation (Finnegan et al., 2003**)**. In the mechanism that miRNA takes place, target mRNA is bound by miRNA and its

translation is inhibited (Tang et al., 2003). miRNAs play an important role during embryogenesis and the development of the organism (Yu and Kumar, 2003). The overview of the mechanism is presented in the diagram below (Figure 1.5).

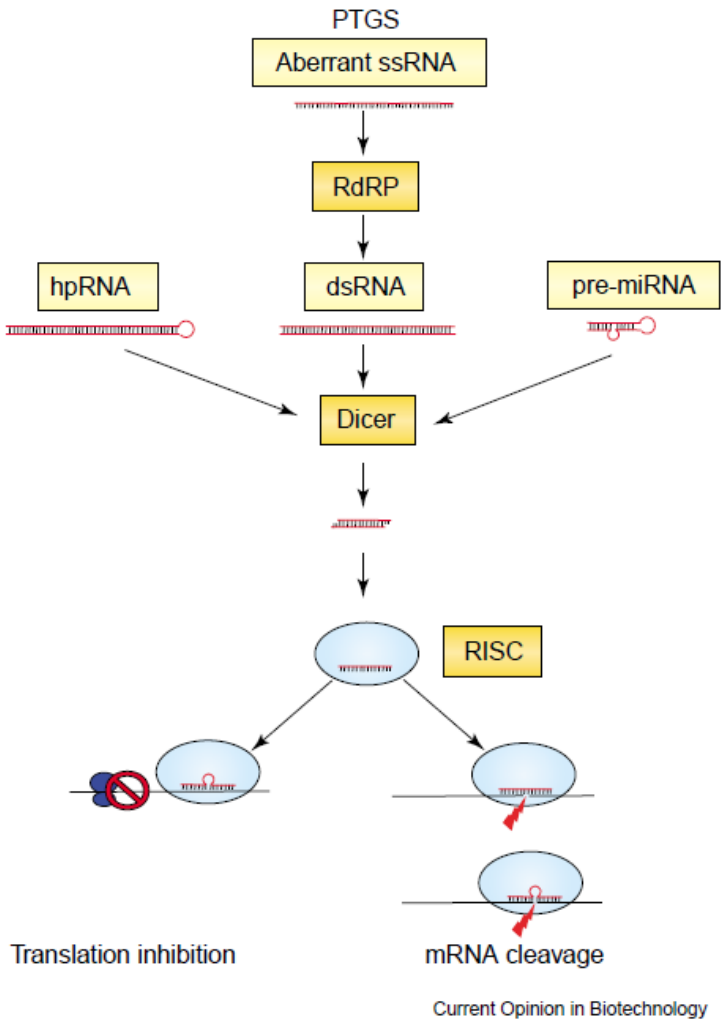


Figure 1.5 Molecular mechanism of RNAi system in crop plants. PTGS: Post transcriptional gene silencing, RdRP: RNA dependent RNA polymerase, hp: hairpin, ds: double-stranded. Image is directly taken from Kusaba, 2004.

Unlike siRNA, which binds to its target messenger RNA (mRNA) with complete compatibility, miRNAs do not show complete compatibility to their target mRNAs (Aukerman and Sakai, 2003). However in plants, the compatibility of miRNA to its target mRNA is much better than in animal RNAi mediated gene silencing system (Palatnik et al., 2003). RNAi mechanism has been exploited to understand the mechanisms of the genes of interest in many organisms, due to its specificity. In plants, RNAi technology is emerged as a promising tool to silence plant genes, since unlike animals, gene-directed mutagenesis may not always be a suitable method for plants (Chuang and Meyerowitz, 2000). RNAi induction can be introduced to plants by creating transgenic plants, or only dsRNA can be inserted into the plant (Chuang and Meyerowitz, 2000). Another approach is to introduce viral agents to produce dsRNA in host plant, which is known as Virus Induced Gene Silencing (VIGS) (Waterhouse and Helliwell, 2003).

1.3.2 Virus Induced Gene Silencing (VIGS)

Virus Induced Gene Silencing technique takes advantage of RNAi mechanism to knock down a gene of interest in order to investigate its functions (Lu et al., 2003). In this technique a viral origin of specifically modified complementary DNA (cDNA) is introduced to the plant (Lu et al., 2003). As the virus replicates, dsRNAs are encoded and cleaved into siRNAs by Dicer enzyme, then siRNA molecules target their complementary mRNA fragments and successfully prevent their translation (Gould and Kramer, 2007). This technique is preferred for its convenience and ability to target specific gene loci (Lu et al., 2003). VIGS phenotype can be established in 1 to 2 weeks after the infection of plant (Lu et al., 2003). *Via* VIGS technique, one can identify the genes that are responsible of plant defense against virus, durability to harsh environmental conditions such as high salinity, and tolerance to climate changes.

VIGS efficiency cannot reach %100 most of the time due to poor spreading of the virus or the inappropriate choice of the vector (Liu and Page, 2008). Also, some plants are more succeptible to virus infection such as cotton and tobacco, whereas others show resistance (Qu et al., 2012). Size of host insert is another matter that affects VIGS efficiency, inserts longer than 1500bp dramatically reduces VIGS efficiency (Liu and Page, 2008). In addition, decreased or limited plasmodesmatal activity in a plant results in inefficient VIGS application (Gould and Kramer, 2007). To increase the VIGS efficiency up to %100, environmental conditions must be set correctly in order to increase the viral spread on plants, insert length should not exceed 200bp, and the most effective viral vector such as Tobacco Mosaic Virus (TMV) must be used (Velasques et al., 2009). Also the succeptibility of the target plant to infection should be considered beforehand (Velasques et al., 2009).

Nicotiana benthamiana is well studied in VIGS research, by which 5000 different phenotypes of *Nicotiana benthamiana* are defined so far (Lu et al., 2003). *Nicotiana benthamiana is* found to be especially prone to viral infections, thus VIGS application is effortlessly processed on this species (Lu et al., 2003). One example of VIGS application on *Nicotiana benthamiana* is shown in Figure 1.6. Also the target genes remain silenced longer compared to other plants (Lu et al., 2003). Reason for this susceptibility is thought to be due to increased plasmodesmatal activity which is required for RNAi mechanism to function properly (Lu et al., 2003).

One of the most efficient roles of VIGS is to detect the roles of genes in plant defense. Elucidation of the role of the gene Rar1 was first identified in barley (Shirasu et al., 1999). Three years later, the role of *Rar1* in tobacco was found to be similar. Silencing of barley genes are performed by using Barley Stripe Mosaic Virus (BSMV) (Holzberg et al., 2002). Silencing of *PDS* (Phytoene desaturase) gene in barley was the first example of gene silencing in monocots (Burch-Smith et al., 2004). In our laboratory, F-box proteins are found to be positive regulators of disease resistance against powdery mildew in barley (Dağdaş et al., 2009).

An alternative to siRNA based VIGS was found in 2010, which uses virus-based miRNA expression for plant functional analysis (Tang et al., 2010). This method enables not only to silence genes related with the defense mechanism as well as general metabolism, but also to analyze the function of endogeneous plant miRNAs (Tang et al., 2010).

VIGS can be used to understand the function of any gene of a plant as long as its function can be detected phenotypically. This gene can translate to a protein related to stress response, a transporter protein (Eybishtz et al., 2010), or a protein that helps plant to survive against boron toxicity (Voxeur et al., 2011). First of all, partial sequence of the gene to be silenced needs to be cloned into a suitable virus of the plant. Then, the plant must be infected with the virus, followed by phenotypic change assessment. Finally, the presence of silencing should be detected by using methods like qRT-PCR and western blot.

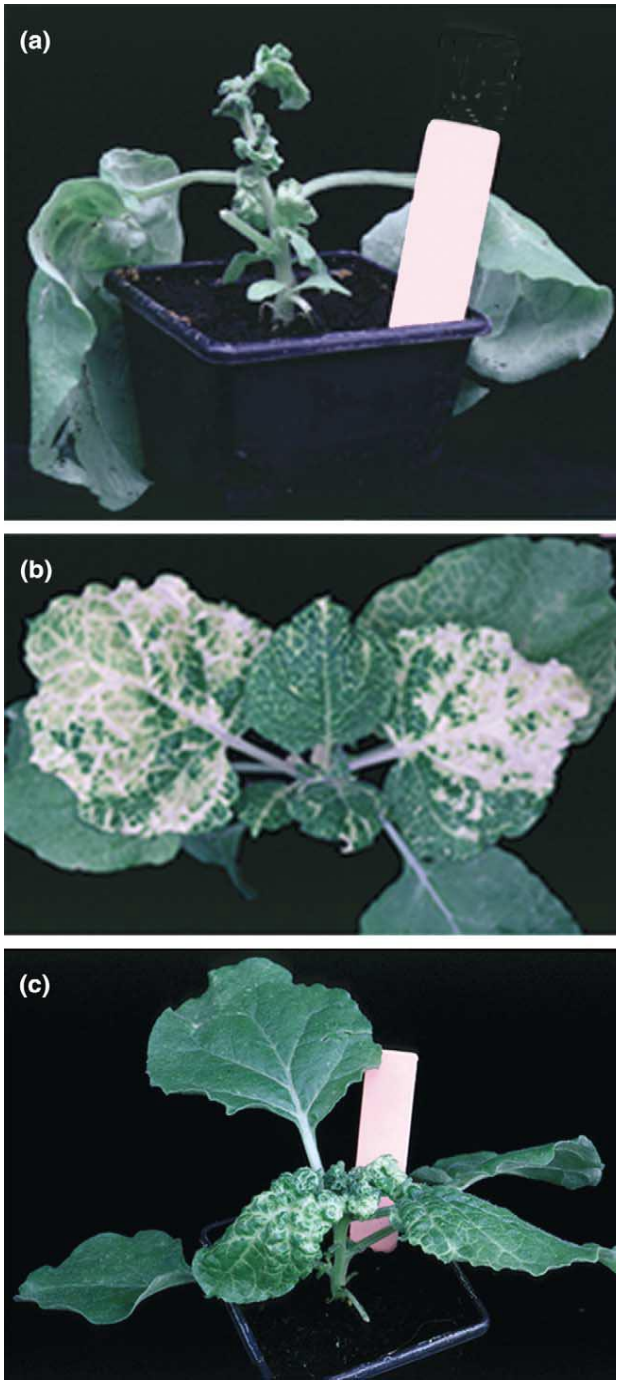


Figure 1.6 *Nicotiana benthamiana* phenotypes. Their growth rates are decreased *via* VIGS. **a)** Ubiquitin silencing, **b)** Magnesium chelatase silencing, **c)** Unknown gene silencing. Image is directly taken from Lu et al., 2003.

1.3.3 Barley Stripe Mosaic Virus (BSMV)

Barley Stripe Mosaic Virus (BSMV) is a virus of the *Hordeivirus* group which infects barley, and the virus shapes are rigid rods in 20 x 100 mm to 20 x 150 mm. Even though their major host is barley, they can also infect wheat (*Triticum aestivum*), and their infection status on oats (*Avena sativa*) is unknown (ODA commodity inspection division, 2012; Holzberg et al., 2002; Pacak et al., 2010). Their distribution is based on mechanical inoculation, as well as rubbing of the infected leaves with new host. There are no known BSMV vectors (ODA commodity inspection division, 2012). The symptoms of BSMV resemble to fungal barley stripe disease, such as the yellow stripes or dots on the host plant, which in time turn brown (Thomson and Ockey, 1998; Pacak et al., 2010). Usually BSMV infection does not lead to great yield loss in agriculture (Holzberg et al., 2002; Brunt et al., 1998).

BSMV induced gene silencing is basically VIGS with a specifically selected vector, in this case barley stripe mosaic virus (BSMV) (Pacak et al., 2010). BSMV genome is segmented into three compartments; α, β and γ (Holzberg et al., 2002). This system is also used to understand the functions of genes of interest. The virus vector is transformed as required by the nature of the experiment, and the host plant (mostly wheat and barley) is infected with this modified vector (Holzberg et al., 2002). The infection application is carried out by directly applying the modified BSMV onto the leaves of the host (Holzberg et al., 2002; Pacak et al., 2010). BSMV induced gene silencing is able to silence genes expressed on the leaves of the plant, but it is also possible to apply BSMV induced gene silencing on the roots to understand the functions of certain genes that are specifically expressed on the roots of the plant (Pacak et al., 2010).

1.4 *GCN2* gene

General Control Non-Depresible-2 (*GCN2*) gene has several reasons to be used as VIGS target to study its effects on plant defense mechanism. GCN2 was found to be physically interacting with Yr10 resistance protein of wheat (Ersoy et al., 2011). In addition, GCN2 protein is the enzyme that phosphorylates α subunit of the eukaryotic translation initiation factor, eIF2α. Phosphorylation of eIF2α results in inhibition of eIF-2-GDP to eIF-2-GTP conversion, subsequently leading to cell death (Hershey, 1991). *Arabidopsis thaliana* homologue of GCN2, *AtGCN2* is essential for growth under stress conditions including wounding, starvation, exposure to methyl jasmonate, salicylic acid, or the ethylene precursor 1-Aminocyclopropane-1-carboxylic acid (ACC). The *AtGCN2* activation because of the stress conditions suggests that it could play a role in plant defense mechanism, since disease is one of the major stress conditions (Lageix et al., 2008). Programmed cell death (PCD) is one of the main defense mechanisms of plants. On the other hand, GCN2 activity in mammalian cells was found to be affected by elevating levels of reactive oxygen species (ROS). However, this role of GCN2 is unrelated with its eIF2α kinase activity. It was suggested that amino acid deprivation causes decrease in intracellular ROS levels in mammalian cells by activation of GCN2, resulting in a protective effect on the cell (Arriazu et al., 2010). In addition, it was found out that budding yeast *Saccharomyces cerevisiae* molecular chaperone Hsp90 regulates GCN2 (Donzé and Picard, 1999). Hsp90 is known to regulate proteins of NB-LRR class, which are needed by plants for proper immune cell function (Hubert et al., 2009).

1.5 Aim of the study

Diseases caused by the pathogens on plants of agricultural importance results in loss of tons of plant products. Resistance related molecular mechanisms are very important to be understood in order to decrease the loss in the yield. For example,

powdery mildew disease causes significant losses in the world as well as Turkey, and understanding resistance related molecular mechanisms can help finding better strategies in fighting against this pathogen.

In this thesis, the role of *Hordeum vulgare* homologue of *GCN2* in plant defense mechanism was aimed to be studied. For this, BSMV mediated VIGS method was planned to be used. Three control and six silencing treatment samples were prepared and phenotypic changes were aimed to be observed by comparing the powdery mildew growth rates between control and silenced groups, and the level of silencing was planned to be confirmed *via* Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

CHAPTER II

MATERIALS and METHODS

2.1 Plant and pathogen materials

2.1.1 Plant growth conditions

Barley seeds of Bülbül-89 or Pallas-01 were planted on soil and grown for 16 hr light and 8 hr dark cycles in Sanyo Versatile Environmental Test Chamber (Model MLR-351H) at 18 ^{o}C. The seedlings were watered with tap water once in every 2 days. Then, 30 seeds were planted on one 650 cm^3 pot. Neither seeds nor soil was sterilized beforehand.

2.1.2 Powdery mildew maintenance

Since *Blumeria graminis* f. sp. *hordei* is an obligate parasite, it can only be kept alive on the inoculated leaves. Thus, fungus must be kept growing all the time. Inoculation of the pathogen, whose strains have been received from Prof. Dr. Mogens Støvring Hovmøller, Aarhus University, Denmark in year 2008, for the maintenance of powdery mildew races on Bülbül-89 (no known resistance to all known races) detached barley seedlings were carried out after the seedlings were 10-days old and placed on water agar.

Water agar plates were prepared by adding 3.5 g agar (LabM, Lot no: Q34567/129) and 225 mL ddH_2O in a bottle, after autoclaving for 20 min and cooling to 50 ^{o}C, adding 25 mL benzamidazole solution (0.2 g Benzamidazole (Aldrich, Lot no: 23968-

222), 200 µL DMSO (Merck, Lot No: S23526 744), and ddH_2O to a final volume to be 200 mL), and distributing the solution to sterile plates. The pathogen races were separately inoculated by whiffing on the leaves. The leaves were incubated in the growth chamber in the above conditions (Section 2.1.1). When the pathogen develops conidia, fresh conidia were used to re-inoculate new set of detached Bülbül-89 leaves on water agar containing benzamidazole.

Table 2.1 The pathogen races used in the thesis. Infection was conducted on the *Hordeum vulgare* lines; Pallas-01 and Bülbül-89.

Cultivar	*Bgh* pathotype	Plant-Pathogen Interaction Type	Resistant / Susceptible
Pallas-01	*Bgh*-95	Compatible	Susceptible plant response
Pallas-01	*Bgh*-103	Incompatible	Resistant plant response
Bülbül-89	*Bgh*-95 / *Bgh*-103	Compatible	Susceptible plant response

2.2 VIGS

2.2.1 Preparation of BSMV

Proviral DNA of the Barley Stripe Mosaic Virus (BSMV), being a tripartite genome RNA virus, was cloned into three plasmids by Holzberg in 2002, allowing generating viral RNA by *in vitro* transcription. The BSMV genomes were cloned into high copy plasmid vectors such that the respective BSMV RNA can be *in vitro* transcribed from the T7 RNA polymerase promoter (Figure 2.1). Our laboratory obtained BSMV vectors pα, pβ, pβΔβa in which, there is a deletion of coat protein of pβ, pγ, pγ.bPDS4S (sense direction), and pγ.bPDS4As (anti-sense direction) from "Large Scale Biology Corporation, USA" in 2003 (Figure 2.2). In this study, we have used pα, pβΔβa, pγ obtained from Large Scale Biology and pSL039B-1 obtained from Steven R. Scofield of Purdue University, USA. The sequences of the BSMV genomes and the plasmid DNA sequences are presented in Appendix A. The *E. coli* DH5α

strains were already transformed with all the plasmids and stored in 15% glycerol stock solutions. To conduct *in vitro* RNA transcription, plasmids must have been amplified and pSL039B-1 must have been engineered to possess the silencing target gene fragment.

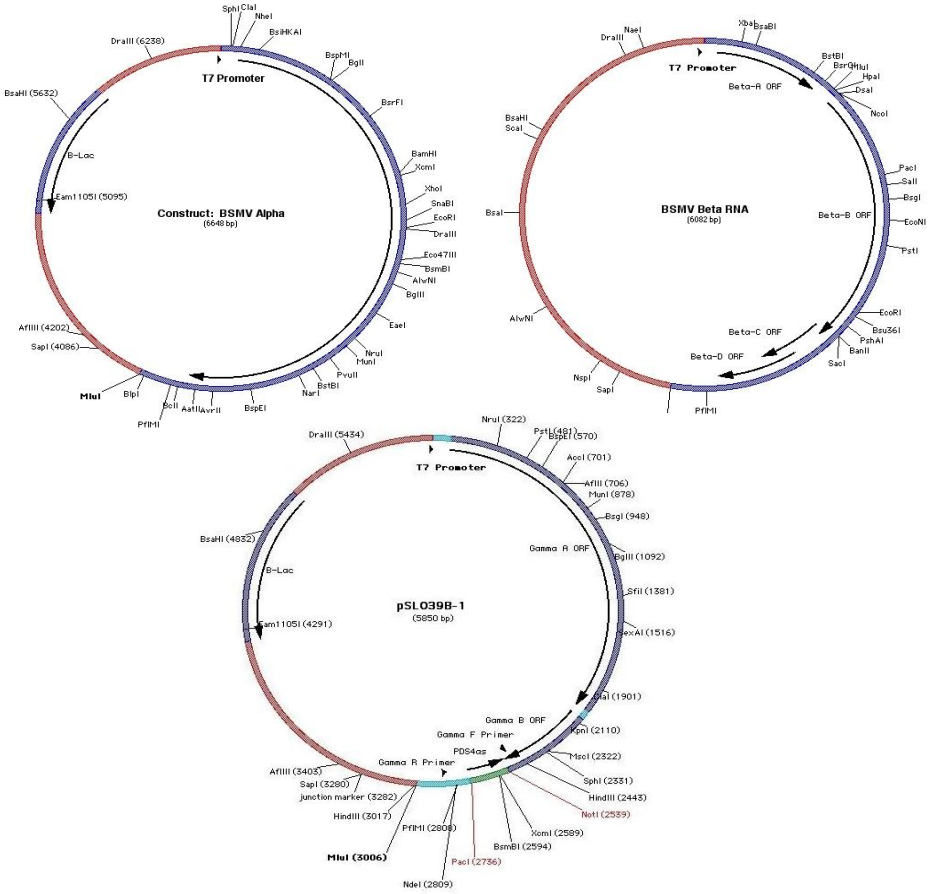


Figure 2.1 Maps of the plasmid vectors containing BSMV clones. **a)** BSMV ND18 α genome proviral DNA cloned into a plasmid vector. **b)** BSMV ND18 β genome proviral DNA cloned into a plasmid vector. **c)** BSMV ND18 γ genome adjacent to a 185 bp fragment of the barley PDS gene at anti-sense orientation was cloned into a high copy plasmid vector. *Not*I and *Pac*I enzymes can be used to clone new fragments by replacing the PDS fragment. Images are directly taken from Scofield, 2007.

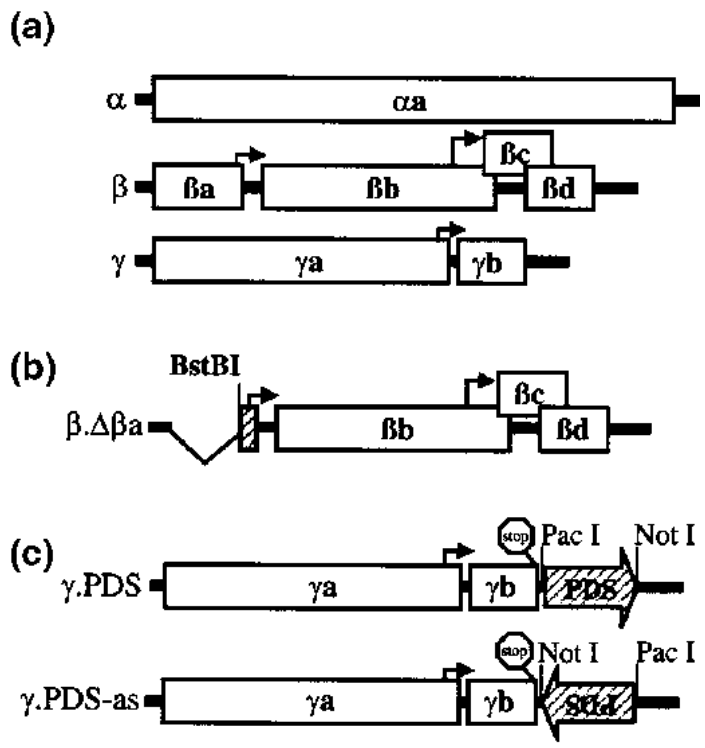


Figure 2.2 Vectors derived from BSMV RNA. **a)** BSMV pα, pβ, pγ genomes. **b)** pβΔβa, showing deletion in pβ genome. **c)** Orientations of pγ.bPDS4S and pγ.bPDS4As. Image is directly taken from Holzberg et al., 2002.

Plasmid isolation has been performed according to the manufacturer's protocol of QIAGEN, QIAprep Spin Miniprep Kit (Lot No: 124104532). Firstly, the DH5α *E. coli* cell stocks that were kept in -80 ^{o}C, which contain the plasmids pα, pβΔβa, pγ and pγPDS were inoculated into 4 mL liquid broth (LB) medium (5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 1.6 mL NaOH (0.5 M)), in 500 mL), ampicillin to a final concentration of 100 µg/mL. The inoculates were grown overnight (16-18 hours) at 37 ^{o}C and shaking at 250 rpm. Then the cells were collected and suspended in 250 µL resuspension buffer, (P1), (+4 ^{o}C) including RNase A (100 µg/mL) in final concentration of 100 µg/mL. Then, 250 µL lysis buffer, (P2), was added and the tube was gently inverted for 4–6 times to mix. After that, 350 µL neutralization buffer, (N3), was added and the tube was inverted immediately but gently for 4–6 times.

Centrifugation was performed for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge (Continental Lab Products, Model 3410 Microcentrifuge). The supernatant was applied to a QIAprep spin column by pipetting. Centrifugation was conducted for 1 min at 13,000 rpm, and the flow-through was discarded. The binding buffer, (PB), of 500 µL was added to wash the column. The flow-through was discarded. The column was washed by adding 750 µL wash buffer, (PE) and centrifuged for 1 min. The flow-through was discarded and centrifuged for an additional 1 min to remove any possible residual wash buffer. After that, the column was put in a clean 1.5 mL microcentrifuge tube. The elution buffer, (EB), of 30 µL was added to the center of each column, incubated at room temperature for 1 min and centrifuged for 1 min at maximum speed.

2.2.2 Cloning of *GCN2* into pγ

Cloning of *Hordeum vulgare* homologue of *GCN2* gene fragment into pγ was previously carried out in our laboratory. Since we don't have full length sequence of *GCN2* of barley in databases, the cloning had to be performed based on the EST sequence of barley *GCN2* homologue (Figure 2.3). Since there is no need for full length sequence of *GCN2* to target the gene of interest in VIGS, the EST sequence was used to amplify *HvGCN2* DNA using primers specific to the gene. Then, the amplified DNA was used for cloning into pγ fragment of BSMV, at the *Not*I and *Pac*I sites of the pSL039B-1 vector after releasing PDS fragment in sense direction (Figure 2.4).

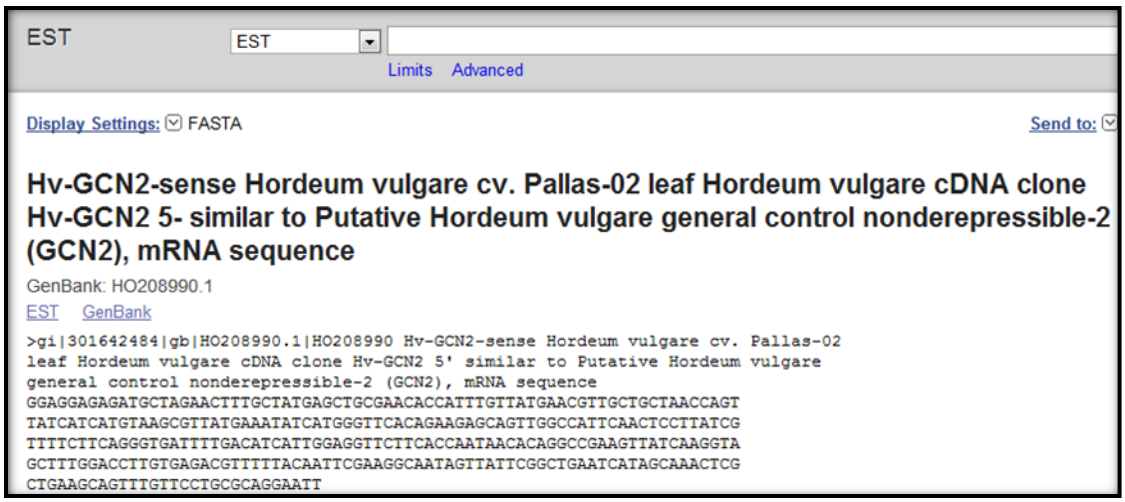


Figure 2.3 Expressed sequence tag (EST) sequence of *GCN2* in barley.

Figure 2.4 *GCN2* in the pγ sequence of BSMV. Highlighted region represents the cloned *GCN2* fragment. The underlined sequences are restriction enzyme cutsites (*Pac*I and *Not*I).

The common strategy in determining the level of silencing from VIGS studies by qRT-PCR is to use at least one primer outside the cloned fragment, either upstream or downstream. For that, the sequence of *Triticum aestivum GCN2* homologue was used. Because there is a very high homology between wheat and barley *GCN2* sequences, it was a reasonable choice (Figure 2.5). The sequences of qRT-PCR primers are given in Table 2.5. In our situation, both primers are taken from outside of the *GCN2* cloned fragment. The cloned fragment is from the 2542nd to 2850th position on *TaGCN2* (Figure 2.5), while the primers fit in 615th-635th and 500th-519th positions on *TaGCN2* (Figure 2.6). The BLAST results of the primers show where the primers fit, the size of the product, and if the primers are unique to the sites (Figure 2.7).

Figure 2.5 Sequence alignment of *Hordeum vulgare GCN2* EST and *Triticum aestivum GCN2* homologue.

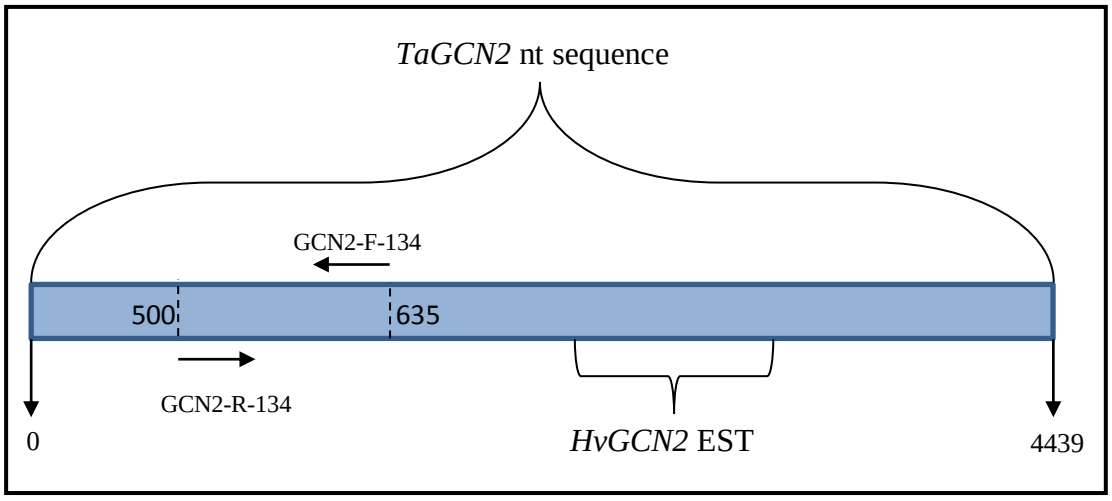


Figure 2.6 *HvGCN2* primers for qRT-PCR and *HvGCN2* EST on *TaGCN2*.

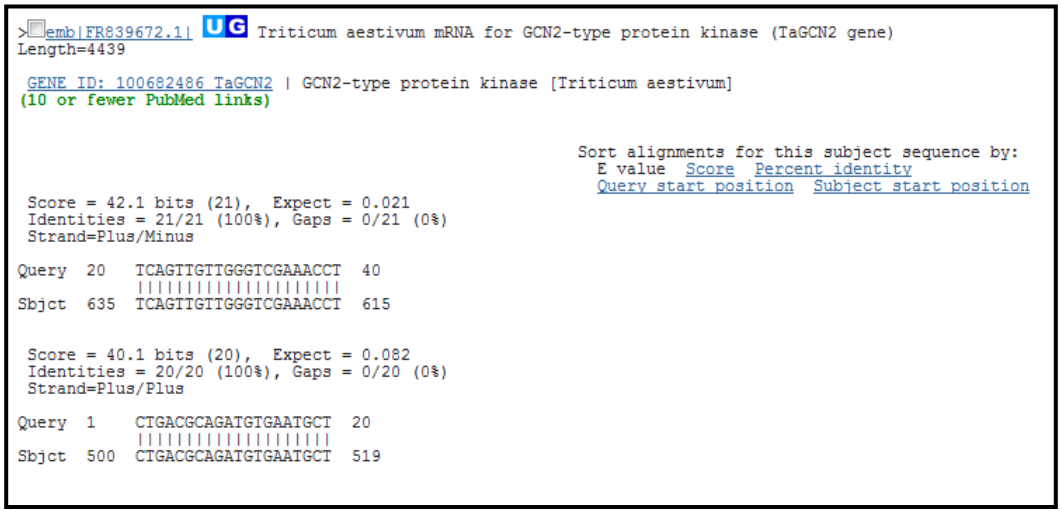


Figure 2.7 Sequence alignments of qRT-PCR primers to *TaGCN2*.

2.2.3 Restriction enzyme digestion of the plasmids for linearization

The required volume for 4 µg of each plasmid was placed for pα, pβΔβa, pγ, pγPDS and pγBSMV:*GCN2*. Then, the volume was completed to 43 µL. Afterwards, 5 µL of Buffer R (Fermentas-Lot:00040013) for pα, pγ, pγPDS and pγBSMV:*GCN2*; and 5 µL of Buffer Tango (Fermentas-Lot:9215) for pβΔβa was added. Then, 2 µL restriction enzyme *Pau*I (*BssH*II) (Biolabs-Lot:028011) for pγPDS, and pγBSMV:*GCN2*; 2 µL *Bcu*I (*Spe*I) (Fermentas-Lot:00054530) for pβΔβα; and 2 µL *Mlu*I (Fermentas-Lot:00053835) for pα and pγ were added to complete the volume to 50 µL. The mixtures were spinned briefly. Following the enzymatic digestion for 4 hours at 37 ^{o}C, enzyme inactivation for 20 min at 80 ^{o}C was performed. Afterwards, 1µL of the the linearized plasmids were analyzed in 1% agarose gel prepared in TAE (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA) buffer and 0.5 µg/µL EtBr. Electrophoresis was conducted at 60 V for 1 hour.

2.2.4 *In vitro* transcription from the linearized plasmids

The linearized plasmids were *in vitro* transcribed according to the manufacturer's protocol of Ambion mMessage mMachine T7 *in vitro* transcription kit, (Lot:1009036). Firstly, 5 µL 2x NTP, 1 µL 10x Buffer, 1µL T7 RNA Polymerase Enzyme (suggested as 2 µL / reaction) were mixed for all tubes, and then 3 µL of the linearized plasmid was added. *In vitro* transcription was conducted by incubating the linearized plasmids at 37 ^{o}C for 2 hours, followed by a hold at 7 ^{o}C. After *in vitro* transcription, the products were analyzed in 1% agarose gel electrophoresis at 70 V for 100 min using 0.01 M sodium phosphate buffer (13.4 g $Na_2HPO_4.7H_2O$, 6.58 g $NaH_2PO_4.H_2O$, add 1 L ddH_2O, adjust pH 6.8 with orthophosphoric acid), and replacing the buffer for every 20 min.

2.2.5 Rub-inoculation of the mixture of α, β, and γ RNA transcripts

First leaves of 10 days old Pallas-01 plants were rub-inoculated from bottom to top with the transcripts. RNAs of α fragment, βΔβa fragment, and γ fragment (naked γ or γGCN2 or γPDS) were mixed in 1:1:1 ratio using 1.5 µL of transcription products for each reaction. Then, 27.5 µL FES solution (50 mL 10x GP (18.77 g glucose; 26.13 g K_2HPO_4, ddH_2O up to 500 mL and autoclaved for 20 min.); 2.5 g sodium pyrophosphate, 2.5 g bentonite, 2.5 g celite, ddH_2O up to 250 mL and autoclaved for 20 min) was added. Besides, 6 seedlings were inoculated with FES alone, marked as "Mock inoculated".

2.3 qRT-PCR

14 days after the BSMV inoculation, the leaves were cut into 4 different pieces, among which two of them were used for RNA isolation (Figure 2.8).

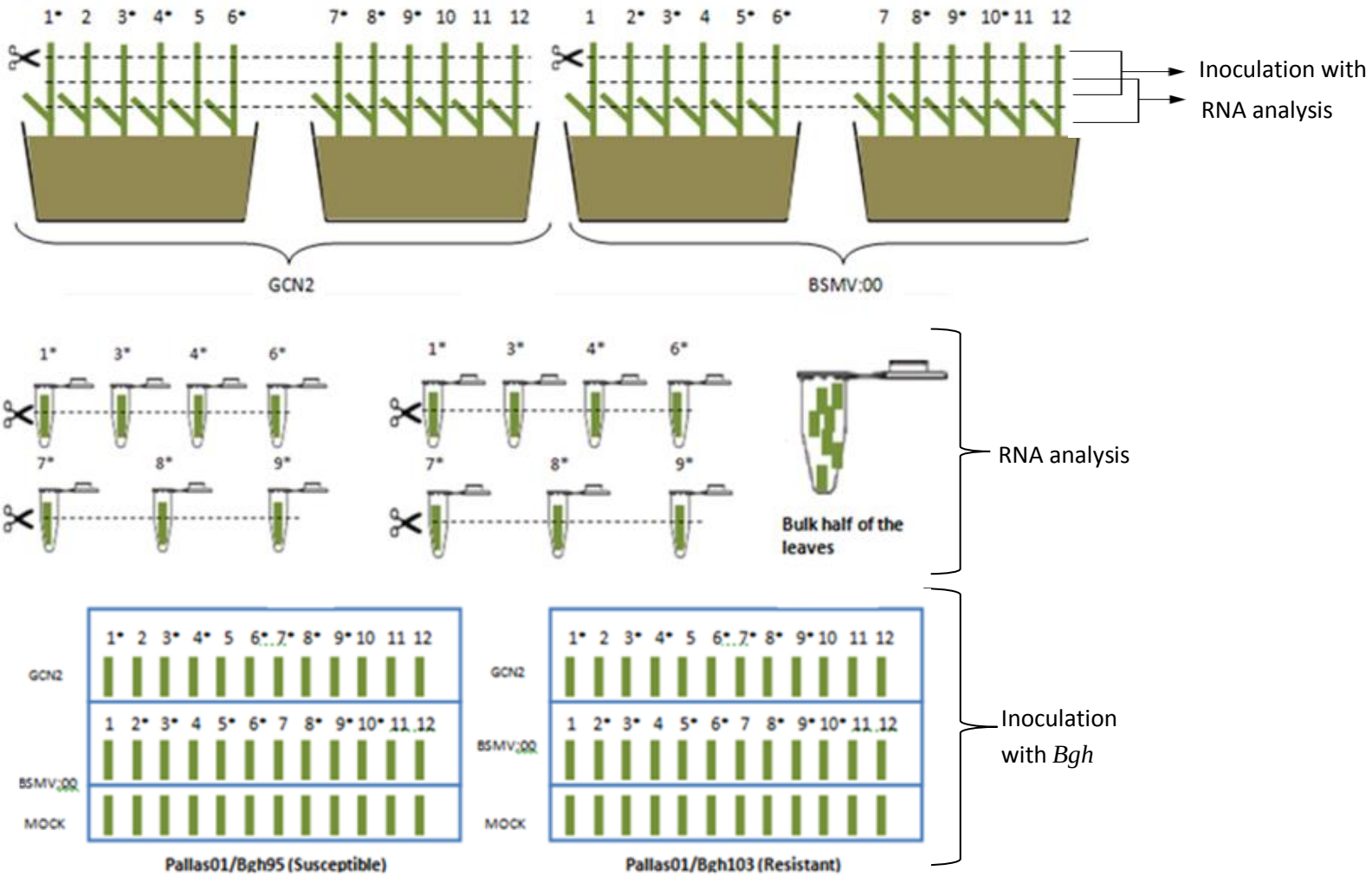


Figure 2.8 Overall VIGS plan (Prepared by İbrahim Kutay Öztürk)

2.3.1 RNA isolation from the leaves

RNA isolation from the leaf fragments were performed according to the manufacturer's protocol of QIAGEN, RNeasy Plant Mini Kit (Lot No: 136265242). The remaining two leaf fragments mentioned in Section 2.3.1 were put into same mortar and powdered in liquid nitrogen. The powdered leaf fragments were transfered to an RNase free microcentrifuge tube, into which immediately, 450 µL lysis buffer, (RLT), (including 10 µL β-mercaptoethanol per 1 mL buffer RLT) was added and vortexed vigorously. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 mL collection tube, and then centrifuged for 2 min at full speed. The flow-through may have both pellet and supernatant. Supernatant was transferred to a new microcentrifuge tube without disturbing the pellet. Then, 0.5 volume of ethanol (99.5%) was added to the cleared lysate, and mixed immediately by pipetting. The sample was transferred to an RNeasy spin column (pink) placed in a 2 mL collection tube. The lid was closed gently, and centrifuged for 15 sec at 10,000 rpm. The flow-through was discarded. The wash buffer, (RW1), of 700 µL was added to the RNeasy spin column. The lid was closed gently, and centrifuged for 15 sec at 10,000 rpm to wash the spin column membrane. The flow-through was discarded. The wash buffer, (RPE) including 4 volumes of 96% ethanol, of 500 µL was added to the RNeasy spin column. The lid was closed gently, and centrifuged for 15 sec at 10,000 rpm to wash the spin column membrane. This step was repeated twice. The RNeasy spin column was placed in a new 2 mL collection tube, and the old collection tube with the flow-through was discarded. The lid was closed gently, and centrifuged at full speed for 1 min. This step was conducted to remove any residual carryover of ethanol or buffers. The RNeasy spin column was placed in a new 1.5 mL collection tube. RNase-free ddH_2O of 30 µL was added directly to the spin column membrane. The lid was closed gently, and centrifuged for 1 min at 10,000 rpm to elute the RNA. Then, the RNA samples were analyzed in agarose gel electrophoresis as indicated in Section 2.2.4, but the samples were run for 60 min.

2.3.2 DNase treatment

DNase treatment has been performed according to manufacturer's protocol of Fermentas, DNase I, RNase-free (Lot no: 93831). RNA of 550 ng was placed in to a nuclease-free microcentrifuge tube. The volume was completed to 4.4 µL with ddH_2O. Afterwards, 0.55 µL of 10x reaction buffer and 0.55 µL of DNase I (1u/µL) were added. The samples were incubated at 37 ^oC for 30 min. To inactivate the enzymes, 550 µL 50 mM EDTA (Fermentas, Lot no: 93072) was added and incubated at 65 ^oC for 10 min. Then, the samples were stored in -80 ^oC.

2.3.3 Assessment of DNA contamination free RNA samples

The PCR reaction was designed to amplify Glyceraldehyde 3-phosphate dehydrogenase homologue of barley, *HvGAPDH*. PCR reaction was performed according to the manufacturer's protocol of Fermentas, Pfu DNA Polymerase (Lot no: 59381). Firstly, 8.7 µL ddH_2O was added to a nuclease-free 0.2 µL tube. Then, 0.5 µL 10 mM dNTP, 2 µL forward primer (Hv-GAPDH-cw1: 5'-CGT TCA TCA CCA CCG ACT AC-3'), 2 µL reverse primer (Hv-GAPDH-ccw1: 5'- CAG CCT TGT CCT TGT CAG TG-3'), 1.6 µL 10x Pfu Buffer with $MgSO_4$ (Fermentas, Lot no: 30556), 0.2 µL Pfu DNA polymerase (2.5u/µL), and finally, 1 µL DNase-treated RNA was added. PCR reaction was conducted in Eppendorf Master Cycler Gradient under these conditions; 94 ^oC for 5 min in 1 cycle, 94 ^oC for 30 sec followed by 50 ^oC for 30 sec and 72 ^oC for 45 sec in 35 cycles, and finally 72 ^oC for 5 min followed by hold at 4 ^oC. Then, 1 µL of the PCR products were analyzed in agarose gel electrophoresis. Agarose gel electrophoresis was conducted as indicated in Section 2.2.3.

2.3.4 cDNA synthesis

cDNA synthesis from the DNase-treated RNA samples was conducted according to manufacturer's protocol of Invitrogen, SuperScript ® II Reverse Transcriptase (Lot no: 1038858). DNase-treated RNA of 5 µL (454.5 ng) was placed to a nuclease-free 0.2 mL microcentrifuge tube, 1 µL of 10 µM random hexamer primers, 1 µL of 10 µM oligo-dT primers, and 1 µL of 10 mM dNTP were added, the volume was completed to 12 µL with 5 µL ddH_2O. The mixture was incubated at 65 ^oC for 5 min, and chilled on ice for 5 min. The tube was briefly spinned to collect the contents at the bottom of the tube; and 4 µL 5x First-Strand Buffer (Invitrogen, Lot no: 1005582); 2 µL 0.1 M DTT (Invitrogen, Lot no: 1005609); 1 µL RiboLock RNase Inhibitor (40u/µL) (Fermentas, Lot No: 96098) were added. The contents of the tube were mixed gently. Incubations at 25 ^oC for 2 min and 42 ^oC for 2 min were performed. Afterwards, 1 µL (200 u) of SuperScript ™ II Reverse Transcriptase (Invitrogen, Lot no: 1049412) was added and mixed gently by pipetting. The contents of the tube were collected at the bottom by brief centrifugation and the cDNA synthesis reaction was conducted under these conditions; 25 ^oC for 10 min, then 42 ^oC for 50 min and enzyme inactivation at 70 ^oC for 15 min.

2.3.5 Confirmation of the cDNA synthesis

PCR reaction procedure as in 2.3.3 was performed to see if cDNA synthesis worked efficiently. The only difference was that this time ubiquitin primers of *Hordeum vulgare* (*HvUbi*) were used. The sequences of the primers were as follows; Ubi-F: 5'-GCC GCA CCC TCG CCG ACT AC - 3' and Ubi-R: 5'- CGG CGT TGG GGC ACT CCT TC - 3'.

2.3.6 Determination of reference genes for qRT-PCR expression analyses

cDNAs synthesized from BSMV:00 rub-inoculated leaves (control) and *HvGCN2* silenced leaves were used as templates of the qRT-PCR reaction. Actin (GenBank: AY145451.1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank: M36650.1), Elongation Factor (EF) (GenBank: Z50789.1), Ubiquitin (Ubi) (GenBank: M60175.1), and α-tubulin (GenBank: Y08490.1) (Table 2.2) were tested as candidate reference genes. The sequences of the reference genes were given in Appendix B. The reactions were prepared as triplicates for each cDNA, also for Non-Template Controls (NTC). The reaction was carried out as indicated in the manufacturer's protocol of Sigma, SYBR Green JumpStart Taq Ready Mix (Lot No: 031M6054). Master mix for all the cDNA sets were prepared (Table 2.3) and 8 µL of it was distributed to each reaction tube. The master mix sets were prepared so that each reaction contains 4.545 ng of cDNA. The plate setup is shown in Table 2.4. Then, 2 µL of 1 µM primer mix (forward & reverse) was added to each well, mixed well by pipetting. The tubes were spinned briefly and the qRT-PCR reactions started in Stratage Mx3005P Real-Time PCR System. Experiment type was selected as SYBR Green (with Dissociation Curve). After the plate sets were selected, the well type was chosen as "Unknown" and the option of "collect the flourescence data" was selected as "FAM". Thermal profile was adjusted as follows: 95 ^{o}C for 10 min in 1 cycle, 95 ^{o}C for 30 sec followed by 55 ^{o}C for 1 min at the end of which fluorescence determination was performed, and 72 ^{o}C for 1 min in 35 cycles. Then, incubation at 95 ^{o}C for 1 min, followed by 55 ^{o}C for 30 sec, and continuous fluorescence determination until reaching 95 ^{o}C to form dissociation curve was conducted. Finally, the samples were incubated at 95 ^{o}C for 30 sec. At the end, qRT-PCR products were analysed also via agarose gel electrophoresis to confirm the presence of the desired product. Agarose gel electrophoresis was conducted as indicated in Section 2.2.3. For the reference gene determination, GeNorm software which works on MS Excel was used (http://www.biogazelle .com/genormplus), and M values were calculated using GeNorm.

Table 2.2 Primer sequences for the amplification of the candidate reference genes in qRT-PCR.

Primer name	5'-3' Sequence
Actin-F:	AAT GGT CAA GGC TGG TTT CGC
Actin-R	CTG CGC CTC ATC ACC AAC ATA
Hv-GAPDH-cw1:	CGT TCA TCA CCA CCG ACT AC
Hv-GAPDH-ccw1:	CAG CCT TGT CCT TGT CAG TG
EF-F:	ATG ATT CCC ACC AAG CCC AT
EF-R:	ACA CCA ACA GCC ACA GTT TGC
Ubi-F:	GCC GCA CCC TCG CCG ACT AC
Ubi-R:	CGG CGT TGG GGC ACT CCT TC
α-tubulin-F:	AGT GTC CTG TCC ACC CAC TC
α-tubulin-R:	AGC ATG AAG TGG ATC CTT GG

Table 2.3 The components of the master mix.

Reaction components	Single reaction	Master mix (10 reactions)
cDNA (1/10 diluted)	2 µL	20 µL
ddH_2O	1 µL	10 µL
SYBR mix	5 µL	50 µL

Table 2.4 The plate setup for qRT-PCR.

	1	2	3	4	5	6
A	00/ Actin	00/ Actin	00/ Actin	NTC/ EF	NTC/ EF	NTC/ EF
B	GCN2/ Actin	GCN2/ Actin	GCN2/ Actin	00/ Ubi	00/ Ubi	00/ Ubi
C	NTC/ Actin	NTC/ Actin	NTC/ Actin	GCN2/ Ubi	GCN2/ Ubi	GCN2/ Ubi
D	00/ GAPDH	00/ GAPDH	00/ GAPDH	NTC/ Ubi	NTC/ Ubi	NTC/ Ubi
E	GCN2/ GAPDH	GCN2/ GAPDH	GCN2/ GAPDH	00/ α-tubulin	00/ α-tubulin	00/ α-tubulin
F	NTC/ GAPDH	NTC/ GAPDH	NTC/ GAPDH	GCN2/α-tubulin	GCN2/α-tubulin	GCN2/α-tubulin
G	00/ EF	00/ EF	00/ EF	NTC/ α-tubulin	NTC/ α-tubulin	NTC/ α-tubulin
H	GCN2/ EF	GCN2/ EF	GCN2/ EF			

00:BSMV:00-Naked virus inoculated leaf cDNA, **GCN2**: *HvGCN2* silenced leaf cDNA, **Actin**: using Actin primer set, **GAPDH**: using Glyceraldehyde 3-phosphate dehydrogenase primer set, **EF**: using Elongation factor primer set, **Ubi**: using Ubiquitin primer set, **α-tubulin**: using α-tubulin primer set

2.3.7 qRT-PCR to assess the silencing level of *HvGCN2*

The procedure was carried out same as Section 2.3.6, with only two differences. One difference is the use of primers, and the other is that the reaction was carried out for 40 cycles. In silencing level determination, actin-F / actin-R and EF-F / EF-R primers were used for normalization analysis. The gene specific primers used were indicated in Table 2.5. Six different cDNAs from silenced samples and three different cDNAs from leaves inoculated with BSMV:00 (naked virus) as control were used for comparisons. The calculations for silencing level determination were performed according to Pfaffl (2001). This method makes use of ΔCp values between control and silenced groups for both gene of interest and reference genes. In this equation, ΔCp (crossing point) means the difference between the Ct (threshold cycle) values of control and silenced groups.

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

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

This calculation method is also mentioned as delta-delta method (Pfaffl, 2001). This formula gives you the result of sample compared to control. By modifying this equation, the silencing levels were calculated as follows;

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

In addition, the expression level differences can be mentioned as fold change. Fold change in the expression level of the control groups with respect to silenced groups can be calculated as follows;

$$\text{Fold change} = \frac{2^{\Delta Cp}\ \text{Reference gene (control – sample)}}{2^{\Delta Cp}\ \text{Gene of interest (control – sample)}}$$

After qRT-PCR, the products were again analyzed in agarose gel electrophoresis to check if there was only one product with desired size.

Table 2.5 Sequences of gene specific primers for qRT-PCR.

Primer name	5'-3' Sequence
GCN2-F-134	CTG ACG CAG ATG TGA ATG CT
GCN2-R-134	CAG TTG TTG GGT CGA AAC CT

2.4 Pathogen Inoculation

2.4.1 Powdery mildew infection of the silenced and control plants

As mentioned in Section 2.3, the leaves were cut into 4 different pieces 14 days after the BSMV inoculation. Two of the pieces were stored for RNA isolation, and the other two were used in powdery mildew infections with *Bgh*-103 (avirulent) and *Bgh*-95 (virulent) (Table 2.1) (Figure 2.8).

The powdery mildew infections have been performed as mentioned in Section 2.1.2 (Figure 2.9).

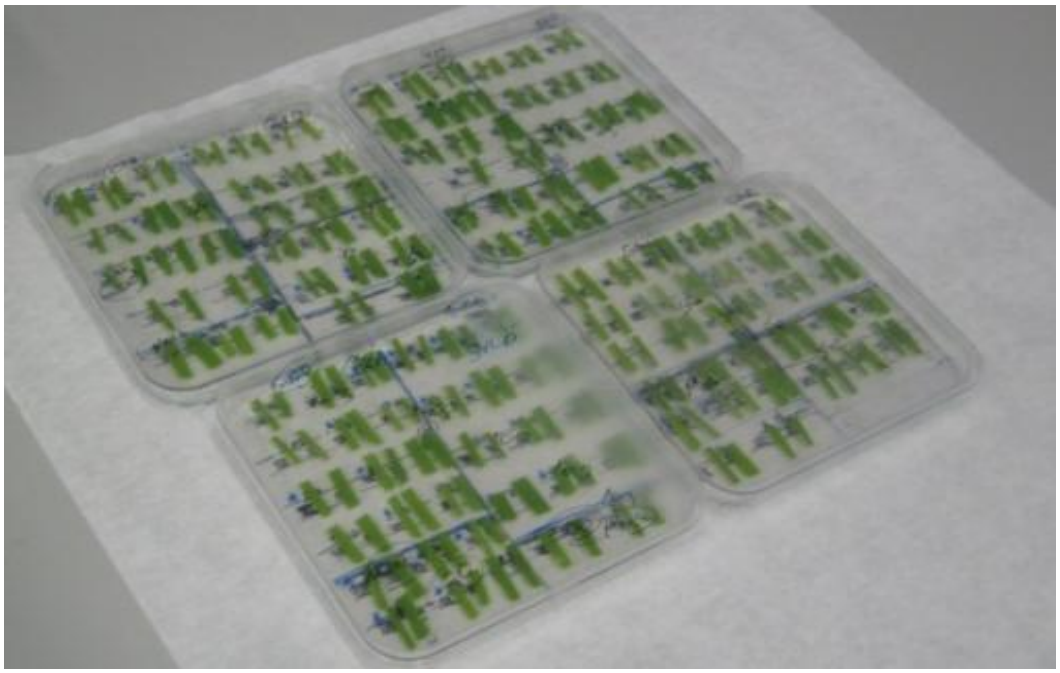


Figure 2.9 The detached leaves on the water agar plates ready for infection by powdery mildew races.

2.4.2 Assessment of powdery mildew infection levels on the inoculated leaves

After 3 days post inoculation (dpi) and 5 dpi, the leaf fragments were stained by trypan blue. The leaf fragments were kept in 96% ethanol overnight to remove green background. Afterwards, the fragments were kept in trypan blue solution (5 mL lactic acid (Fluka; Lot no: BCBC5008); 5 mL 87% glycerol; 5 mL ddH_2O; 0.00375 g trypan blue (Applichem; Lot number: 7D008258)) for 15 min at room temperature. Then, the leaves were transferred into rinsing solution (5 mL lactic acid (Fluka, Lot no: BCBC5008), 5 mL 87% glycerol, 5 mL ddH_2O) for 3 min. Then, the leaf fragments were put onto glass slides with a drop of 60% glycerol. After the staining, the pathogen infection levels were observed under light microscope, Leica DM4000B microscope / DFC 280 camera. The lenghts of all hyphae were measured and classified as primary hyphae, secondary hyphae, and longest hyphae. The primary

hyphae are the ones that emerge from the spore itself, and secondary hyphae are the ones that emerge from the primary hyphae (Figure 2.10). Then, the average hyphae sizes in all classifications were calculated.

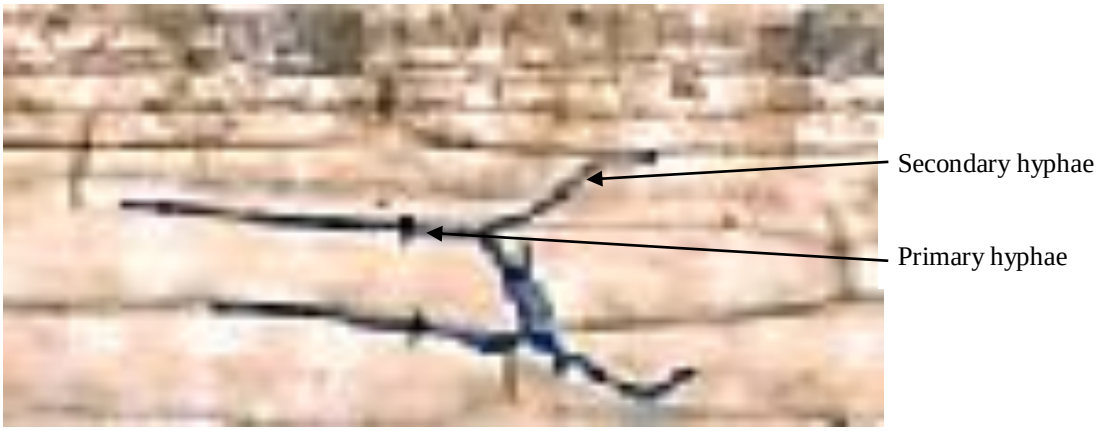


Figure 2.10 Hyphae types of a typical powdery mildew spore at 3 dpi.

CHAPTER III

RESULTS and DISCUSSION

3.1 Powdery mildew maintenance

The inoculation of powdery mildew strains on Bülbül-89 barley leaves was conducted as in Section 2.1.2. After 10 dpi, the white velvety pustules were easily detectable on the leaf surfaces (Figure 3.1).

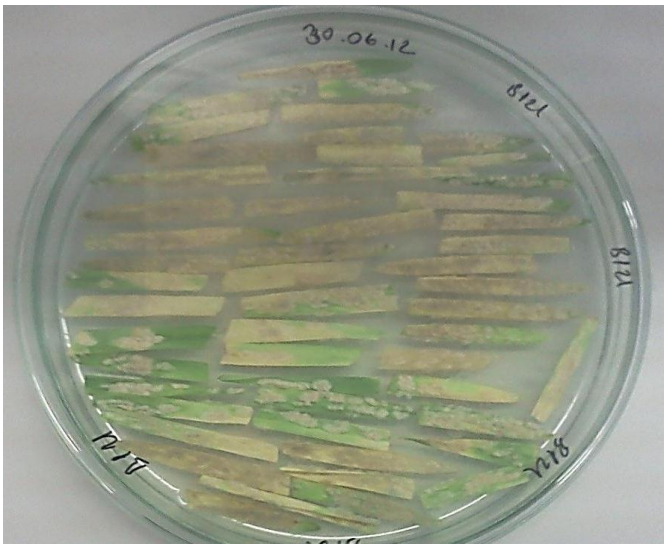


Figure 3.1 Powdery mildew infected detached Bülbül-89 leaves. The spores are ready to infect new leaves (10 dpi).

The powdery mildew spores were also analyzed under light microscope after trypan blue staining. Trypan blue is a vital dye that stains only dead cells. The chromopore

of the trypan blue is negatively charged and only interacts with the cell with damaged cell membranes (Freshney, 1987). Besides, trypan blue is selective for chitin, which is present in the hyphae cell walls of the fungi (Virant, 1988). The trypan blue staining of the spores at different time points are indicated in Figure 3.2.

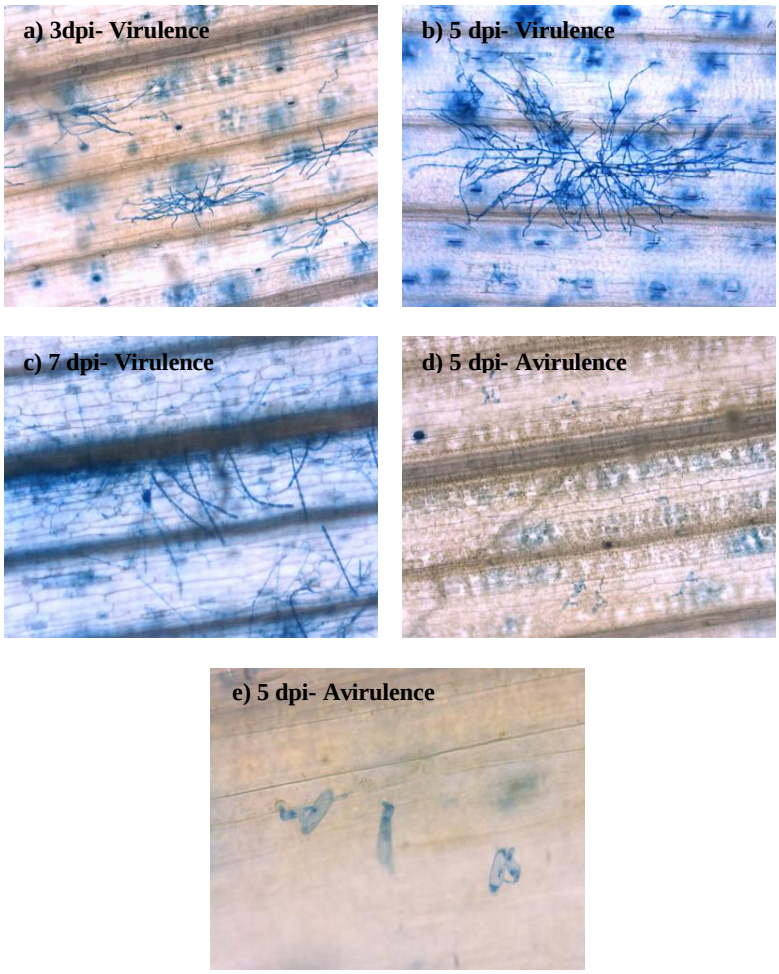


Figure 3.2 Trypan blue stained powdery mildew inoculated plant leaves. **a)** Powdery mildew growth (*Bgh*-95/Pallas-01) at 3 dpi, 10X magnification **b)** Powdery mildew growth (*Bgh*-95/Pallas-01) at 5 dpi, 10X magnification **c)** Powdery mildew growth (*Bgh*-95/Pallas-01) at 7dpi, 10X magnification **d)** Resistance against powdery mildew (*Bgh*-103/Pallas-01), 10X magnification **e)** Resistance against powdery mildew (*Bgh*-103/Pallas-01), 40X magnification.

3.2 Preparation of BSMV

Preparation of BSMV began with plasmid isolation. Purity and integrity of plasmid is important for efficient restriction enzyme digestion. For purity determination, absorbance values in 230 nm, 260 nm, and 280 nm are important. Polysaccharides give peaks in absorbance at wavelength of 230 nm, while proteins do the same at 280 nm. Nucleic acids absorb strongly at 260 nm. A_{260}/A_{280} should be between 1.80-1.90 and A_{260}/A_{230} should be around 2.00. Higher A_{260}/A_{280} value indicates too much RNA presence in the plasmid isolate, and if A_{260}/A_{280} value is lower, it means there is protein contamination. There are several sources causing high absorbance at 230 nm, such as carbohydrates, phenolic compounds, EDTA and guanidine HCl from TRIzol® reagent. As a result of plasmid isolation, if A_{230}/A_{260} is too low, it means there is polysaccharide contamination. In addition, the plasmids must be concentrated enough for digestion. Since 43 µL is the maximum amount of plasmid DNA used to have 4 µg of plasmid, the concentration should be at least;

$$\frac{4000 \text{ ng}}{43 \text{ µL}} \cong 93 \text{ ng/µL}$$

NanoDrop measurements can provide both concentration and purity information, which makes it a very useful tool. For NanoDrop measurements, NanoDrop ND-1000 spectrophotometer was used. NanoDrop measurements of the isolated plasmids are shown in Table 3.1.

Table 3.1 NanoDrop measurements of isolated plasmids

Plasmid	**Concentration (ng/µL)**	**A_{260} / A_{230}**	**A_{260} / A_{280}**
pα	321.8	2.38	1.98
pβΔβa	242.2	2.48	2.03
pγ	472.3	2.27	1.93
pγb.PDSAs	498.5	2.28	1.93
pγBSMV:*GCN2*	390.8	2.25	1.95

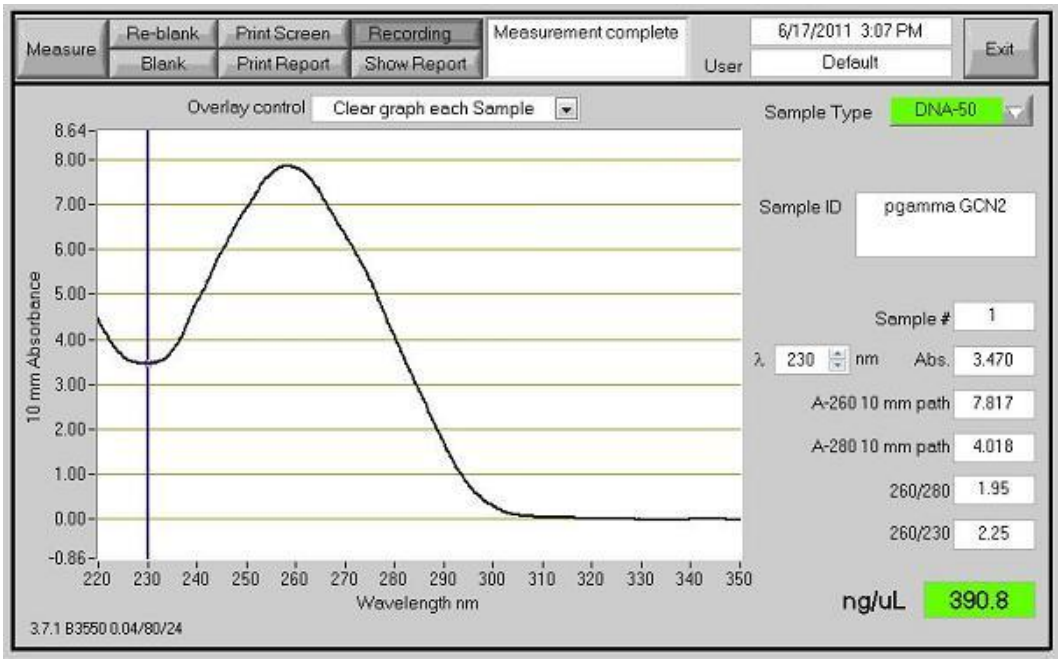


Figure 3.3 Concentration determination of pγBSMV:*GCN2*.

Once the plasmids were isolated with accepted quality, restriction enzyme digestion was conducted. Plasmids can be in different 3D-forms, thus migrate at different speeds on gel electrophoresis: closed circular (supercoiled) plasmids both of the strands are intact, open circular (nicked) where only one of the strands is cut, linear where both strands are cut. In addition, the plasmids may be in supercoiled denatured formation in which they have unpaired regions, mostly because of the damage during plasmid isolation, and relaxed circular where both strands are intact but supercoils are removed enzymatically. Therefore, the resulting gel might show up to five different bands; covalently closed circular, open circular, relaxed circular, linear, and supercoiled denatured forms. On the other hand, after linearization, only one band should be observed. Open circular plasmid moves slowest, followed by relaxed circular, linear, covalently closed circular and supercoiled denatured plasmids. Our results proved both

integrity of the plasmids and efficiency of the linearization (Figure 3.4). The bands were clear and there was no smear formation on both uncut and linearized plasmids.

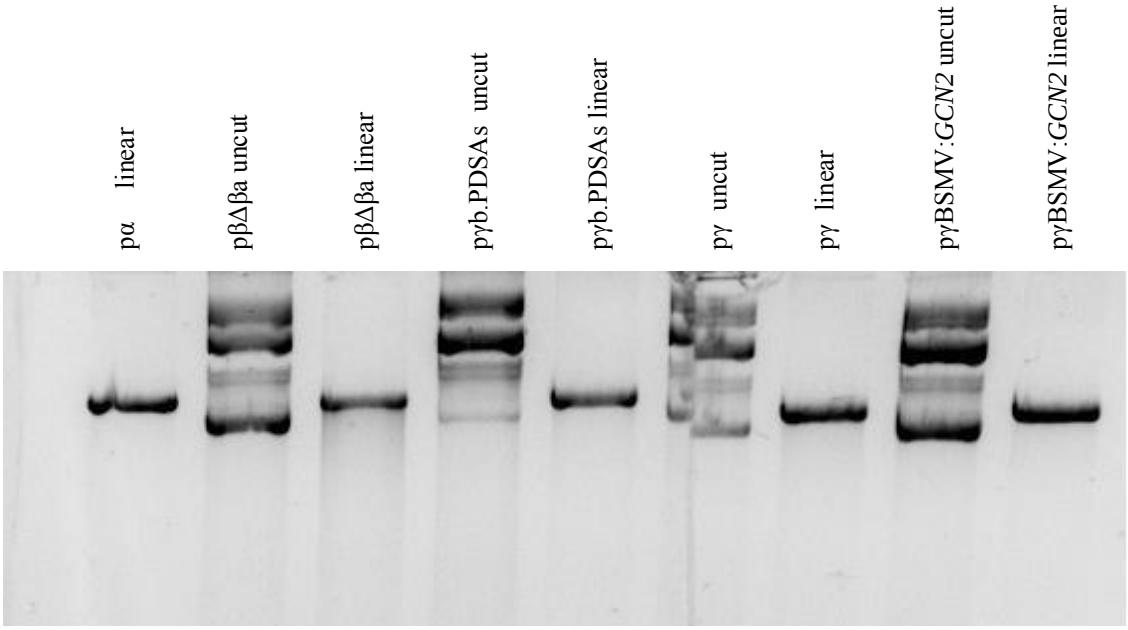


Figure 3.4 Agarose gel image of linearized pα, pβΔβa, pγb.PDSAs, pγ, and pγBSMV:*GCN2* plasmids. Cut and uncut plasmids (0.8-1.0 µg/lane) on the labeled wells of 1 % agaorose (Note: The gel was broken on the 6th lane).

After the plasmids were linearized, *in vitro* transcription was performed. In this step, T7 RNA Polymerase binds to the T7 promoter, and transcribes RNA until reaching the end of the DNA (restriction cut site). After *in vitro* transcription reactions, the products were analyzed with agarose gel electrophoresis to assess the efficient RNA transcription (Figure 3.5). The RNA gel electrophoresis was conducted by replacing the buffer for every 20 minutes to prevent formation of pH gradient.

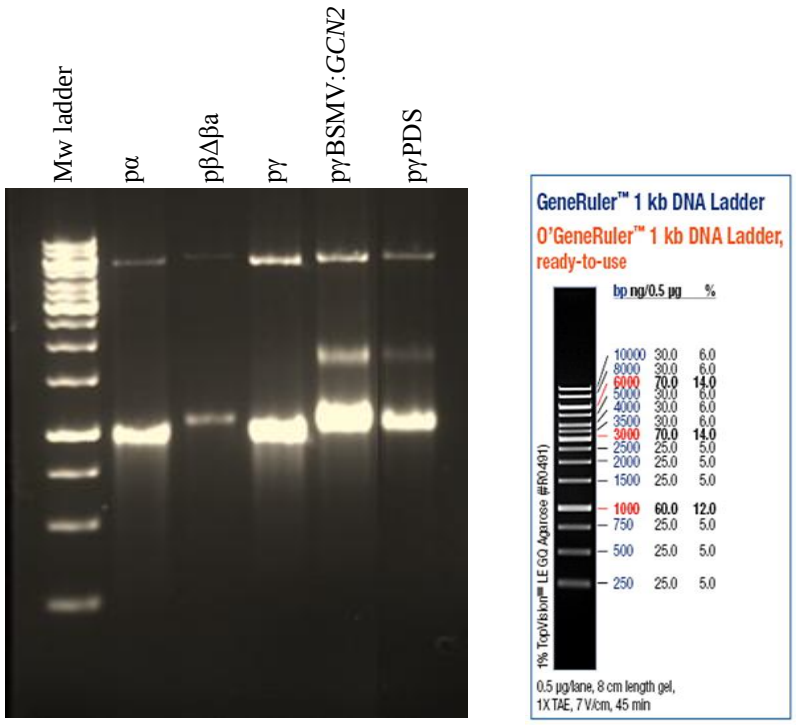


Figure 3.5 RNA gel electrophoresis image of *in vitro* transcription products. *In vitro* transcription products of 1 µL from 10 µL reaction were loaded to each well. On the right: GeneRuler, 1kb DNA ladder molecular weight banding pattern.

3.3 qRT-PCR

14 days after BSMV inoculation, next step was to assess the level of silencing on the same plant samples. Thus, the silencing levels were determined with qRT-PCR analyses. The very leaf samples presented in the Figure 2.8 were used for qRT-PCRs.

Leaf total RNA isolation was performed as indicated in Section 2.3.1. In order to use the samples in qRT-PCR analysis, the quality of RNA isolation is critical such as; the purity, quantity, and integrity of the RNA samples. RNA purity can be determined by looking A_{230}, A_{260}, and A_{280} values. Nucleic acids give absorbance peaks at 260 nm.

At 230 nm; some proteins, salts, and phenolic compounds give absorbance peaks. At 280 nm, proteins give absorbance peaks. A pure RNA must have A_{260}/A_{230} and A_{260}/A_{280} values close to 2.00. RNA samples with $A_{260}/A_{230} > 1.60$ can be used, but the ones lower than this value should be avoided. The RNA concentration should be at least 125 ng/µL for efficient DNase treatment because in lesser concentrations, the DNase becomes too concentrated. The manufacturer's protocol suggests not to use more than 1 u of DNase for 1 µg of RNA. Four of our samples had slightly lesser concentrations but this didn't cause any problem.

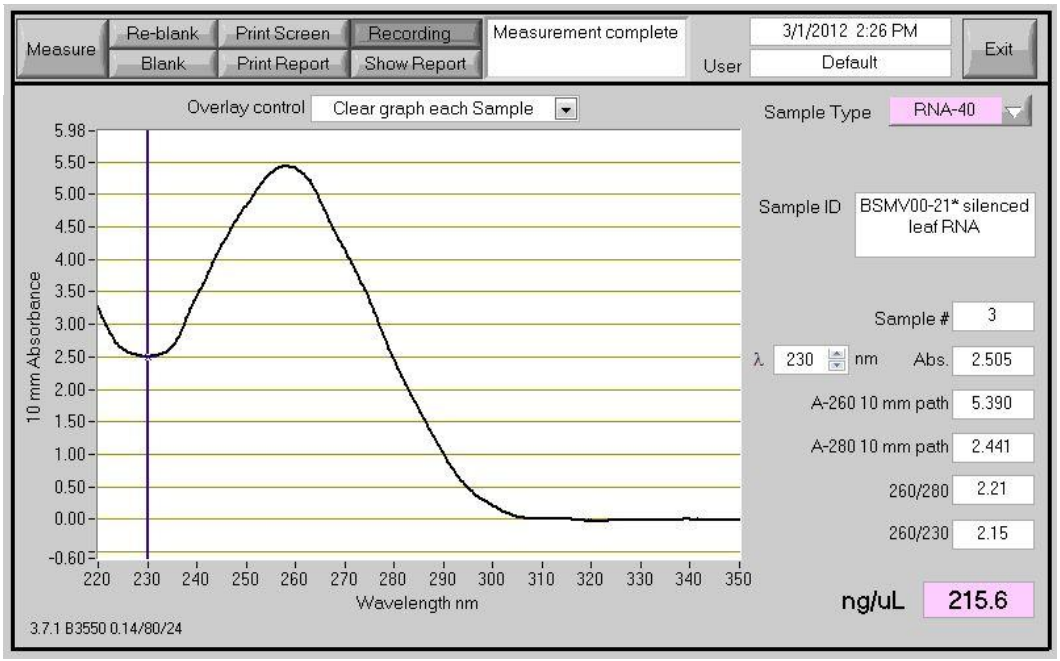
In my experiments, I faced some problems with obtaining adequate A_{260}/A_{230} values in some of the samples, since those RNA isolations were not satisfactory to use in qRT-PCR analyses. Unfortunately, "Bulk" set was also lost because of this reason. Therefore, RNA isolations from the individual leaf samples had been performed. From them, the average silencing level was aimed to be determined. The list of RNA isolation NanoDrop results are indicated in Table 3.2.

Table 3.2 NanoDrop measurements of isolated total RNAs.

Samples	**RNA silencing type**	**Concentration (ng/µL)**	**A_{260} / A_{230}**	**A_{260} / A_{280}**
R_C1	BSMV:00 replicate 1	215.6	2.15	2.21
R_C2	BSMV:00 replicate 2	117.3	1.92	2.09
R_C3	BSMV:00 replicate 3	121.9	1.94	2.16
R_S1	BSMV:*HvGCN2* replicate 1	168.9	1.68	2.06
R_S2	BSMV:*HvGCN2* replicate 2	173.0	2.00	2.23
R_S3	BSMV:*HvGCN2* replicate 3	178.1	1.65	2.06
R_S4	BSMV:*HvGCN2* replicate 4	121.6	2.00	2.10
R_S5	BSMV:*HvGCN2* replicate 5	118.7	1.94	2.08
R_S6	BSMV:*HvGCN2* replicate 6	311.6	2.00	2.01

The concentrations and absorbance values at different wavelengths of the RNAs suggest that the concentrations and the purities of the RNA samples were adequate. R_C2, R_C3, R_S4, and R_S5 had concentrations slightly lower than 125 ng/µL, however, no problem was faced regarding these samples.

a)



b)

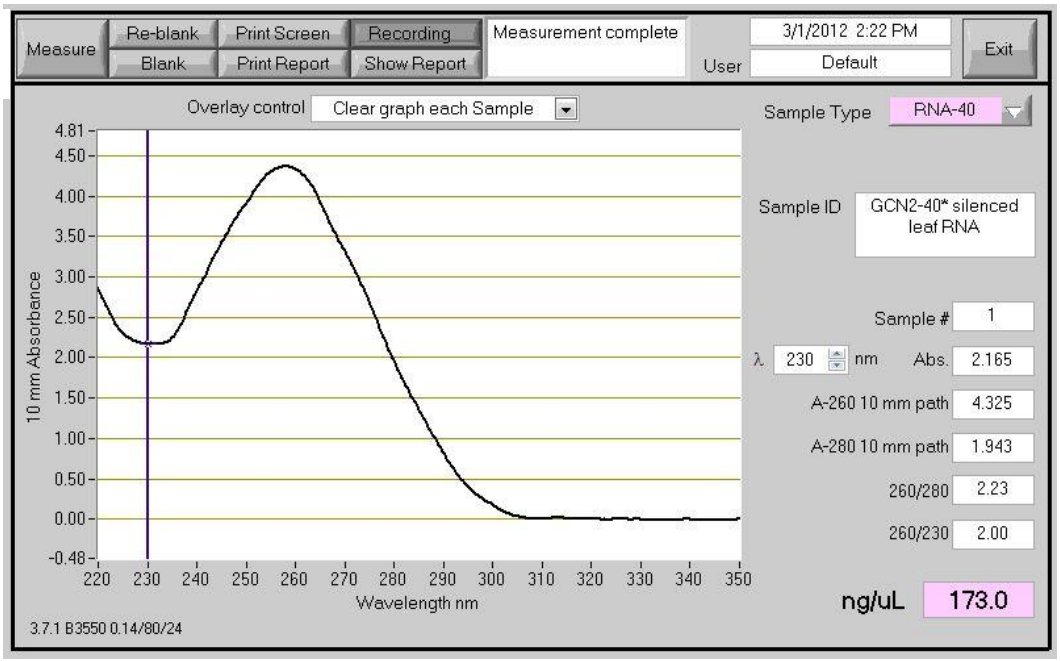


Figure 3.6 Concentration determinations of isolated total RNAs. **a)** BSMV:00 replicate 1, also indicated as R_C1. **b)** BSMV:*HvGCN2* replicate 2, also indicated as R_S2.

For integrity determination, 2 µL of each RNA isolate were analyzed by agarose gel electrophoresis as indicated in Section 2.3.1. In the gel electrophoresis, only ribosomal RNAs are concentrated enough for visualization. Plants have 4 different types of ribosomal RNAs that can be visualized on gel: 26S, 18S, 5.8S, and 5S. At the end of gel electrophoresis, we obtained clear bands in all of the RNA samples, the integrity of all of the samples were acceptable (Figure 3.7).

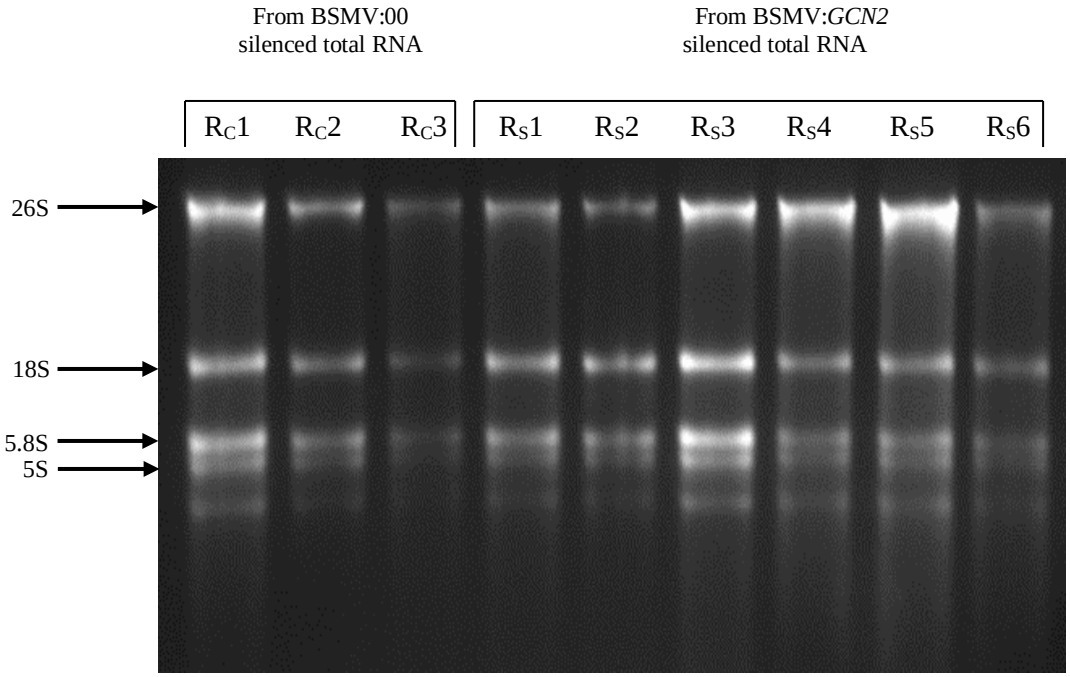


Figure 3.7 Agarose gel (1 %) electrophoresis of the isolated RNAs. Total RNA of 2 µL of varying concentrations were added to each well. The lane definitions are the same as in Table 3.2.

After RNA isolation, DNase treatment was performed to remove any residual genomic DNA (gDNA) from the RNA preparations. They are all at very low concentrations;

however, there must be no genomic DNA contamination at all. Even a very low concentration of gDNA contamination may cause huge difference in qRT-PCR analysis since every signal is amplified 2^{40} fold at the end of a qRT-PCR experiment. After DNase treatment, EDTA is used to prevent the RNA samples from degradation. EDTA is a chelating agent and it binds to Mg^{2+} ions in the solution, which may cause non-specific degradation of RNA molecules at elevated temperatures. Phenol-chloroform extraction is especially avoided because of having very low amount of RNA samples. Even though it is a more reliable method to obtain pure RNA at the end of DNase treatment, phenol-chloroform extraction may cause significant losses in RNA quantity, in which we couldn't take risk.

After DNase treatment, *HvGAPDH* primers were used in the PCR reactions which were performed to see if there is any gDNA left. If any amplification product is observed on the gels, this would have been the indication of gDNA contamination. Even if there is a very small amount of genomic DNA left after DNase treatment, PCR amplifies it and we can see clear bands. Nevertheless, there was no amplification of DNA in DNase treated group of samples as well as in the negative controls, while having amplification in positive control, in which a previously prepared barley cDNA is used (Figure 3.8). The gel picture showed that DNase treatment removed all of the contaminating gDNA. In the positive control, the band was expected to be observed at 224 bp, and it also proved that PCR worked efficiently and amplified *HvGAPDH*.

As an alternative to check the DNase treatment efficiency using PCR, use of intron-spanning primers in qRT-PCR can enable us to detect the presence of gDNA contaminations. In case we use intron spanning primers, gDNA contaminations would result in products with different sizes, which leads in two peaks in dissociation curves and two bands in gel electrophoresis results provided that there are one or more introns within the amplified sequence. Nevertheless, this method requires full length sequence of the gene of interest.

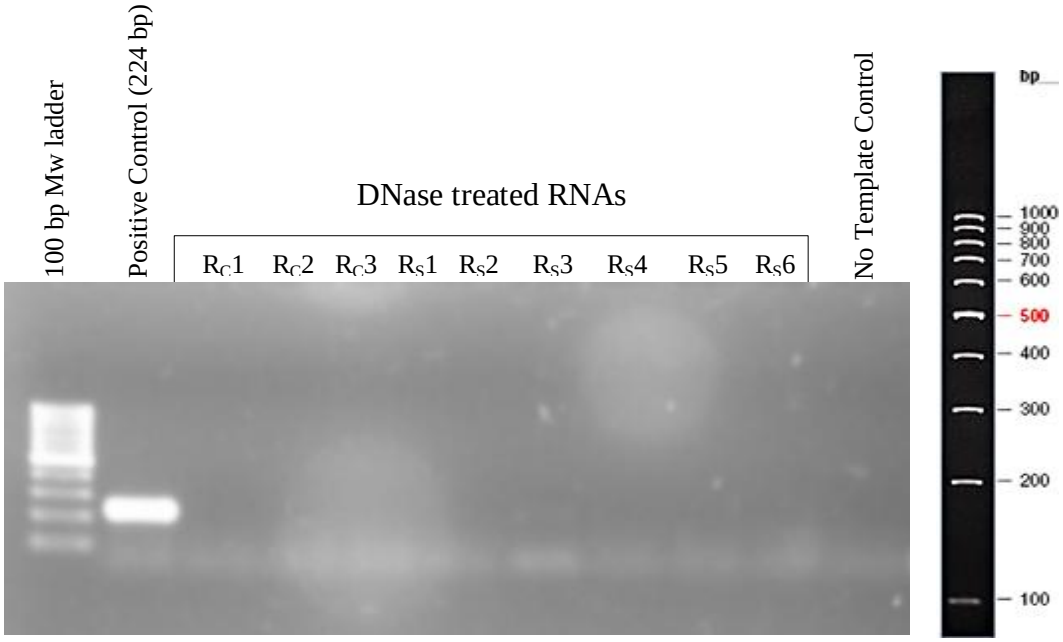


Figure 3.8 Agarose gel of PCR products from DNase treated RNA samples for *HvGAPDH* amplification. Each lane contains 1 µL of PCR products of 1 % agarose gel. The lane definitions are the same as in Table 3.2.

Once the RNAs were proved to be free of any DNA contamination, cDNA synthesis was conducted. During cDNA synthesis, oligo $(dT)_{20}$ and random hexamer primers were used. Oligo $(dT)_{20}$ primers were aimed to bind poly-A tails of the mRNA molecules, while random hexamer primers bind anywhere possible. Both primers were used together to ensure complete coverage of the RNA for cDNA synthesis. During cDNA synthesis, two pre-incubations were performed before starting the reverse transcription. It is extremely important to perform these incubations, since they are needed to remove the secondary structures in the RNA and to ensure binding of the primers to the RNA template.

Following the cDNA synthesis, PCR was conducted to make sure if conversion of RNA to cDNA is successful. This way, cDNA presence and integrity was confirmed

before performing many qRT-PCR of samples together with the PCR replicates. The PCR was performed using ubiquitin primers which amplifies products of 218 bp length, then the products were analyzed on an agarose gel. The results showed that cDNAs were adequately synthesized from RNAs (Figure 3.9).

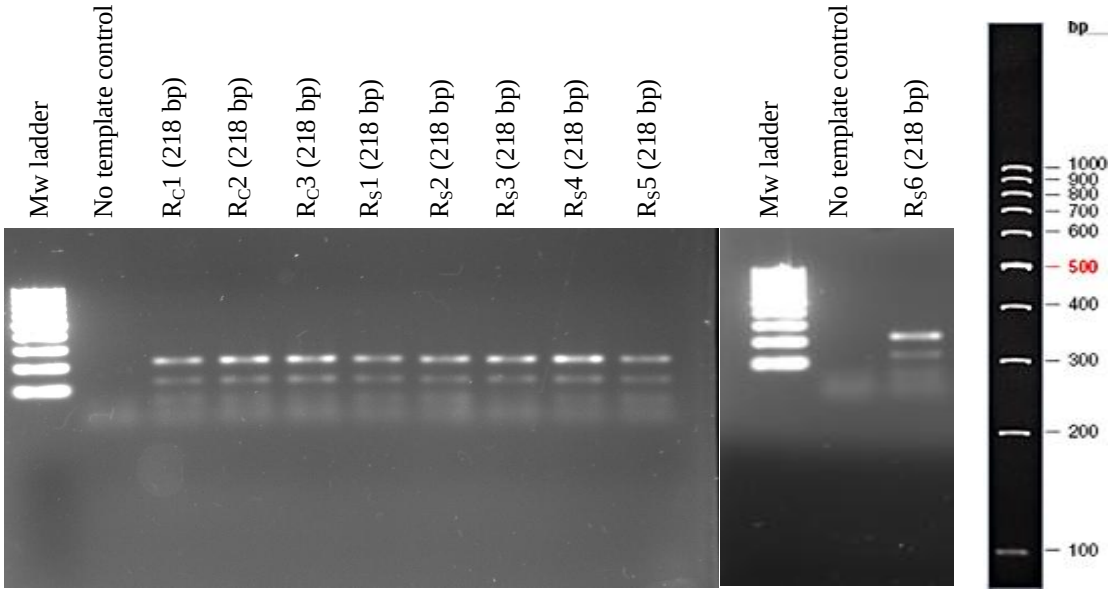


Figure 3.9 Agarose gel of PCR products from cDNA samples for *HvUbi* amplification. The samples were analyzed in agarose gel electrophoresis, 1 µL of PCR products were added to each well.

The presence of double bands on gel electrophoresis result might have two reasons. Either the specificity of the polymerase we have used wasn't high enough, resulting in non-specific amplifications, or the high primer concentration causes such non-specific amplifications.

One of the most critical points in qRT-PCR is to use reliable reference genes. Reference genes are housekeeping genes whose expression level is not affected by the silencing of gene of interest. For that reason, 5 different candidate reference genes

were selected: Actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Elongation Factor (EF), Ubiquitin (Ubi), and α-tubulin. They all are housekeeping genes that were used in various silencing experiments in barley. In order to find out the best two reference genes, a GeNorm program which works on MS Excel was used. Loading the relative expression values of the reference genes, the program determines the M values for each of the reference gene. The smaller M value is the better. As long as M value is less than 1.5, it can be acceptable. In this experiment, all of the normalization primers except GAPDH resulted in M < 1.5 (Table 3.3 and Figure 3.10). The ones with smallest M values, Actin and EF were selected to use in silencing determination by qRT-PCR analysis. The amplification plots and dissociation curves were given in Figure 3.11. The amplification plots also show that *GAPDH* expression level significantly changes between control and silenced groups, which is consistent with the M values. Dissociation curves indicate the presence of only one product, because each product corresponds to one peak. In this case, *HvUbi* amplification resulted in only one peak in dissociation curve (Figure 3.11). As mentioned before, this is because of either using very low concentration of primers or using a much more sensitive DNA polymerase in qRT-PCR experiment. Therefore, all of the products resulted in only one peak as expected. On the other hand, in order to ensure if qRT-PCR product has the desired size, the products were analyzed in agarose gel electrophoresis (Figure 3.12). The gel electrophoresis results also showed both the presence of only one product and the correct sizes.

Table 3.3 M values of the reference genes

Reference Gene	M value
Actin	0.582
GAPDH	1.563
EF	0.578
Ubi	1.186
α-tubulin	0.603

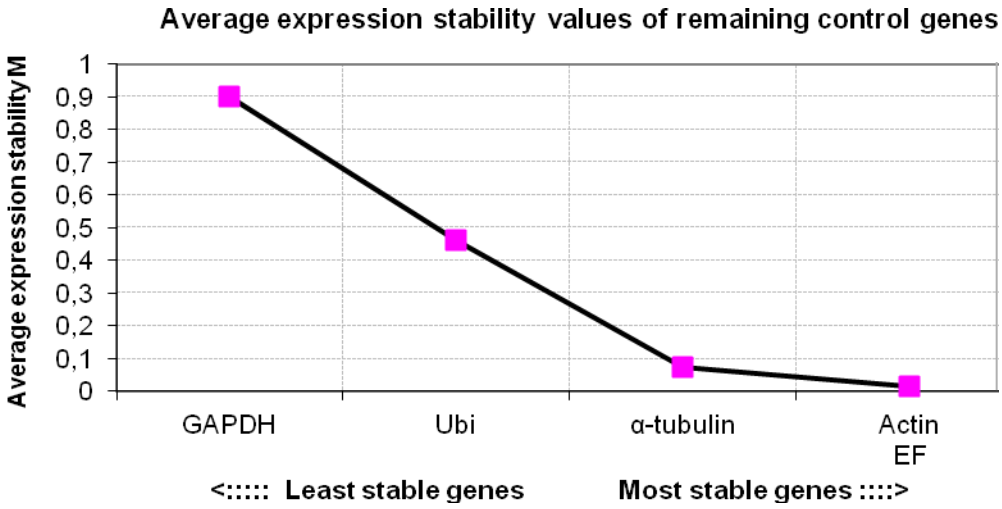


Figure 3.10 Graphic representation of the M values of candidate reference genes.

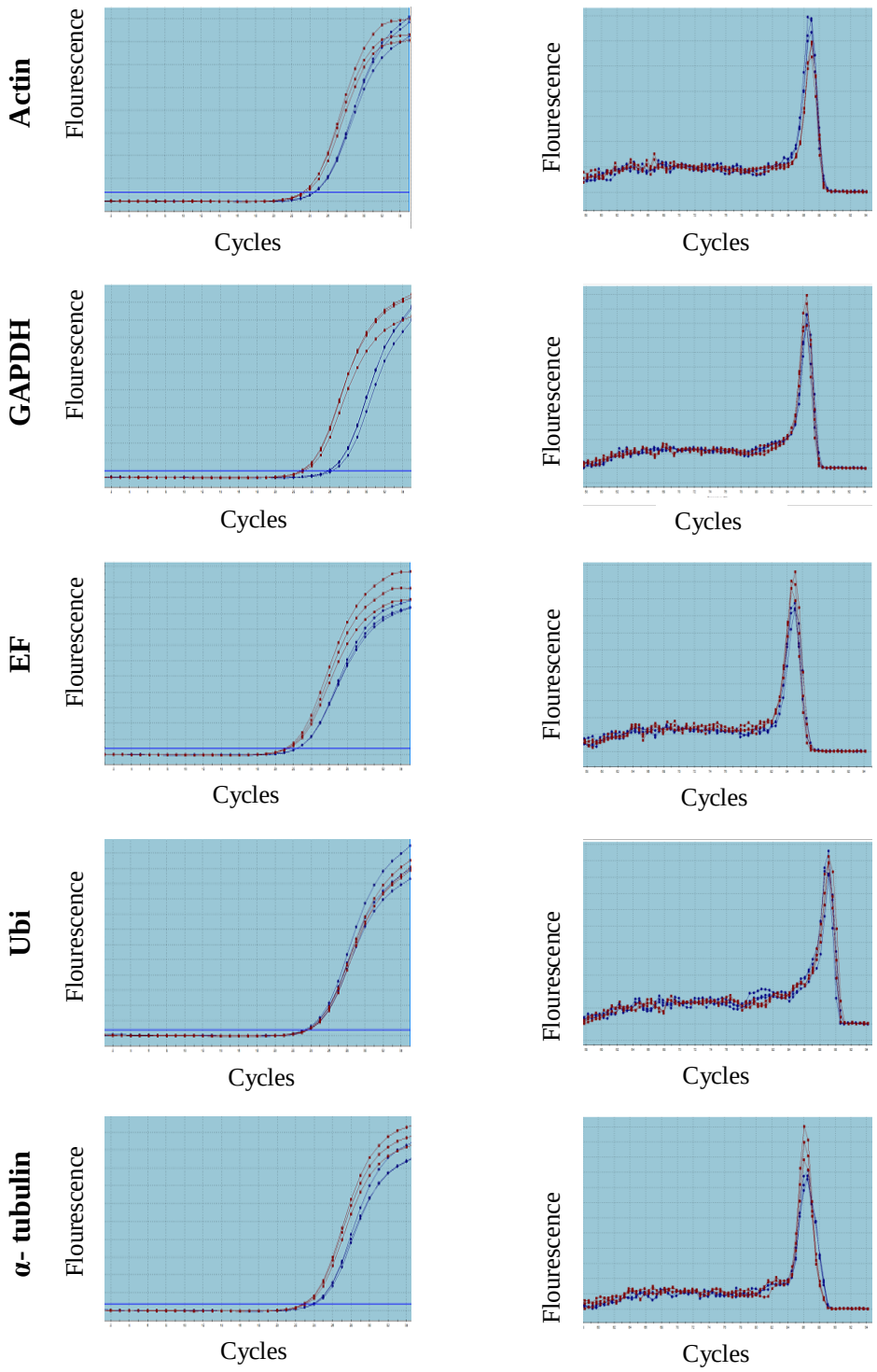


Figure 3.11 The amplification plots (left) and the dissociation curves (right) of the candidate reference genes.

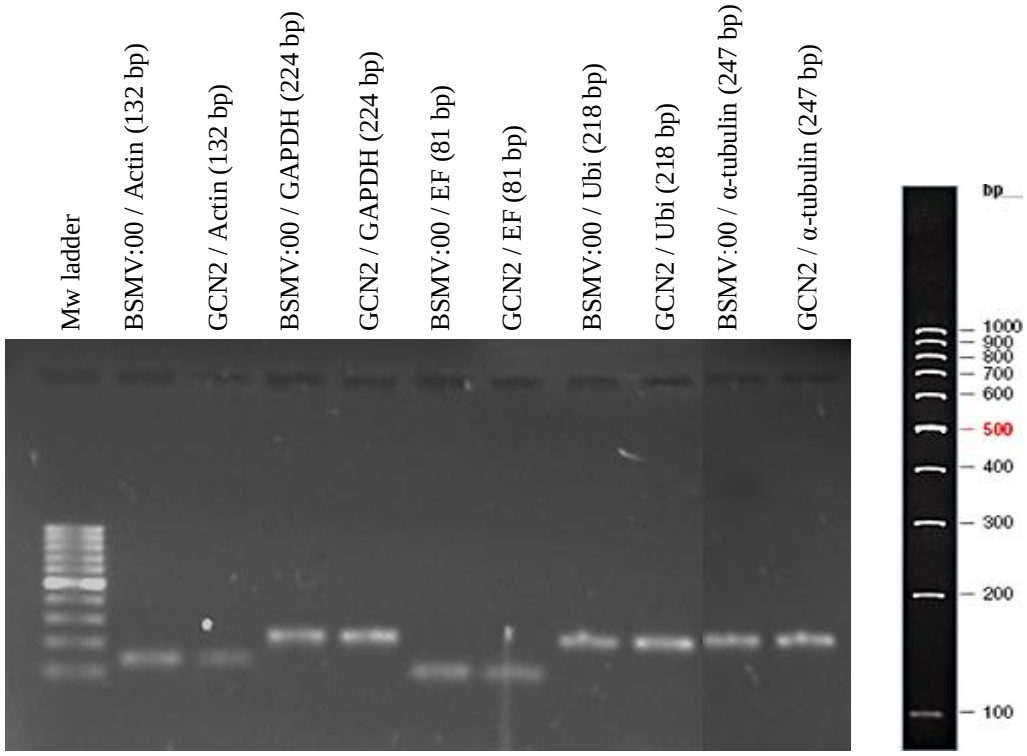


Figure 3.12 Agarose gel of qRT-PCR products of the reference genes. qRT-PCR products of 1 µL were loaded on each well of 1 % agarose gel.

After selecting the reference genes, qRT-PCR for silencing level determination was performed as in Section 2.3.7. Silencing levels for each sample were calculated (Table 3.4). In this step, the arithmetic mean of the average Ct values within R_C1, R_C2 and R_C3 were calculated and taken as one Ct value. In addition, geometric means of the Ct values obtained via using Actin and EF primers were calculated and taken as one Ct value. Then, the silencing calculations were performed as mentioned in Section 2.3.7.

Table 3.4 Silencing levels of individual *HvGCN2* gene silenced plant samples.

Sample	Silencing level (%)	Fold change
R_S1	No silencing	-
R_S2	33 %	1.49
R_S3	43 %	1.75
R_S4	39 %	1.64
R_S5	71 %	3.45
R_S6	30 %	1.43
Average	**43.2 %**	**1.76**

R_S1, the only sample with no silencing, had no viral symptoms at the beginning either. Besides, the hyphae sizes on this sample as a result of staining were similar to BSMV:00 control group instead of other silenced samples. This confirms that the rub-inoculation on sample 1 was unsuccessful. Nevertheless, having silencing on 5/6 of the samples with 43.2 % is enough to confirm that *HvGCN2* has a role in plant defense mechanism (Figure 3.13).

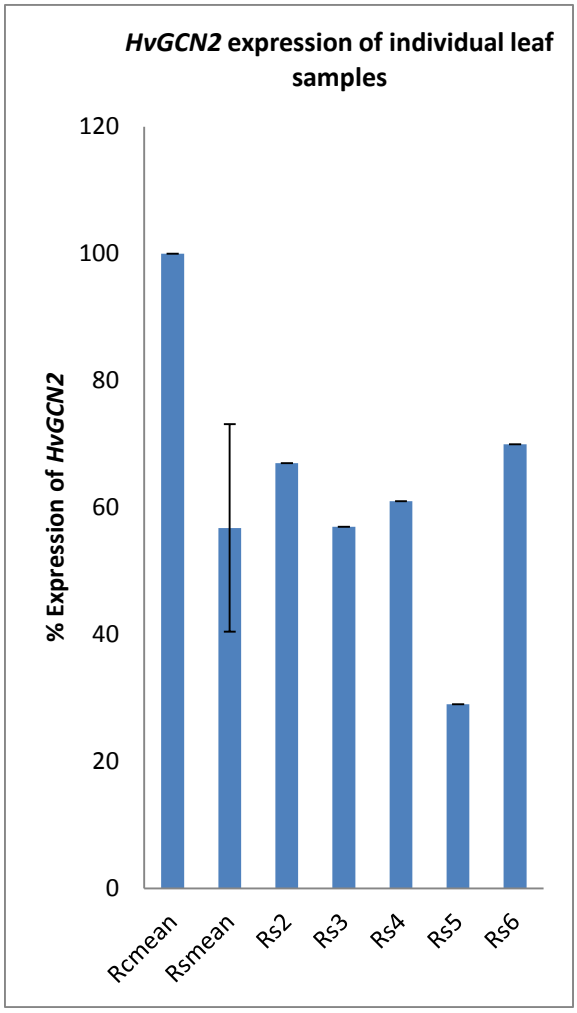


Figure 3.13 The expression levels of *HvGCN2* in individual silenced plant samples.

After qRT-PCR, the products were analyzed in agarose gel electrophoresis. The electrophoresis result also proved that there is only one product with desired size (Figure 3.14).

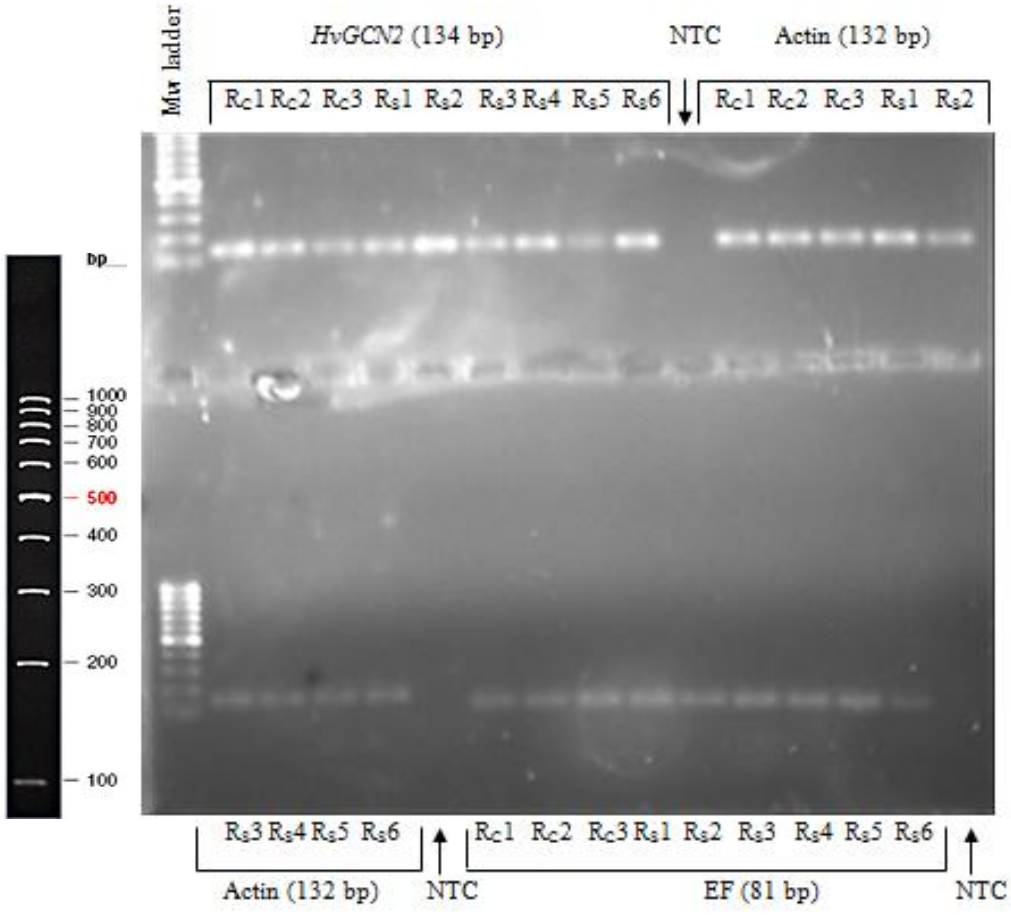


Figure 3.14 Agarose gel electrophoresis of qRT-PCR products for *HvGCN2* silencing level determination. *HvGCN2*, Actin, and Elongation Factor qRT-PCR products of 1 µL were added to each well.

3.4 Pathogen inoculation

After silencing level determination, the powdery mildew infection rates were analyzed to correlate them with the quantitated silencing levels. The assessment of infection levels is based on the hyphae growth rate between control and silenced groups. There was no difference between control and silenced groups on *Bgh*-103/Pallas-01

inoculations: there was no pathogen growth at all in both control and silenced groups as expected, since Pallas-01 is resistant to *Bgh*-103. Losing disease resistance of a plant species and responding as susceptible by silencing a single gene requires silencing of a very critical gene. Unfortunately, *HvGCN2* is not that much critical.

On the other hand, in *Bgh*-95/Pallas-01 inoculations in naked virus (BSMV:00) inoculated and *HvGNC2* silenced samples (BSMV:*HvGCN2*), very clear differences between pathogen growth rates were observed. These differences in the concentration and the size of the hyphae were even more pronounced at 5 dpi. It was impossible to measure the hyphae sizes at 5 dpi in order to quantitate the differences, due to the long length and high concentration. For that reason, the measurements were performed at 3 dpi. Results of 5 dpi were used to observe the difference between the pathogen growth rates without quantitative assessment (Figure 3.15). As indicated in Figure 3.6, there is a very clear difference in pathogen growth rates between a sample of BSMV:00 control group and a sample of *BSMV:HvGCN2* silenced group.

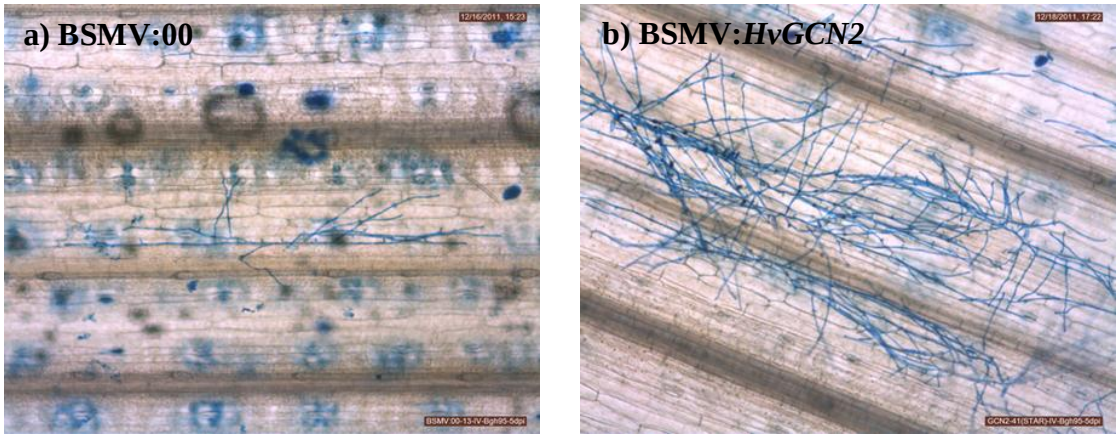


Figure 3.15 Pathogen growth rate differences at 5 dpi on the control and silenced group samples. Powdery mildew inoculation was performed 14 days after silencing of 10 days old plant seedlings of Pallas-01. **a)** BSMV:00 rub-inoculated control sample. **b)** *HvGCN2* gene silenced: BSMV:*HvGCN2* rub-inoculated sample.

The images of all staining results are indicated in Figure 3.16. The overall powdery mildew staining results also show that the pathogen growth rates differ dramatically between control and silenced groups, and the differences are much easier to observe at 5 dpi. On the other hand, there is not much difference between mock-inoculated and BSMV:00 control groups, which means that the spread of BSMV doesn't have much impact on plant's resistance against powdery mildew infection.

a) *Bgh*-95 inoculated samples at 3 dpi on Pallas-01 mock silenced (FES treated) samples

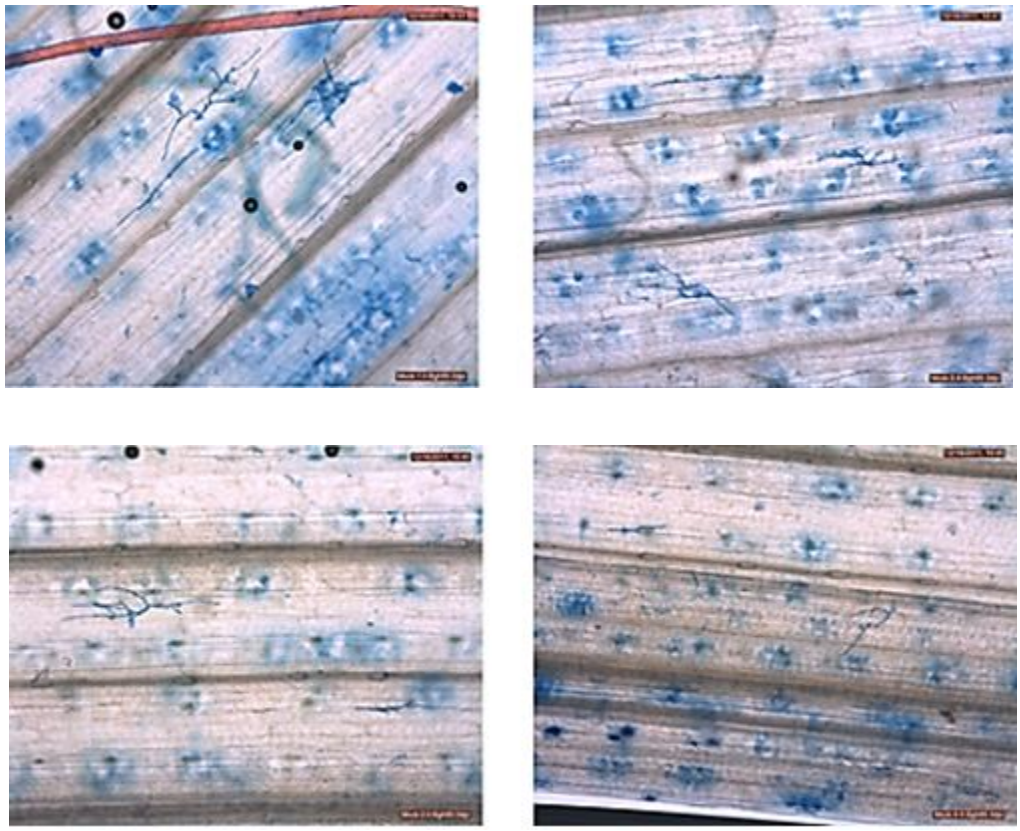


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **a)** Powdery mildew infection on mock inoculated (FES only) seedlings at 3 dpi (Cont'd).

b) *Bgh*-95 inoculated samples at 3 dpi on Pallas-01 BSMV:00 inoculated (naked virus) samples

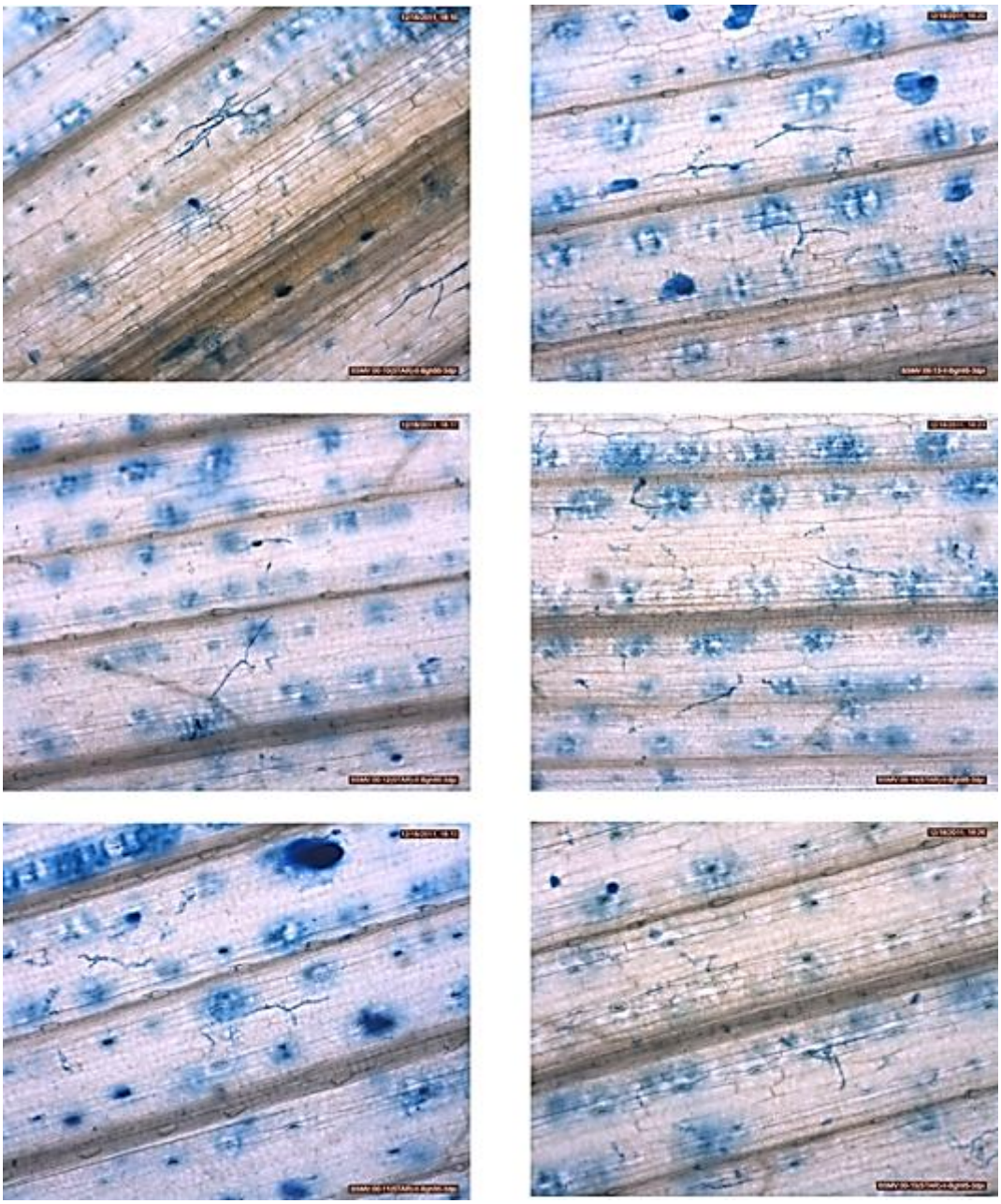


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **b)** Powdery mildew infection on BSMV:00 inoculated (naked virus) seedlings at 3 dpi (Cont'd).

c) *Bgh*-95 inoculated samples at 3 dpi on Pallas-01 *HvGCN2* silenced samples

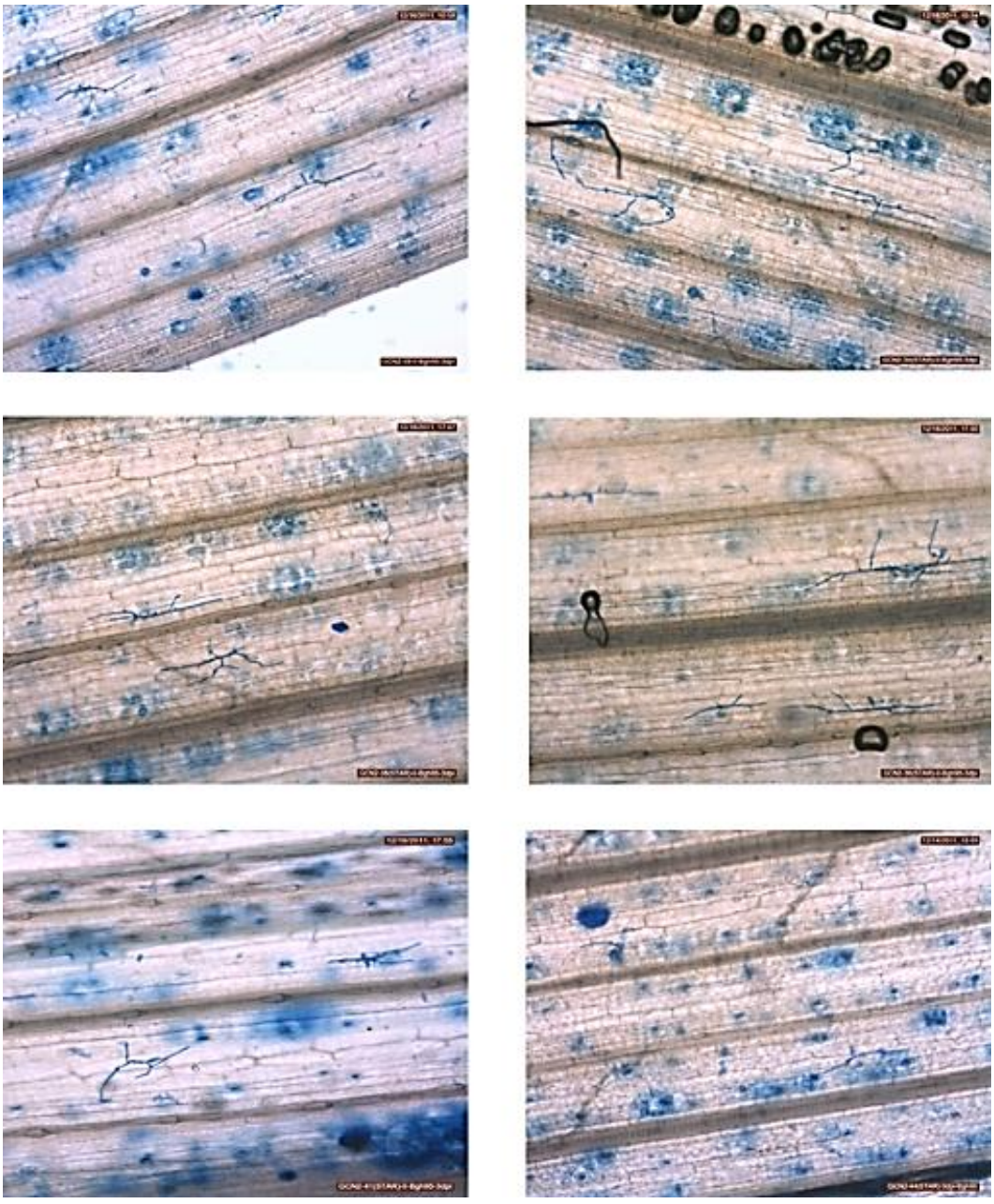


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **c)** Powdery mildew infection on BSMV:*GCN2* inoculated (*GCN2* silenced) seedlings at 3 dpi (Cont'd).

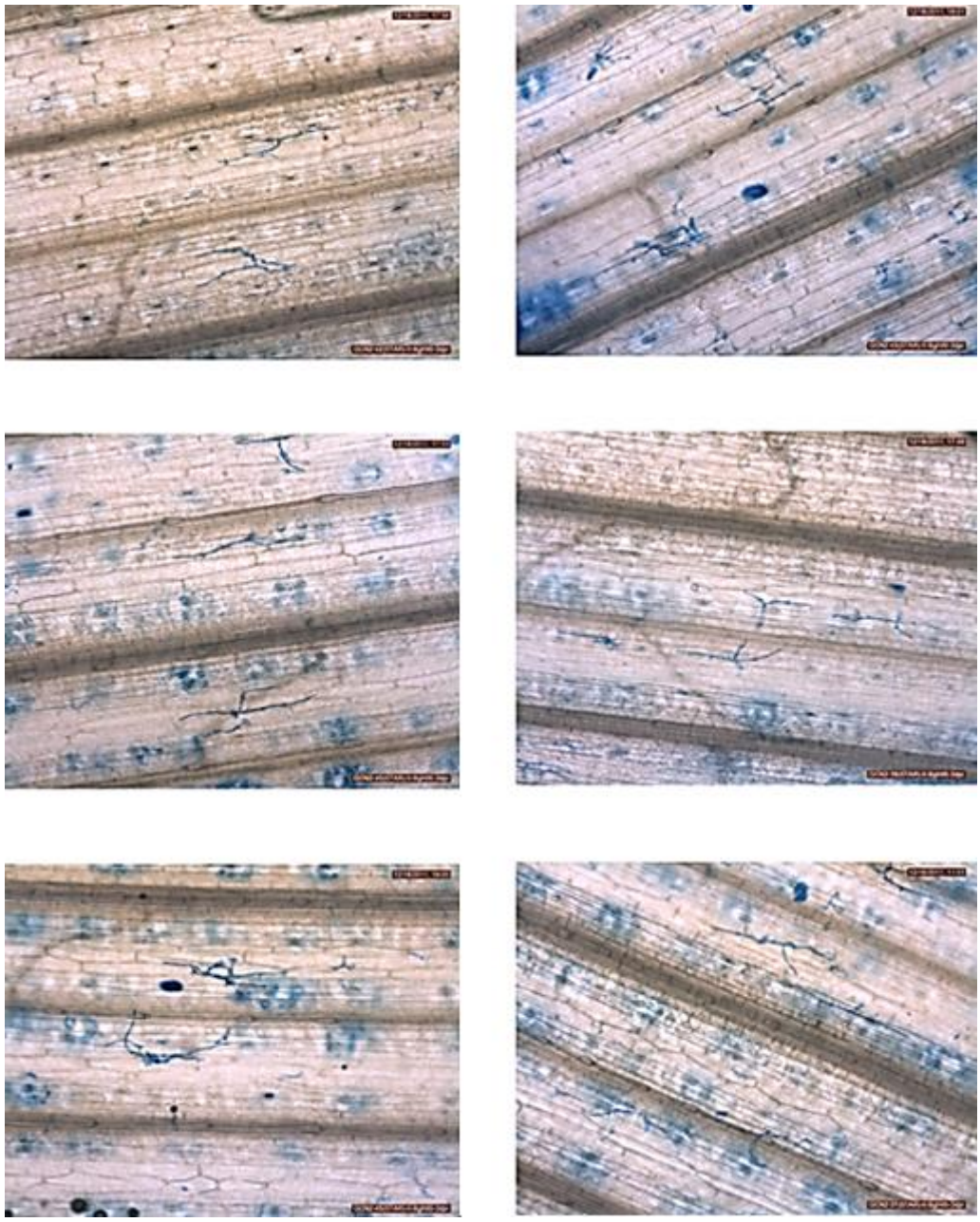


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **c)** Powdery mildew infection on BSMV:*GCN2* inoculated (*GCN2* silenced) seedlings at 3 dpi (Cont'd).

d) *Bgh*-95 inoculated samples at 5 dpi on Pallas-01 mock silenced (FES treated)

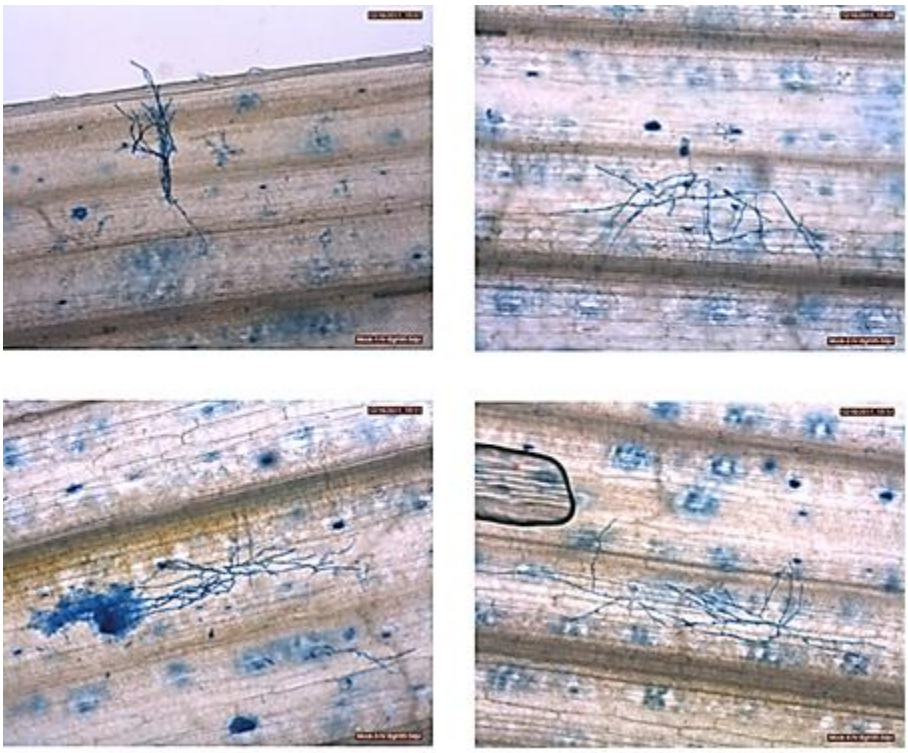


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **d)** Powdery mildew infection on mock inoculated (FES only) seedlings at 5 dpi (Cont'd).

e) *Bgh*-95 inoculated samples at 5 dpi on Pallas-01 BSMV:00 inoculated (naked virus) samples

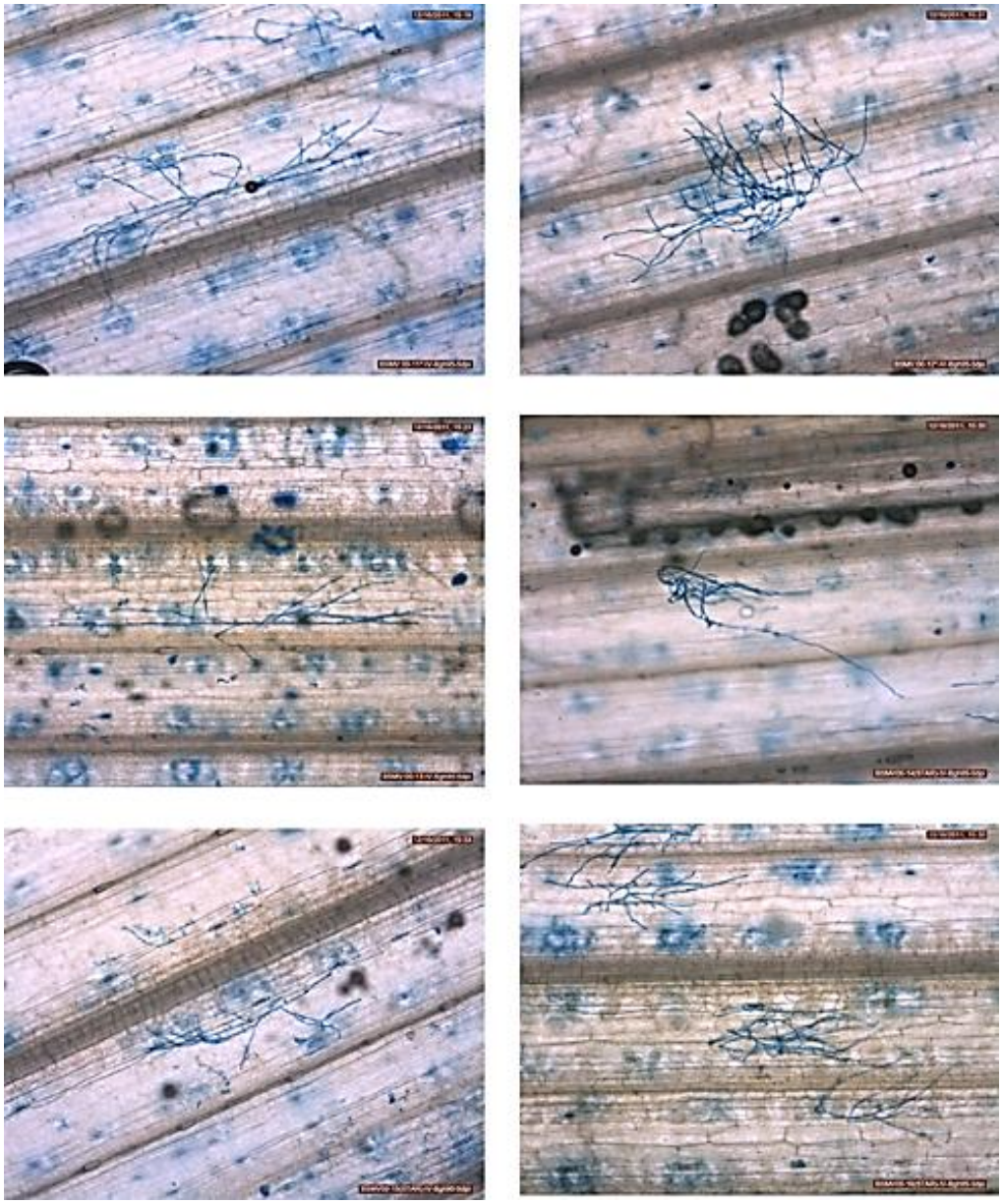


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **e)** Powdery mildew infection on BSMV:00 inoculated (naked virus) seedlings at 5 dpi (Cont'd).

f) *Bgh*-95 inoculated samples at 5 dpi on Pallas-01 *HvGCN2* silenced samples

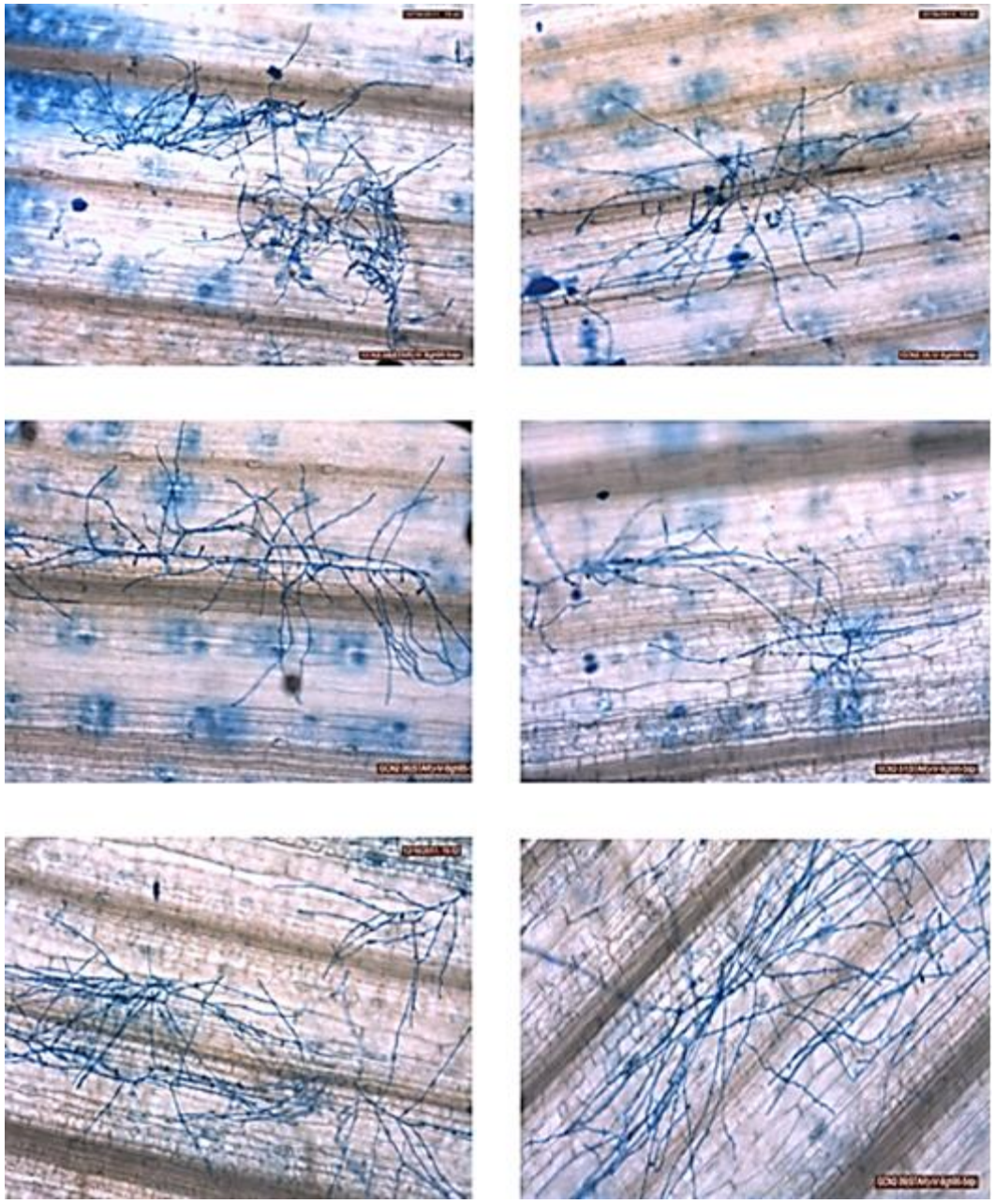


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **f)** Powdery mildew infection on BSMV:*GCN2* inoculated (*GCN2* silenced) seedlings at 5 dpi (Cont'd).

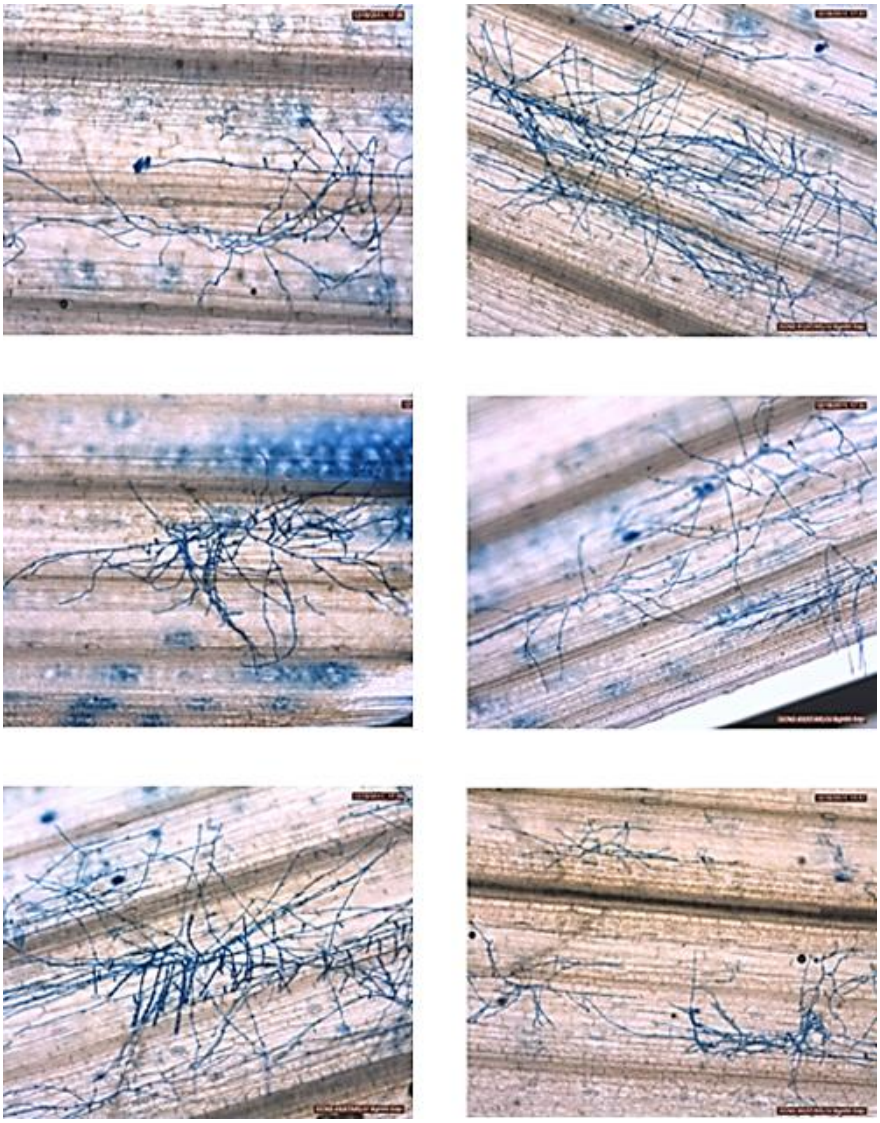


Figure 3.16 Trypan blue staining of all the powdery mildew infections. All the pictures were taken in 10X magnification by Leica DM4000B microscope / DFC 280 camera. **a)** Powdery mildew infection on mock inoculated (FES only) seedlings at 3 dpi. **b)** Powdery mildew infection on BSMV:00 inoculated (naked virus) seedlings at 3 dpi. **c)** Powdery mildew infection on BSMV:*GCN2* inoculated (*GCN2* silenced) seedlings at 3 dpi. **d)** Powdery mildew infection on mock inoculated (FES only) seedlings at 5 dpi. **e)** Powdery mildew infection on BSMV:00 inoculated (naked virus) seedlings at 5 dpi. **f)** Powdery mildew infection on BSMV:*GCN2* inoculated (*GCN2* silenced) seedlings at 5 dpi.

All the hyphae lengths were measured separately, and then the classifications were performed as mentioned in Section 2.4.2. In this step, a total of 292 *Bgh* spores were analyzed. First, the average hyphae lengths within each leaf were calculated by following the classifications of primary and secondary hyphae mentioned in Section 2.3.2. Then, these mean values were used to calculate the hyphae average lengths used in Figure 3.17. The quantitative analysis results showed that there is ~16% difference in longest hyphae lengths between control and silenced group (n=292, p=0.0084), ~20% difference in primary hyphae lengths between control and silenced group (n=603, p= 0.00614) and ~25% difference in secondary hyphae lengths between control and silenced group (n=106, p=0.029336). This result is consistent with qualitative analyses indicated in Figure 3.17.

The t-test was performed by using the means of the hyphae sizes on individual leaves, and selecting t-test function in MS Excel. The t-test was performed as one-tailed, since we can predict the direction, increased pathogen growth rate in silenced group with respect to control group. The type of the t-test was selected as type-III since there are unequal variants, silencing of *GCN2* may cause many changes within the plant cells.

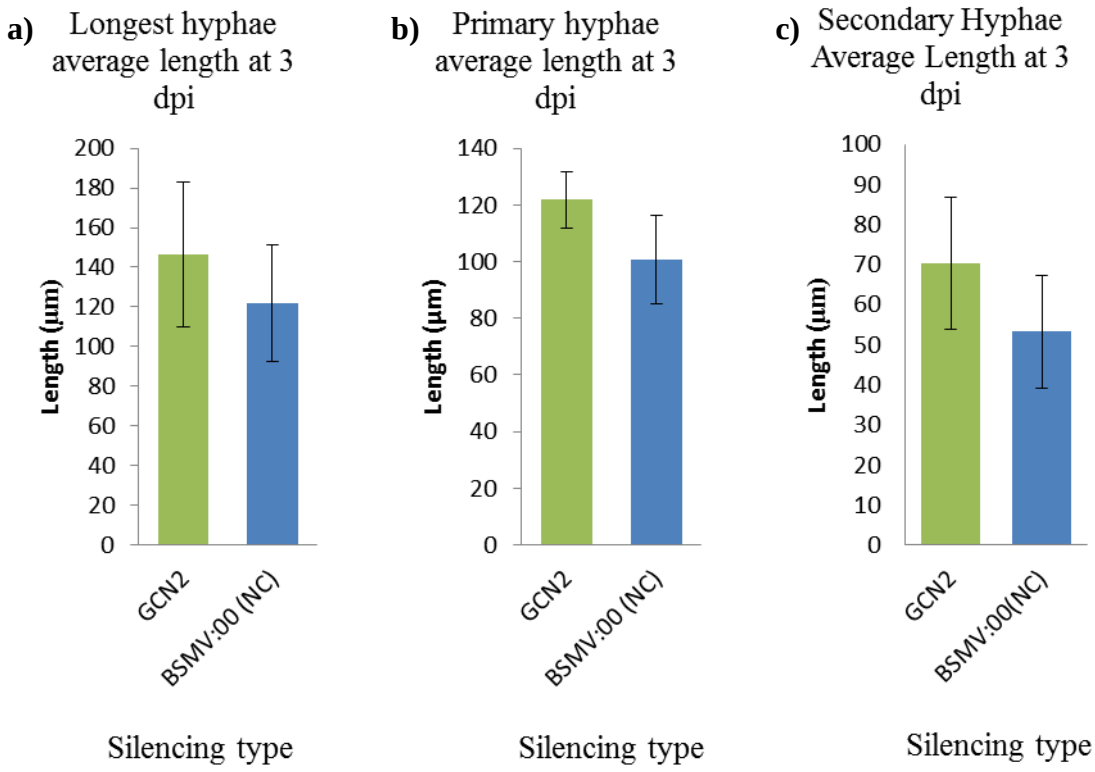


Figure 3.17 Graphic representation of powdery mildew growth differences at 3 dpi. **a)** Averages of the longest hyphae (n=292, p=0.0084). **b)** Averages of the primary hyphae (n=603, p=0.00614). **c)** Averages of the secondary hyphae (n=106, p=0.029336).

CHAPTER IV

CONCLUSION

Barley is a very important crop species for the World as well as Turkey. Nevertheless, functional genomics and gene function analysis studies are currently inadequate in order to further improve the studies in the crop molecular biology discipline. Powdery mildew is one of the major diseases of barley, and finding genes responsible for resistance against the powdery mildew infection are of great significance to that end. In this thesis, the functional analysis of a hypothetically important gene in disease defense mechanisms of plants was studied.

The role of *HvGCN2* in barley defense mechanism against powdery mildew infection was studied using VIGS. The analyses were performed in two steps; first the level of silencing was analyzed using qRT-PCR, then the pathogen growth rate assessment was performed on treated samples.

Silencing level determination was performed using qRT-PCR. It was observed that 5 of the 6 samples were silenced at an average silencing level of 43.2 % with respect to naked virus inoculation.

As a result of pathogen growth rate assessment, even though there was no change if the plant was resistant against the pathogen, it was observed that the silencing of *HvGCN2* gene increases the susceptibility of the already susceptible plant, making it extremely vulnerable to the disease. This was shown by both hyphae growth measurements at different time points and also by visual inspection of the powdery mildew spores under microscope. As a result, it was found out that there is up to 25% difference in pathogen growth rate between control and silenced groups.

These two findings show that silencing of *HvGCN2* in barley increases susceptibility of the plant against powdery mildew infection in susceptible plant. Therefore, it can be proposed that *HvGCN2* is a positive regulator of plant defense against the powdery mildew pathogens. In the literature, homologues of *GCN2* are found to be taking several roles associated with stress conditions. One of these functions is that *GCN2* is invoked as a result of increased ROS concentrations. In addition, HSP90, which is crucial for resistance in several occasions, of budding yeast was found to be a regulator of *GCN2*. *Arabidopsis thaliana* homolog of *GCN2* was also found to be activated by wounding and exposure to key hormones involved in plant defense.

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APPENDIX A

BSMV GENOME SEQUENCES

1. **BSMV α genome (GenBank: U35767.1,** http://www.ncbi.nlm.nih.gov/nucleotide/1016775?report=genbank&log$=nucltop&blast_rank=1&RID=2RCHTS9H01N**):**

GTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCACATAATCGTAATCGATTC
TTCTTGATCTCTAAACAACACTTTCCCGTTAGCATGGCTAGCGATGAGATTGTCCGCA
ATCTGATCTCCCGTGAGGAGGTGATGGGTAATTTGATTAGCACAGCTTCTAGCTCAGT
AAGGTCACCCTTACATGACGTACTGTGCTCGCACGTAAGGACCATCGTCGATTCCGTG
GATAAGAAAGCGGTCAGTCGCAAGCATGTTGATGTACGGCGCAACATCTCCTCTGAAG
AGTTACAGATGTTGATAAATGCATATCCTGAATATGCCGTTTCATCCTCAGCTTGTGA
ATCTGGTACTCATAGCATGGCGGCTTGTTTTCGATTTCTGGAGACAGAATACCTCTTA
GATATGGTTCCAATGAAAGAGACTTTTGTTTATGACATTGGTGGTAACTGGTTTTCTC
ATATGAAGTTTCGTGCTGATAGAGAAATTCATTGTTGCTGTCCGATCTTATCTATGAG
AGATTCTGAAAGACTGGAAACACGCATGATGGCAATGCAAAAATATATGCGTGGATCG
AAAGACAAACCGTTACGCTTGTTAAGCCGTTATCAAAATATCCTGCGTGAACAAGCGG
CGAGAACAACTGCCTTTATGGCAGGTGAGGTGAATGCGGGTGTTCTCGATGGAGATGT
GTTTTGTGAGAACACTTTTCAAGACTGTGTGAGACAGGTGCCCGAAGGTTTTTTGAAG
ACAGCTATAGCAGTTCATAGCATCTACGATATCAAAGTGGAAGAATTTGCGTCTGCAT
TGAAAAGAAAAGGTATAACACAGGCTTATGGGTGCTTCCTGTTTCCTCCTGCTGTATT
GATAGGTCAGAAGGAAGGTATTTTACCTTCCGTGGACGGTCATTACTTGGTGGAGAAT
GGCAGGATTAAGTTCTTCTTTGCGAATGATCCGAATGCCGGTTACTCTCATGACCTTA
AGGATTATCTGAAGTATGTGGAAAAAACCTACGTGGATATAAAGGATGGAGTGTTTGC
TATTGAGCTGATGCAAATGCGAGGTGATACCATGTTCTTTAAGATCACGGATGTCACC
GCAGCAATGTATCATATGAAATACAGAGGTATGAAACGTGATGAAACATTCAAATGCA
TTCCGTTGCTAAAAAATTCATCCGTTGTCGTACCTCTATTTTCGTGGGACAACCGTTC
TTTAAAGATCACAAGTGGTTTATTACCACGAACTTTGGTCGAGCAAGGTGCGGCGTTT
ATTATGAAAAACAAGGAGAAGGACTTGAACGTTGCTGTGTTGAAGAACTATCTTTCCG
CTGTGAACAACTCATACATTTTCAACGGATCCCAGGTTAGAGATGGTGTGAAAATTGC
CCCGGATTTAATCTCCAAATTGGCAGTGACTCTGTACCTGAGAGAAAAGGTCTATCGA
CAAAGAGAAAATTCAATCATAAGTTATTTCGAGCAAGAAATGCTTCACGATCCCAACT
TGAAAGCCATGTTTGGAGACTTTCTGTGGTTTGTTCCAAATACTCTCTCGAGTGTCTG
GAAGAACATGCGAAAATCACTGATGGAATGGTTTGGCTACGCAGAATTTGACTTGACT
ACTTTTGATATTTGCGATCCCGTTCTCTACGTAGAGATAGTGGATCGGTATAAGATCA
TTCAAAAAGGGCGAATTCCACTTGGTGAGTTTTTTGATTGTCATGAAGAATGCGAGAA
TTACGAACTGCGTGAGAAGGAGAAAAATGACCTAGCGGTGAAAATGGCCCAGAAGGTA

ACAGGGACGGTGACCGAATGCGAGAAGGACCTGGGACCTCTTGTTCAACCGATAAAAC
AGATATTGGTTCAACTTGTGATGCCCAATTTGGTCAGAGCGCTGTGTAGACCTCGTAG
CCCAACGTCTCCTTTGGACTTAAATATCCCAGGGTCAACTCCATCACACTCAAGTTCA
GATTCTGAACAATCTATGACTGAAGAAGCGAGCTGCGCCATTGCGGGTAGCGTACCAA
CATGGGAAATTGCGACTAAGAAAGATCTAACCTTTCAGCGAATTGATGAAGATATGTC
TCGACGAACTGGTATGCCTCCAAGACCAAAAGTAACTTCTAGTTACAACATGAATGCC
AGAGCTGAGTTTCTCTACTATCAACTGTGTAGCGTGATTTGTGAAAGGGCTCAGATTT
TGAGTGTCATCGAAGACTTTCGTCAGAATTTGATATTCTCAGATAAAGTGGCCGTTCC
ATTGAACGCTAGATTCTACAGTTTTCAGTCATTGCAACCCGGATGGGTGTTCAAGACT
CCATCGCATAGTGAAGTAGGCCACAGTTATGCAGTACATTTTGACTTCAAGACAGTTG
GAACCGATTTGGAAGAGAGCCTAGCTTTTTGCCGAATGGTACCGATTTCATGGGATAA
AAGCGGCAAATACATCGCGACAACTCCTCATTTTCCCGAGAGACATGGTTACTACGTG
ATTTGTGACAACACTAAATTGTGTAACAATTGGCTTATTTACAATAAGTTAGTTGATG
TCTACGCACGAGTGGCTGATAGACCTCTGAGATTTGAGTTGATTGACGGAGTTCCTGG
CTGCGGAAAGTCAACCATGATTTTAAACAGCTGTGATATTCGACGCGAAGTTGTTGTT
GGTGAAGGAAGGAATGCAACTGATGACTTAAGGGAGAGGTTCAAGCGTAAGAAAAATT
TGAATAGTAAGACTGCTAATCATAGAGTTCGAACGCTTGACAGCTTATTACTTGCTGA
AGGACCTTGTGTACCGCAAGCTGATAGGTTTCATTTTGATGAAGCTCTAAAAGTTCAT
TACGGCGCCATAATGTTCTGTGCTGATAAGCTTGGTGCCTCAGAAATTCTCGCTCAGG
GAGATAGGGCTCAACTGCCGATGATCTGTCGTGTAGAAGGTATTGAACTTCAATTTCA
ATCTCCTGATTACACGAAGACGATCATAAATCCTAAGCTACGATCATACCGTATCCCT
GGAGATGTTGCCTTCTATTTGAGTGCTAAGGAATTTTACAAAGTTAAAGGAATACCTC
AAAAGGTTACAACTTCTAACAGTGTGAAACGTTCCCTGTACGCTAGAGGCGAAACAAC
TCCGGAAAGATTCGTGAGTTTGCTTGATGTTCCGGTGAGAAAAAACACCCATTATCTA
ACCTTCTTACAAGCTGAGAAGGAAAGTTTGATGAGTCATTTGATTCCAAAGGGTGTGA
AGAAAGAGTCTATTTCAACGATTCATGAGGCGCAGGGTGGTACCTATGAAAATGTGAT
TCTGGTCCGTTTGCAACGGACGCCCAATGAAATTTATCCGGGTGGACCTAGGTCCGCC
CCTTACATTGTGGTTGGGACTTCAAGGCATACAAAAACTTTCACTTATTGTAGTGTTA
CGGACGATAAGTTGCTTTTAGATATCGCCGACGTCGGTGGTATTGCACATACACCTAT
TCGTACTTTTGAATCTCATATAGTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAATGTTTGATCAGATCATTCAAATCTGATGGTGCCCATCAACCATATGA
TGGGAGTGTTTGCAAGTCCACTATAATCGAACTTGAAAACGATGCCTGAATTGGAAAC
CATGAATCTTAACGGATTCTGGAGAGAAAATTTAGGAATTGGTATGTAAGCTACAACT
TCCGGTAGCTGCGTCACACTTTAAGAGTGTGCATACTGAGCCGAAGCTCAGCTTCGGT
CCCCCAAGGGAAGACCA

2. BSMV β genome (Scofield, 2007):

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT
ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGA
AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAG

TCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC
TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC
TCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGT
GTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC
TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGC
CACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC
AGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATC
TGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA
AACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAG
AAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGG
AACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCT
AGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAAC
TTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTA
TTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGG
GCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCC
AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCA
ACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTT
CGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACG
CTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
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3. BSMV γ genome (Scofield, 2007):

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4. Sequence of γ.bPDS4As (Scofield, 2007):

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GCT

PLASMID SEQUENCES

1. pα plasmid (Scofield, 2007)

GTAAAAGAAAAGGAACAAGTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCA
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AAATATATGCGTGGATCGAAAGACAAACCGTTACGCTTGTTAAGCCGTTATCAAAATA
TCCTGCGTGAACAAGCGGCGAGAACAACTGCCTTTATGGCAGGTGAGGTGAATGCGGG
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CCCGAAGGTTTTTTGAAGACAGCTATAGCAGTTCATAGCATCTACGATATCAAAGTGG
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GTTTCCTCCTGCTGTATTGATAGGTCAGAAGGAAGGTATTTTACCTTCCGTGGACGGT
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CCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACG
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CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACC
CGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGC
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TATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT
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CATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC
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GTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGT
AAAACGACGGCCAGTGAATTAATACGACTCACTATA

2. pβ plasmid (Scofield, 2007)

GTAAAAGAAAAGGAACAACCCTGTTGTTGTTCGACGCTATACTAAATATATATTATCT
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TTGACGATTGGTGGGTCCACGTAGAAGCCTGGAATAAATTTCTAGACAATTTACGTGG
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GATCGTGATCTACCTGCTGATGTAGACAGACGGTTTGCAGGTGCTAGAGGACAAATTG
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CGTGTTGATTGACCTACAGAGTGCGAGACTCCCAGTGTATACCAGGAAGACTTTCGAA
CGTGAACTCGCTTTGGAATGGATCATTCCAGATGCCGAGGAAGCGTGACCTGCTGTTG
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GCTTTTACTGTCGATACAGTTCGAGATGTGAAAGATTGCGGGTACGATTGTGCCCTGG
CAATTGATGTGCAAGGGAAAGAATTCGATTCAGTGACTTTATTCCTAAGGAACGAAGA
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GATGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCG
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CAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAG
CTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCC
AGTCACGACGTTGTAAAACGACGGCCAGTGAATTAATACGACTCACTATA

3. pSL39B-1 plasmid (Scofield, 2007)

GTATAGCTTGAGCATTACCGTCGTGTAATTGCAACACTTGGCTTGCCAAATAACGCTA
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GTTTGGAACAGGCTTAATTGCCGTATCTTTGGTGAAACGGTTGCTACAGGAACAACCC
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GTAGTGGGAGTTGAACGTGGTCTTTACCGGGACATTTTTCAGGACAACGAAATCCCAT
CAGTCATGGAAGAGAAACTGCAGAAACTCCTTTACTCTGAGGGTGAGAAGATTCGAAG
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GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATC
CTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGAT
TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGA
AGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCT
TAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTG
ACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT
GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGC

CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC
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TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAA
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GCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCG
ACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACT
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CGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATC
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AAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATT
ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA
GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGAAATT
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TTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGAT
AGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCC
AACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCAC
CCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGG
GATGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCG
TAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATT
CAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAG
CTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCC
AGTCACGACGTTGTAAAACGACGGCCAGTGAATTAATACGACTCACTATA

APPENDIX B

SEQUENCES of CANDIDATE REFERENCE GENES

1. Actin (GenBank: AY145451.1, http://www.ncbi.nlm.nih.gov/nuccore/AY145451.1**)**

GAATTCCATTCTGTAGGAAATGGCTGACGGTGAGGACATCCAGCCCCTTGTCTGCGAC
AATGGAACCGGAATGGTCAAGGCTGGTTTCGCTGGAGATGATGCGCCAAGGGCTGTTT
TCCCTAGCATAGTTGGTCGCCCTCGGCACACTGGTGTCATGGTAGGGATGGGGCAGAA
GGATGCTTATGTTGGTGATGAGGCGCAGTCCAAGAGAGGTATCCTCACGCTCAAGTAC
CCCATCGAGCACGGTATCGTAAGCAACTGGGATGACATGGAGAAAATCTGGCATCACA
CTTTCTACAATGAGCTCCGTGTGGCACCCGAGGAGCACCCTGTGTTGCTCACTGAGGC
CCCTTTGAACCCAAAAGCCAACAGAGAGAAGATGACCCAGATTATGTTTGAGACTTTC
AATGTTCCTGCCATGTACGTCGCTATTCAGGCCGTGCTTTCCCTCTATGCAAGTGGTC
GTACTACTGGTATCGTTCTCGACTCTGGTGATGGTGTCAGCCACACTGTGCCCATTTA
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ACGGACTCCCTTATGAAGATCCTCACCGAGAGAGGTTACTCCTTCACAACCTCAGCTG
AGCGGGAAATTGTAAGGGACATCAAGGAGAAGCTTGCGTACGTTGCCCTTGATTATGA
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GATGGTCAGGTGATCACGATTGGCGCAGAGAGGTTCAGGTGCCCTGAGGTCCTCTTCC
AGCCATCCATGATCGGCATGGAGTCTTCTGGAATCCACGAGACGACCTACAACTCCAT
CATGAAGTGTGACGTGGATATCAGGAAGGACTTGTATGGAAACATCGTGCTCAGTGGT
GGCACAACTATGTTCCCAGGTATCGCTGACCGTATGAGCAAGGAGATCACCGCCCTTG
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AATGTATTGCGTATTTGCAAGCTATGTTTTTTTCCAGACGTGACGTGGGTACTCTTGG
GATACGCCACCTATATACGTGGCGGCTCCATGGTGCAAGTGCAAGTACACTATCTATG
TTTGTGCATTGTCAGTGTGTTTGTGGGATCAGTTGTCAAACTTGGGTTGGCTTGATTT
GTTGTTGGGAATTGTCTGTAAAGGAATTC

2. GAPDH (GenBank: M36650.1, http://www.ncbi.nlm.nih.gov/nuccore/ M36650.1**)**

GTCAACGACCCGTTCATCACCACCGACTACATGACCTACATGTTCAAGTATGACACTG
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CTGCAGCTCACATTAAGGGTGGTGCCAAGAAGGTCATCATTTCTGCTCCCAGCAAGGA
CGCTCCCATGTTTGTCTGTGGTGTCAACGAGAAGGAATACAAGTCAGACATCGACATT
GTCTCCAATGCTAGCTGCACCACCAACTGTCCTGCTCCTCTTGCTAAGGTTATCAATG
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GAAGACTGTTGATGGTCCTTCAAGCAAGGACTGGAGAGGTGGAAGGGCTGCTAGCTTC
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GCATGAAGATACAGGGAGTGTGGTTTGCCCCAGAGAAGAGAAGAGTGTACAACCTCTT
CCGAGAATAAATTTTTGTATGGAATTATGGCAACTAAAAAAAACCTTTTATTGGATGA
TCCTGATGGTTGGTTGAGCTTAGCGGCTCACATTTTGGTGGTATTATGTACTTGCTTG
AACTAAATCATGAGTTATTTTCACTCATCGTCATGGGTT

3. Elongation Factor (GenBank: Z50789.1, http://www.ncbi.nlm.nih.gov/ nuccore/ Z50789.1**)**

CGGCCTCGCTTTGCGACCCCTCCGTTCGTTCTTCTTCTTCGAGTTTGTTATCAGCCAT
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GCTTGACAAGCTCAAGGCTGAGCGTGAGAGAGGTATCACCATCGATATTGCCCTCTGG
AAGTTCGAGACCACCAAGTACTACTGCACCGTCATTGATGCCCCTGGTCACCGTGACT
TCATCAAGAACATGATCACGGGTACCTCCCAGGCTGACTGTGCTGTTCTCATCATTGA
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GCTCTCCTTGCTTTCACTCTTGGAGTGAAGCAGATGATCTGCTGCTGCAACAAGATGG
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CAACCCTGCTTGAGGCGCTTGACCAGATCAACGAGCCCAAGAGGCCCTCAGACAAGCC

CCTCCGTCTTCCCCTCCAGGACGTTTACAAGATTGGTGGCATTGGAACTGTGCCTGTT
GGCCGTGTTGAGACTGGTGTCATCAAGCCTGGTATGGTTGTGACCTTTGGTCCCACTG
GTCTGACAACTGAGGTCAAGTCCGTTGAGATGCACCATGAGTCTCTCCTGGAGGCGCT
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TTGAGTACTGTCGTGTTATTTCGGCTTTCGGGAAAACATGGATGCTTCTGAAGACTAA
TCATTTTATCATCTGTCTGCTTTCGAAAAAAAAAAAAAAAAA

4. Ubiquitin (GenBank: M60175.1, http://www.ncbi.nlm.nih.gov/nuccore/M60175.1**)**

GAATTCATGAGCATGTATACATCTCACTAACCTGTTAACTTCGATGCTACCTCGGAAA
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TAACCAATTTTCGAATCTGTAGTCTTCCTAGTTTATGTATATCTTATTAATTGCGAGG
CCAATTCTTTTGCTGGACAAAATGTAGAACTTACACATTTACTCTTATATAAAAATAA
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CCCAAAATAGCGTCCACGAAAGCCAGCCGTCCGATCTGGAACCCCTAGATCCAACAGC
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GGCCAAGATCCAGGACAAGGAGGGCATCCCGCCGGACCAGCAGCGCCTCATCTTCGCC
GGCAAGCAGCTCGAGGACGGCCGCACCCTCGCCGACTACAACATCCAGAAGGAGTCCA
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CACCAAGCCCAAGAAGCAAAAGCACAAGCACAAGAAGGTGAAGCTCGCCGTCCTCCAG
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CCATCATGGGATATATCTTGTTGATACAAGTGTTCTTGAGCTTAACCCTTTATTGCAG
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TGGTTTATGATATGTTGATTGTTGATGTGTGTGATCTTTGTCACTTGCAATTATAATT
CAGTTCATCCCAAGTGATTGCGTGGTTGGTTTGTTTTAGATACATGTGTTATTTCATC
CCTAGATGTGTCTGAAACTGTGGCTGTAGACAGTTGCAGTGTGATGATTGCTAGTGTG
GAGCATATTGGTGCCCTGTGGTTTATCCACCATTCACCATTGCCTACCCTGTGGTGCT
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TGTAGTTGAGTTTTGCTGTTCTCTTTTCATTTTGTTCTTGGGTTGTGATAAAGTCATT
TCACATACGCCGATGATATGTAATTTACAACTAATACATAAGATGGCTCAAATGCATT
GTATGTGAATGCAAACTTGTCACAAATACTACGAGAATTATCTAAACTGCAACATCAC
TTCATGCATTTGAATTC

5. α-tubulin (GenBank: Y08490.1, http://www.ncbi.nlm.nih.gov/nuccore/ Y08490.1**)**

GGAAAGGCGTCTTCGTACTCGCCTCTCTCCGCGCACACGAGCTCTCGCCCCCTTCCTC
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GTGATGATGCTTTCAACACCTTCTTCAGCGAGACTGGTGCTGGGAAGCACGTCCCCCG
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