APPLICATION OF VIRUS INDUCED GENE SILENCING OF *BRACHYPODIUM DISTACHYON*, A MODEL ORGANISM FOR CROPS

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ABSTRACT

APPLICATION OF VIRUS INDUCED GENE SILENCING OF BRACHYPODIUM DISTACHYON, A MODEL ORGANISM FOR CROPS

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Grass family is most important family in plant kingdom due to intensive usage of crops in agriculture. To date, molecular biology researches on grass family have had limitations because of inappropriate characteristics of barley and wheat to conduct experiments on them. *Brachypodium distachyon* that belongs to grass family has recently emerged as a model organism for crops. It shares common characteristics for a model plant due to its small genome, small physical plant size, a short lifecycle, and less demanding growth requirements; as other model organisms; *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays* (Draper et al. 2001). Especially after appreciating, the genetic distance of *O. sativa* to grasses (Garvin et al. 2008), it become a key organism to understand complicated genomic organization of agriculturally valuable grasses. Virus-induced gene silencing (VIGS) is one of the revolutionary methods allowing a rapid and effective loss of a gene function through RNA interference (Holzberg et al. 2002; Liu et al. 2008). Barley stripe mosaic virus (BSMV) is still the most effective vector used in monocot gene silencing. It has a tripartite RNA genome having a wide range of infection ability for monocots including barley, oats, wheat, and maize as host (Holzberg et al. 2002; Scofield 2005). In this thesis, *Phytoene desaturase* (PDS) gene of *Brachypodium distachyon*

was silenced *via* BSMV mediated VIGS. Additionally, with *Green fluorescence protein* (GFP) bearing BSMV transcripts, GFP expression was observed under fluorescent microscope. To our best knowledge, this is the first report demonstrating a VIGS *via* BSMV in *Brachypodium distachyon*. The success of virus induced gene silencing method in *Brachypodium distachyon*, will be a new convenient tool for evaluating functions of crop genes in this model organism.

Keywords: *Brachypodium distachyon*, Gene silencing, GFP expression, *Phytoene desaturase* (*PDS*) gene, qRT-PCR, Reverse genetics, VIGS

ÖZ

TAHILLAR İÇİN YENİ MODEL ORGANİZMA OLAN,
BRACHYPODIUM DISTACHYON'DA VİRÜS İNDÜKLEMESİ
YOLUYLA GEN SUSTURULMASI UYGULAMASI

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Tahıl ailesi tarımdaki kullanım alanlarının yoğunluğu sebebiyle bitki alemindeki en önemli ailedir. Bu zamana kadar, arpa ve buğdayın uygun olmayan özellikleri, tahıl ailesi üzerinde yapılmak istenen moleküler biyoloji çalışmalarını hep sınırlamış ve tahıl ailesine üye olan *Brachypodium distachyon* bu aile için model organizma olarak önem kazanmaya başlamıştır. Bu organizma, *Arabidopsis thaliana*, *Oryza sativa*, ve *Zea mays* gibi model organizma olarak kullanılan bitkilerle şu benzer özellikleri taşır: küçük genom, küçük fiziksel boyut, kısa yaşam döngüsü, çok emek istemeyen büyüme koşulu. Özellikle çeltik ve tahıl ailesinin diğer üyeleri arasındaki genetik mesafenin büyüklüğü anlaşıldıktan sonra, kompleks genoma sahip tahıl ailesinin incelenmesinde bu organizma anahtar bir bitki olmuştur. Virüs indüklenmesi yoluyla gen susturulması metodu RNA müdahalesini kullanan, hızlı ve etkili bir gen fonksiyonu kaybı yöntemidir (Holzberg et al. 2002, Liu et al. 2008). Barley stripe mosaic virus (BSMV) tek çenekli bitkilerin genlerini susturmak için kullanılan en etkili vektördür. Bu virus üçlü RNA genomuna sahip olup arpa, buğday, mısır ve yulafı enfekte edebilmektedir (Holzberg et al. 2002, Scofield 2005). Bu tezde BSMV aracılığıyla VIGS metodu kullanılıp *Brachypodium*

distachyon bitkisinin *Phytoene desaturase* (PDS) geni susturulmuştur. Buna ek olarak *Green flourescence protein* (GFP) geni taşıyan BSMV enfeksiyonu sonrası, GFP ifadesi floresan mikroskopu altında incelenmiştir. Bildiğimiz kadarı ile, bu çalışma *Brachypodium distachyon*'da BSMV aracılığı ile VIGS metodu kullanılıp gen susuturulabildiğini gösteren ilk rapordur. VIGS'in *Brachypodium distachyon* bitkisinde başarısı tahıl ailesi üyelerinin genlerinin fonksiyon çalışmalarında yeni ve uygun bir yöntem olarak kullanılmasını sağlayacaktır.

Anahtar kelimeler: *Brachypodium distachyon*, Gen susturulması, GFP ifadesi, *Phytoene desaturase* (*PDS*) geni, qRT-PCR, Ters genetik, VIGS

to my dearest and unique family

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LIST OF ABBREVIATIONS

µg	: Microgram
µL	: Microliter
µM	: Micromolar
[a^{32}P]-dATP	: [a32P]-deoxyadenosinetriphosphate
ADP	: Adenosine Di phosphate
APS	: Ammoniumpersulfate
As	: Anti sense
Ath	: *Arabidopsis thaliana*
Avr	: Avirulence
Bgh	: Blumeria graminis hordei
BSA	: Bovine Serum Albumin
BSMV	: Barley Stripe Mosaic Virus
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotidetriphosphate
dpi	: Day post inoculation
ds	: Double stranded
DTT	: Dithiothretiol solution
EDTA	: Ethylenediaminetetraacetic acid
GFP	: Green Flourescent Protein
h	: Hour
Hai	: Hour after inoculation

HR	: Hypersensitive response
kb	: Kilobase
LiCl	: Lithium Chloride
M	: Molar
mCi	: Milicurie
mg	: Miligram
min.	: Minute
mL	: Mililiter
mM	: Milimolar
NB	: Nucleotide binding
NC	: Not Change
ND	: Not Determined
NEB	: New England Biolabs
ng	: Nanogram
nm	: Nanometer
NTC	: No Template Control
Os	: *Oryza sativa*
p	: Plasmid
PCR	: Polymerase chain reaction
PDS	: Phytoene desaturase
pmol	: Picomole
Pst	: *Puccinia striiformis tritici*
PTGS	: Post Transcriptional Gene Silencing

PVX	: Potato Virus X
qRT-PCR	: quantitative Reverse transcriptase PCR
R	: Resistance
RISC	: RNA Induced Silencing Complex
RNA	: Ribonucleic acid
RNAi	: RNA interference
rpm	: Rotation per minute
RT-PCR	: Reverse transcriptase polymerase chain reaction
SiRNA	: Small interfering RNA
Ss	: Single stranded
Ta	: *Triticum aestivum*
Taq	: *Thermus aquaticus*
U	: Unit
UV	: Ultraviolet
v/v	: Volume per volume
VIGS	: Virus Induced Gene Silencing

CHAPTER 1

INTRODUCTION

1.1 *Brachypodium distachyon*

Brachypodium distachyon (*B. distachyon*) is a member of the grasses that constitute a family which is one of the economically most important families among plant kingdom (Huo et al. 2006). Broadly speaking, grasses lie at the center of agriculture on the world we know. We used seeds of this family members to make human foods such as flour, starch, sugar, syrup, oils, and malt for the production of alcoholic beverages, and in addition to this, seeds are used indirectly as animal feed (Opanowicz et al. 2008). Moreover, residues of crops (ie. their processed wastes) have been used in energy production (van Dam et al. 2007) and recently specific interest on members of grass family has expanded for biofuel productions (Fike et al. 2006). Because of this agronomical importance; grass family is under exceptional interest for many groups among the world. Since pathogen invasion to grass family is one of the most important causes of economic losses, it is vital to understand molecular mechanism of plant resistance against pathogens. To understand nature of plant-pathogen interactions and to acquire new arms against pathogen invasion is especially indispensible for barley and wheat, which are very fragile to fungal invasions. Unfortunately, these plants are physically large, and they have relatively long life cycles and huge, complex genomes, features that are not necessarily the most suitable for many research applications (Opanowicz et al. 2008). For this purpose scientists have used *O. sativa* (rice) as a model organism for crops. However, after appreciating distance of rice genome to the barley and wheat; *B. distachyon* has emerged as promising plant model organism for crops (Devos et al. 1999; Tikhonov et al. 1999; Keller and Feuillet 2000; Vogel et al. 2006) especially the EST studies of *B. distachyon*

support the close evolutionary relation with barley and wheat (Vogel et al. 2006). It has many of the characteristics required for an experimental model organism; small genome, short life cycle, small plant size, easy to cultivate and genetically manipulate (Draper et al. 2001) as shown in Table 1 (Opanowicz et al. 2008). Since it has one of the smallest genomes of any grass, a genome sequencing project is on the way for *B. distachyon.* After finishing this project researchers will have incomparable data about genome organization of barley and wheat due to synteny between *B. distachyon* and these plants. (For detailed information about recent improvements in *B. distachyon* sequencing project: "International Brachypodium Initiative", http://www.brachypodium.org/node/8).

Table 1. Comparison of *B. distachyon* with other plants (Opanowicz et al. 2008)

	Brachypodium distachyon	*Arabidopsis thaliana*	*Triticum aestivum*	*Zea mays*	*Oryza sativa*	*Hordeum vulgare*
Number of chromosomes	10 (2n)	10 (2n)	42 (2n)	20 (2n)	24 (2n)	14 (2n)
Genome size (1C)	300 Mb	164 Mb	16 700 Mb	2400 Mb	441 Mb	5000 Mb
Reproductive strategy	Self-fertilizing	Self-fertilizing	Self-fertilizing	Cross-pollination	Self-fertilizing	Self-fertilizing
Life cycle (weeks)	10–18	10–11	12 (spring wheat) 40+ (winter wheat)	10+	20–30	16+
Height at maturity (m)	0.3	0.2	Up to 1	Up to 2	1.2	Up to 1.2
Transformation	Facile	Facile	Possible	Facile	Facile	Facile
Growth requirements	Very simple	Very simple	Simple	Simple	Specialized	Simple

B. distachyon have different ecotypes (with the prefix designation "ABR"; e.g., ABR1, ABR2) with diploid, tetraploid, and hexaploid genome organisations. In diploid ecotypes *B. distachyon* has 10 chromosomes with a genome size of ~300 Mb one 56^{th} of the wheat genome (Bennett et al. 2005, Opanowicz et al. 2008) and majority of the diploid accessions with a haploid genome of five chromosomes grow in northern Spain, France, Italy, Slovenia, Iraq, and Turkey (Garvin et al. 2008). Research on *B .distachyon* such as; mutagenesis (Engvild 2005), cytogenetic studies (Hasterok et al. 2004), BAC library constructions (Hasterok et al. 2006, Huo et al. 2006),

studies about pathogen-host interactions (Draper *et al.* 2001; Routledge *et al.* 2004) have expanded the knowledge about *B. distachyon*. According to these studies to date, *B. distachyon* genus is found to be more closely related to wheat, barley than rice and it can be phylogenetically located between temperate cereals and tropical cereals (Fig. 1.1).

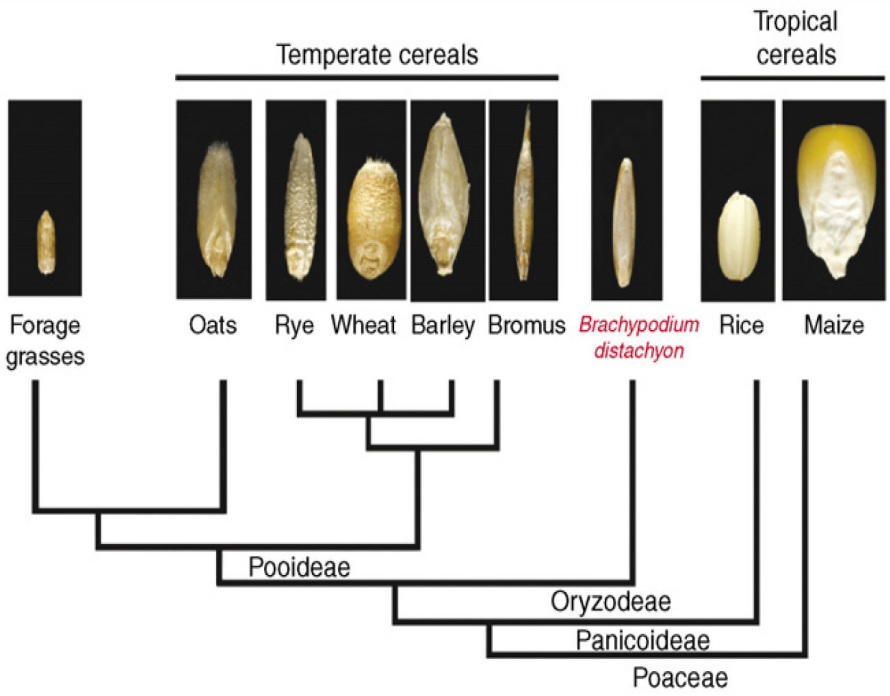


Figure 1.1. Phylogenetic relationship between *B. distachyon* and the other cereals. The Pooideae subfamily includes forage grasses, *B. distachyon* and many economically important species such as wheat (Triticum aestivum), rye (Secale cereale), barley (Hordeum vulgare) and oats (Avena sativa). Rice (Oryza sativa) and maize (Zea mays) belong to distinct subfamilies. (Opanowicz et al. 2008)

Therefore, after recognizing the significance of *B. distachyon* genome organization to understand genome of grasses and functions of genes that

have roles in resistance; one of the important steps for the sake of functional genomics had been developing efficient transformation systems that facilitate gain-of-function and/or loss-of function studies. For this purpose, researchers have developed both bombardment (Draper et al. 2001; Christiansen et al. 2005) and Agrobacterium mediated transformation systems (Vogel et al. 2006; Pacurar et al. 2007). In this study too, due to appreciation of significance of *B. distachyon*'s close genetic relatedness to wheat and barley, we initiated a first gene silencing demonstration on *B. distachyon* using VIGS (Virus induced gene silencing). We believed that importance of using *B. distachyon* as a model organism in this thesis will make a significant contribution to understand roles of genes of grass family using one of reverse genetic tool which is virus induced gene silencing (VIGS) used for the first time in this species.

1.2 Reverse genetic studies in functional genomics

Approaches in identification of biochemical and biological functions of genes can be classified in two genetic analysis methods. In forward genetics, the function of genes is investigated by studying organisms where gene function is altered. As an example of classical forward genetic screening, individuals are treated with mutagens to induce DNA lesions and mutants with a phenotype of interest are sought. After a mutant is found, the gene mutated is identified through standard molecular techniques. In this approach researchers use foreknowledge of mutant phenotype to identify responsible gene for this particular phenotype. However, in reverse genetics, the functional study of a gene starts with the gene sequence rather than a mutant phenotype. In this approach it is aimed to target specific part of DNA or alter function of a specific gene and then observe the phenotype to come up possible roles of genes. For this purpose there are many techniques applied successfully to knock out or knock down the gene that

causes permanent or transient interruption of the specific gene as shown in Fig. 1.2.

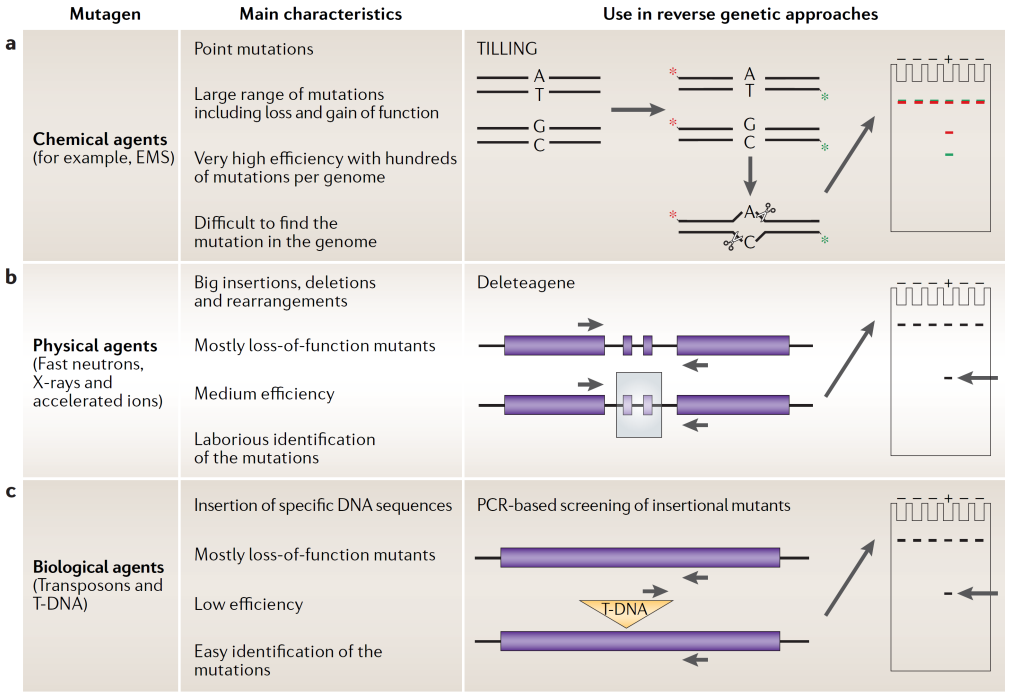


Figure 1.2. Reverse genetic approaches (a) Gene silencing by using chemical agents such as ethyl methanesulphonate (EMS). In this method point mutations are created among the genome and these mutations can be identified using targeting induced local lesions in genomes (TILLING) method. **(b)** Interfering with gene action using physical agents such as X-rays or accelerated ions. In this method deletion of part of gene of interest is verified by PCR using primers that flank the gene of interest after causing damage by physical agents (**c**) Disruption of gene by biological agents such as transferred DNA (T-DNA) or transposons. This method is based on insertional inactivation of gene of interest. (Alonso et al. 2006)

In addition to insertional inactivation of genes there are other methods based on post transcriptional gene silencing (PTGS). Using PTGS as a tool gives chance for stable or transient silencing of targeted gene. In next part of introduction firstly I will discuss transgenic methods to explore gene function and then mechanism of PTGS.

1.2.1 *TILLING*

TILLING has been developed to identify mutations in EMS-mutagenized populations (McCallum et al. 2000; Comai et al. 2006). In this method, EMS can be used to create point mutations in the genome. Detection of mutations is based on the amplification of the gene of interest from the pooled DNA. Fluorescently labeled primers are used and after PCR amplification, the DNA is firstly denatured, then renatured with DNA fragments in the DNA pool and then digested with the mismatch-specific celery nuclease CELI (Oleykowski et al. 1998), which has an exceptional characteristic to recognize base-pair mismatches. DNA fragments are separated on denaturing acrylamide gel and existence of a mutation in a particular DNA pool can be identified by the presence of two DNA fragments on the gel (Stemple 2005; Alonso et al. 2006). If we notice the presence of two different size bands that are labelled with different fluorescent dyes in a given pool; this means that there is a mutation in the gene of interest. These different bands are due to CELI nuclease activity at the site of the mismatch between the wild-type and mutant heteroduplexed DNA fragments as shown schematically in Fig. 1.2(a). In this method we can also have information about the position of mutation in the gene considering specific sizes of the bands on the gel. For further conformation of mutations sequencing can be applied.

The main advantage of this method is opportunity of creating large range of mutations in both loss and gain of functions (Alonso et al. 2006), which

enables a wide range of genetic alterations to be produced: not only loss-of-function alleles, but also hypomorphic (partial loss of gene function), hypermorphic (partial gain of gene function) and neomorphic (suddenly developed function) mutations (Stemple 2005) with a very high efficiency. However, this method demands labor and time to create the mutations and screen the samples. Moreover, using this method may account for different mutations in the same genome in which we can not be sure about contribution of unexpected mutation on phenotype as well as causing lethal mutations.

1.2.2 *Delete a gene*

To asses function of a specific gene, physical agents such as fast neutrons, X-rays and accelerated ions are used to delete a gene from a genome or to cause damage in a gene. Both of these interferences prevent expression of gene that provides observation of phenotypic changes to come up a conclusion about the function of the gene. In this method after exposing the physical agent, detection of damaged DNA from the pools of mutants is PCR-amplified using primers that flank the gene of interest (Li et al. 2002; Zhang et al. 2003a). The amplification products are then analyzed by gel electrophoresis. Recognition of a smaller band in the pool comparing with the wild type gene length is sign of deletion in the gene of interest. For further step secondary screening of individual plants is usually necessary to confirm the presence of the mutation and identification of individual mutants.

High-energy ionizing radiation is also developed for DNA deletions in plants (Li et al. 2002). Although this method is not very popular for *A. thaliana*, because of the other available efficient reverse genetic methods to manipulate *A. thaliana* (Zhang et al. 2003b), Deleteagene application has several attractive advantages. First of all, it can be used for plants that don't

allow efficient transformation to create mutations in the genomic background. Moreover, by this method researchers have chance to provide simultaneously mutate (delete) tandem duplicated genes (Li et al. 2002; Zhang et al. 2003a). Lastly, but equal importantly efficiency of creating mutations in the genome is slightly higher with this method (Alonso et al. 2006). However, this method has some limitations such as need for large labor and time to screen the population to identify mutant individuals. Necessity for sequence knowledge of the gene contributes restriction of usage of this method for the organisms with limited sequence information. Additionally, large deletions may result in disruption of other genes which are not targeted.

1.2.3 Insertional mutagenesis

Biological agents to create mutants for reverse genetic studies, is one of the most effective tool. By this method, loss of function or gain of function studies can be applied easily and very efficiently. Transferred DNA (T-DNA) is used as cargo to carry an exogenous gene into the genome of plant as a part of gain of function studies (Clough et al. 1998) T-DNA is also used for insertional inactivation to disrupt a gene to explore function of that gene (Alonso et al. 2006). Transposons are other effective tools to manipulate genome of organisms (Sundaresan et al. 1995). Transposons use homologous regions called as long terminal repeats (LTRs) to integrate themselves into the genome of organism with the help of factors of host organism (Sundaresan et al. 1995; Feschotte et al. 2002). After integration to genome, it is easy and straightforward to detect presence of T-DNA or transposon using gene specific primers and T-DNA/transposon specific primers. The presence of amplification product by PCR is monitored on the gel that would inform us about precise insertion of biological agent to the genome. For further verification; hybridization after southern blot with gene specific probes can overcome non-specific insertions (Alonso et al.

2006). For plant biology studies *Agrobacterium* mediated T-DNA insertion is by far the most widely used reverse genetic approach in *A. thaliana* (Tzfira et al. 2004). Using this method, we now have mutant lines for *A. thaliana* since the first successful screens were carried out nearly 15 years ago (McKinney et al. 1995).

This method also has its own benefits and drawbacks. Firstly, it is not possible to infect all plants with *Agrobacterium,* therefore host range is a restriction to introduce T-DNA and/or transposon into the genome. This is a big drawback to conduct gene function analysis directly on the crops or a specific crop variety. Secondly, identification of mutants needs construction and assaying of large number of plant pools (Krysan et al. 2002).

1.3 Post transcriptional gene silencing (PTGS)

Post transcriptional gene silencing (PTGS) is an RNA-based silencing used to suppress the expression of targeted genes in plants. It is also described as quelling in fungi (Cogoni et al. 1996; Cogoni et al. 2000) and RNA interference in animals (Fire et al. 1998). Indeed, general mechanism of RNAi is pretty simple and explored well. When dsRNA is introduced into the cells or expressed in the cells dsRNA is recognized by a cytoplasmic RNase type III enzyme, namely Dicer. Dicer converts long dsRNA precursor into small RNA duplexes (~21-25 bp in length) leaving 2 nts overhang at the 3' end with assistance of dsRNA binding protein. RNA loading complex (RLC) formed by Dicer, its dsRNA binding protein partner and dsRNA, loads small duplex RNA into RNA induced silencing complex (RISC). According to 5' stability of short RNA duplex; the strand which has less stable 5', is selected and called as guide short interfering RNA (siRNA). Other strand, called as passenger siRNA, is degraded immediately. Loading of guide siRNA onto Argonaut protein converts pre-

RISC into active RISC. RISC searches transcriptome very efficiently and guides siRNA for alignment to the target transcript. Once the target recognition is achieved, the complex introduces either a cleavage or translational repression due to perfect or non-perfect match between siRNA and its target respectively, and down regulation of expression is accomplished (Further information can be obtained from: Tuschi et al. 1999; Zamore et al. 2000; Caplen et al. 2001; Rana 2007). The siRNA acts as a template that anneals to the target transcript (e.g., a viral gene being replicated, an endogenous mRNA), and when a match is found, it is targeted for destruction. For this sensitive mechanism only few molecules of dsRNA can be used as a substrate for the cells in order to prevent matching RNA molecules from producing proteins efficiently. Although, source and process of micro RNAs (miRNAs) show slight differences, main mechanism is conserved for them as shown in Fig. 1.3. All in all, this mechanism is very effective and fast way to regulate endogenous gene expression, to prevent viral invasion, to protect genome stability by preventing action of transposons. In plants and *C. elegans* an additional protein, RNA dependent RNA polymerase (RDRP) takes role in production of secondary siRNAs. This specific polymerase can amplify targeted single strand RNA (ssRNA) to produce dsRNA using siRNA as primer. By conversion of ssRNA into dsRNA forms a loop in which complementary sequence to siRNA becomes both source and target of siRNAs and this cause systemic silencing of targeted RNA in a very real sense. This excellent silencing mechanism is commonly used in defense against viral invasion by plants. So it can be regarded as plant defense system against viral infection.

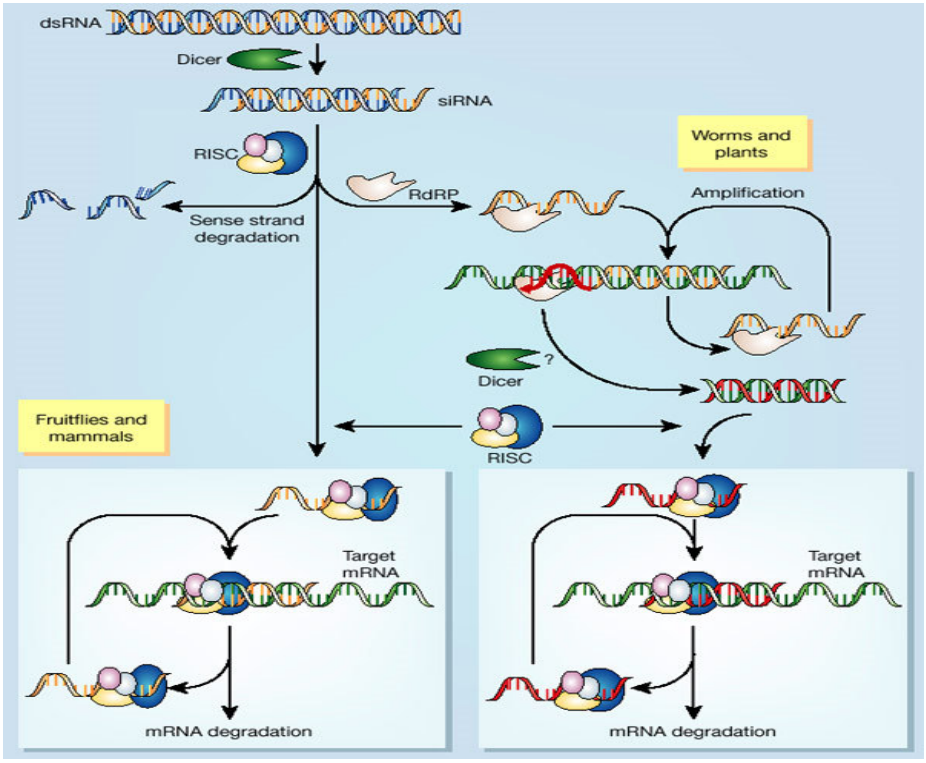


Figure 1.3. Short interfering RNAs— Post-Transcriptional Gene Silencing (PTGS). dsRNAs introduced into or expressed in cells are recognized by Dicer which converts long dsRNA precursor into small RNA duplexes (guide siRNA/ passenger siRNA). Guide siRNA is selected and loaded onto Argonaut protein. This activates RISC and target mRNA is either sliced (commonly in plants) or translationally repressed (commonly in mammals and fruit flies). In worms and plants there is also a distinct pathway to create secondary siRNAs by the action of RDRP (RNA-depended RNA polymerase) whixh uses siRNA as primer and target sequence as template to generate and amplify siRNAs. Novina et al. (2004)

1.4 VIGS as a post transcriptional gene silencing mechanism in plants

In molecular genetics studies, plant defense mechanism against viruses is exploited in order to perform functional analysis of genes. Virus induced gene silencing (VIGS) is one of the transient, fast and effective developed methods based on PTGS (Baulcombe 1999; Dinesh-Kumar et al. 2003). Using this method a short sequence of the plant gene was cloned to be silenced into virus genome to use it as a viral delivery vector. After transformation of virus into plants, spreading of virus provides sequence specific gene knock down to determine the targeted gene function as a reverse genetic approach. General mechanism for RNA interference is also acting in VIGS. Viral RNAs are converted into dsRNA by plant RNA dependent RNA polymerases. Long dsRNA form of virus genome is recognized and cleaved by post transcriptional gene silencing machinery and this also brings about degradation of targeted mRNA of plant as shown in Fig. 1.4.

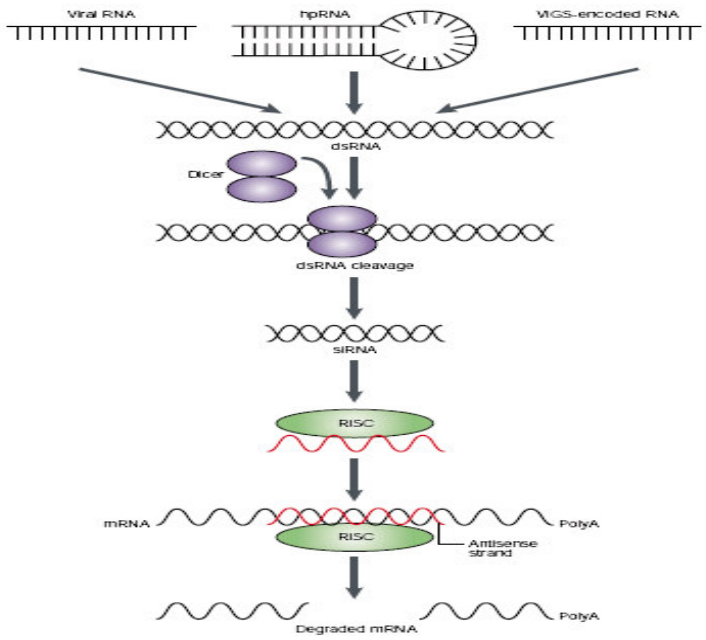


Figure 1.4 The RNA-mediated gene silencing model. Double stranded RNA (ds RNA) is generated from replicating viral RNA. In our case VIGS vectors (viral-vector-derived BSMV vectors) are used to produce viral ds RNA by *in vitro* transcription (Waterhouse and Helliwell, 2003).

In order to use viruses in plant gene silencing they have to be modified to prevent the lethal effect of viruses on plants. For that purpose some viruses that have broad range of infection ability were previously modified and used. One of these modified viruses for effective gene silencing researches is tobacco mosaic virus (TMV). In first particular application it was used to silence the *Pds* gene in *Nicotiana benthamiana* plants (Kumagai et al. 1995). Another modified virus that is more widely used is tobacco rattle virus (TRV) as a silencing tool in *N. benthamiana* (Liu et al. 2002a), and in tomato by Liu and his colleagues (Liu et al. 2002b). The major advantages of TRV-based VIGS in solanaceous species are the ease of introduction of the VIGS vector into plants. This easiness is usually provided by *Agrobacterium tumefaciens* with the VIGS vector placed between RB and

LB sites of T-DNA (Ratcliff et al. 2001; Liu et al. 2002b). Potato virus X (PVX) is also modified and used in virus mediated gene silencing in *N. benthamiana* plant (Lu et al. 2003). Commonly used viruses in virus mediated gene silencing studies are listed in Table 2. The first successful application of VIGS for monocot plants was performed by Holzberg et al, (2002) which meant engineering of a virus system for efficient silencing of monocot plants. They have silenced the *Pds* gene in barley by using barley stripe mosaic virus (BSMV) (Holzberg et al. 2002).

Table 2. Commonly used viruses in VIGS (Robertson 2004).

	Group	Silencing host	Transmission	Pathogen host
RNA viruses				
TMV Tobacco mosaic virus	Tobamovirus	*N. tabacum*, *N. benthamiana*	Mechanical	Tomato, squash, potato, tobacco (mild)
PVX Potato virus X	Potexvirus	*N. benthamiana*, *Arabidopsis*	Mechanical, plant to plant contact	Potato, oilseed rape (mild)
TRV Tobacco rattle virus	Tobravirus	*N. benthamiana*, Tomato, *Arabidopsis*	Nematode, mechanical, seed transmitted (up to 40%)	Spinach, beet, potato, tobacco
BSMV Barley stripe mosaic virus	Hordeivirus	Barley	Mechanical, seed transmitted (90–100%)	Barley, wheat, oat, maize, spinach
Satellite viruses				
STMV Satellite tobacco mosaic virus	RNA satellite virus	*N. tabacum*	Mechanical, with TMV as helper virus	*N. glauca*, pepper; Attenuates symptoms in tobacco
DNA viruses				
TGMV Tomato golden mosaic virus	Begomovirus	*N. benthamiana*	Whiteflies	Tomato[b]
CaLCuV Cabbage leaf curl virus	Begomovirus	*Arabidopsis*	Whiteflies	Cabbage, broccoli, cauliflower

1.5 BSMV & PDS

1.5.1 BSMV

After Holzberg and his colleagues, Scofield et al. used BSMV system to silence the wheat genes (Scofield et al, 2005). Since we also used this virus in our studies, this part of thesis is devoted to genetic characteristics of BSMV. It is a hordeovirus and has wide range of infection ability. BSMV is a positive sense single strand RNA virus and contains tripartite genome named as α, β, γ transcripts. The RNAs that are required for replication are expressed from α and γ genome of virus and RNA β is essential for systemic infection and cell-to-cell movement. The replicase proteins αa (methyl transferase/helicase subunit of the replicase), and γa (polymerase subunit of the replicase) are translated directly from their respective genomic RNAs as well as βa (coat protein) (Petty et al. 1990; Jackson et al. 2009). In addition to this genomic RNA organization there are also subgenomic (sg) RNAs; one for γ and two for β genomes which are due to overlapping sequences as shown in Fig. 1.5. Subgenomic RNAγ, designated γb, encodes a small cysteine-rich protein, translated from sgRNAγ and is dispensable for BSMV replication (Petty et al. 1990; Johnson et al. 2003). Since this part can be removed without any effect to replication, fragments designed for silencing are inserted into this part of γ genome.

The coat protein, βa, is translated directly from RNAβ, and deletion of βa does not have any effect on systemic silencing, on the other hand it prevents necrosis symptoms of plant (Joshi et al. 1990). Therefore, as a proper modification of BSMV in order to use in silencing studies, βa (coat protein) is deleted from genomic background of virus. Following βa open reading frame (ORF), an intergenic region, the overlapping viral movement genes are arranged in a "triple gene block" (TGB) (Petty et al. 1990; Jackson et al. 2009). TGB is encoded by many plant virus genera which is a specialized evolutionarily conserved gene arrangement whose products have roles in the virus movement for both cell-to-cell and longer-distances (Morozov 2003). After expression of these RNAs in a cell, proteins serve as membrane polypeptides and they provide entry of virus genome to plasmodesmata and to surrounding cells. Therefore, TGB based transport system is very crucial for intracellular and intercellular movement of virus. In BSMV, as it is shown schematically in Fig. 1.5, three major TGB proteins; TGB1 (βb), TGB2 (βd), and TGB3 (βc) and one minor protein, TGB2' (βd') are translated from subgenomic RNAs of β genome (Zhou and Jackson 1996). sgRNA1 is site for expression of TGB1 protein and the other overlapping proteins TGB2, TGB2', and TGB3 are translated from sgRNAβ2 (Zhou and Jackson 1996). Since product of these sgRNA β1 and β2 modulate systemic infection of virus through forming transporters for access of virus genome, these genomic part is remained as unmodified.

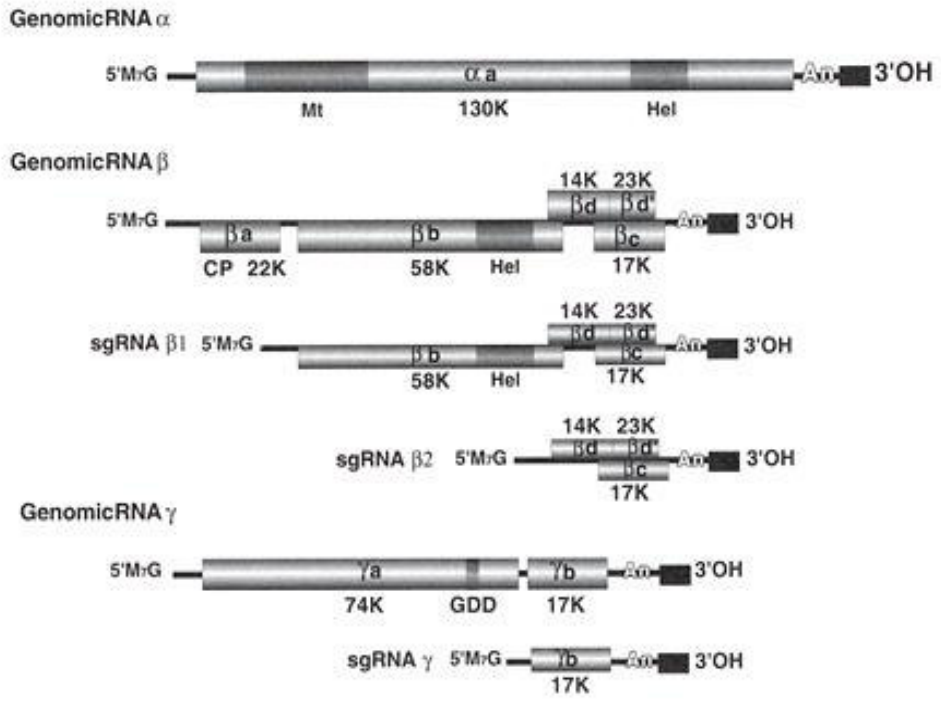


Figure 1.5. Illustration of the BSMV genomic and subgenomic RNAs (sgRNAs). RNA α genome of virus coding mRNA for the αa protein, which is component of the capping and helicase subunits of the RNA-dependent RNA polymerase (RdRp). Coat protein of virus is encoded by RNA βa and the "triple gene block" composed of the TGB1, TGB2, TGB2', and TGB3 proteins are also produced by RNA β genome. From genomic RNA, βa is translated and the TGB1 protein is expressed from sgRNA β1 and the TGB2, TGB2', and TGB3 proteins are translated from sgRNAβ2. Polymerase subunit of RdRP is translated from the genomic RNA γ. This genome also encodes the γb protein, a dispensable protein, expressed from sgRNAγ (Johnson et al. 2003).

1.5.2 PDS

In molecular biology studies marker genes are widely used in order to assure that applications were conducted successfully and effectively. For this purpose, there are many genes that can be transferred into or targeted in cells like green fluorescence protein (GFP), luciferase enzyme, pytone desaturase (PDS) enzyme to verify effectiveness of assays and/or for selection/screening purpose as listed in Table 3. Usually we expect to observe visual changes by using marker gene technology to use them as phenotypic markers.

Table 3. Commonly used marker genes (Godge et al. 2008).

Marker gene	Features	Silencing phenotype
Phytoene desaturase, PDS	Early enzyme of caretenoid biosynthesis, protects plants from photobleaching	Photobleached white spots on green tissue particularly leaves
Green fluorescent protein, GFP	Originally isolated from jellyfish, *Aequorea victoria* Fluoresces green under ultraviolet light	GFP-expressing tissue appear green under UV-light while silenced tissue appear red because of chlorophyll auto-fluorescence
Chalcone synthase, CHS	Key enzyme in anthocyanin biosynthesis, required for coloration of petals of petunia	Silencing leads to either partial or complete white petals
Sulfur gene, SU	Encodes one unit of the chloroplast enzyme magnesium chelatase	Yellow white spots in leaves
Proliferating cell nuclear antigen, PCNA	Highly conserved processivity factor for DNA polymerase δ required for DNA replication, highly expressive in dividing cells	Cessation of shoot extension

Phytone desaturase is widely used as a phenotypic marker in silencing studies. This enzyme lies at the center of caretenoid biosynthesis (Qin et al. 2007). Carotenoids a class of C40 hydrocarbon compounds, the pigments

that are membrane-bound lipid-soluble pigments, consist of carotene and xanthophyll. They are formed by condensation of isoprenoids (Bartley et al. 1995). Carotenoids play many roles in large number of biological processes in plants. One of major usages of carotenoids is serving as precursor in vitamin A and abscisic acid (ABA) synthesis. In addition to this, they have central role as accessory pigments in photosynthesis by forming the basic structural units of photosynthetic antennae. Moreover, carotenoids ability to quench the free radicals in many physiological pathways, (Bendcih et al. 1994) provides them acting as photo-protection agents by neutralizing the singlet oxygen that might bring about chlorophyll damage. Indeed, by inhibition of carotenoid synthesis, we observed color change in leaves due to photo-bleaching meaning destruction of chlorophyll. White patch formation on leaves becomes the proof of inhibition of the enzyme which act in carotenoid biosynthesis. Although carotenoids are synthesized in plastids, all the enzymes involved in their biosynthesis are encoded by nuclear genes. So, mRNA of any gene, whose product acts in biosynthesis; can be targeted by post transcriptional gene silencing. Most commonly targeted gene in this pathway is PDS gene. As shown in Fig. 1.6, phytoene desaturation is an important step in the β carotene biosynthesis pathway. In order to eliminate unwanted plant growth Norflurazon is used as a commercial herbicide whose mode of action is targeting PDS enzyme which shows importance of this enzyme in carotenoid biosynthesis (Chamovitz et al. 1993). After first cloning of PDS gene from cyanobacterium, homolog of gene were cloned and identified in kingdom virida planta, such as soybean, Arabidopsis, maize, wheat and barley (References within: Qin et al. 2007) High homology in both nucleotide and amino acid level between these homologs show evolutionary conservation of PDS enzyme among different organisms. Phytoene desaturation is an important step in carotenoid synthesis in which product of this reaction becomes a critical element for electron carrier in electron transport. In addition to albino phenotype of plants as a result of mutations in PDS gene, dwarfism also

observed in Arabidopsis mutants due to inhibition of carotenoid synthesis (Qin et al. 2007).

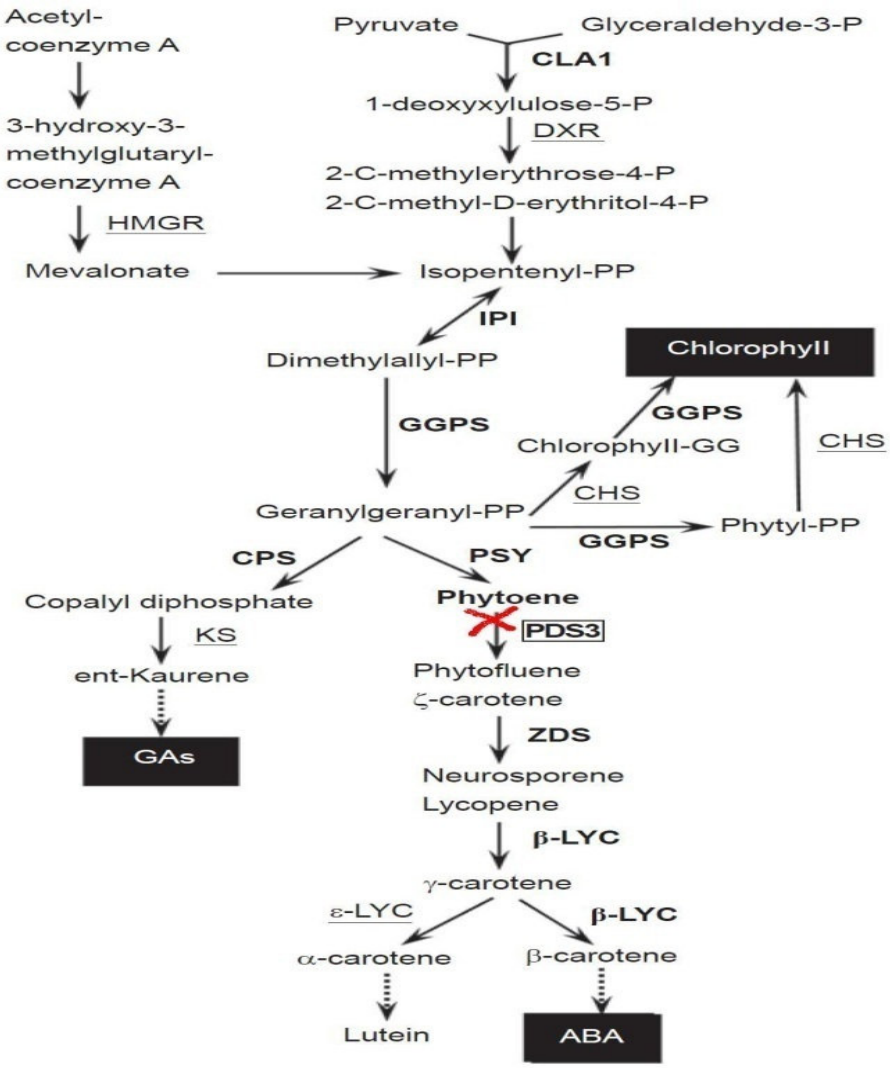


Figure 1.6. Carotenoid, chlorophyll and GA biosynthesis pathways. Inhibition of PDS enzyme caused phytoene accumulation and this interferes with production of α and β carotene (Qin et al. 2007).

1.6 Aim of the study

Crops are economically very important family in plant kingdom and pathogens infection in these plants result in yield and economical loss of in every year. Therefore, it is crucial to understand molecular mechanism of plant-pathogen interactions to prevent pathogen invasion and provide resistance to plants against pathogens. This can be achieved by characterization of plant genes that have roles in resistance. Since it is difficult to study on and manipulate agriculturally important plants such as barley and wheat due to their complex genomes, *B. distachyon* has been regarded as new model organism for such crops.

Since VIGS is fast, effective and a transient method to use in functional analysis of genes, in this study, we aimed to implement BSMV mediated VIGS in *B. distachyon*. As first part of thesis we used recombinant virus that bear GFP to test infection ability of the virus. In second part we tried to silence an endogenous gene of *B. distachyon* by targeting PDS gene using RNAi. For that purpose we used $BdPDS_{(185)}$ fragment to modify BSMV and deliver recombinant virus in *B. distachyon*. By success of such a strategy we aimed to gain a new tool for functional analysis of genes in *B. distachyon* which would help to understand functions of genes in agriculturally important crops.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

B. distachyon ABR-1 ecotype was used in BSMV mediated VIGS in this thesis. This ecotype was collected from Kaman (Kirsehir, Turkey) and provided by Dr. Konstantin Kanyuka from *Biotechnology and Biological Sciences Research Council*: UK (BBRSC). For positive control barley (cv. Bulbul) was used. Both barley and *B. distachyon* seedlings were grown at 24 ˚C and 18 ˚C for 16 hrs light and 8 hrs dark periods with kept relative humidity between ~25-40 % in a growth chamber.

2.2. PDS gene silencing in *B. distachyon* and barley

2.2.1 RNA isolation from *B. distachyon* leaf tissue

Total RNAs of *B. distachyon* were extracted from the collected leaf samples using Trizol® reagent (Invitrogen, CA, USA) according to suggested procedure by the manufacturer. Tissue samples (approximately 100 µg) were powdered using a mortar in liquid nitrogen. Powdered tissues were homogenized in 1 mL of TRIzol reagent per 100 mg of tissue in Eppendorf 2 mL sterile tubes. Homogenized trizol samples were incubated for a nearly 5 minutes at room temperature. Then 0.2 mL of chloroform was added per 1 mL of TRIzol reagent. Sample tubes were capped securely. Tubes were shaked by hand for 10 to 15 seconds vigorously and incubated at room temperature for 5 min. As a next step, the samples were centrifuged at 15,300 rpm for 15 min at 4 ^{o}C. After centrifugation, RNA remained exclusively in the aqueous and colorless upper phase. The upper phase was

taken and transferred to a fresh sterile tube. For precipitation of RNA isopropyl alcohol was used. 0.5 mL of isopropyl alcohol was added per 1 mL of TRIzol reagent. Then, samples were incubated at room temperature for 10 min and centrifuged at 15,300 rpm for 10 min at 4 ^{o}C. RNA pellets were precipitated on the bottom of the tubes. In order to wash RNA pellets, supernatants were removed and the RNA pellets were washed out by adding at least 0.75 mL of 75 % ethanol per 1 mL of used TRIzol reagent. Samples were mixed by hand shaking vigorously and centrifuged at 10,000 rpm for 6 min at 4 ^{o}C. After pouring upper phase, RNA pellets were let to air dry for 10-15 minutes. RNAs were dissolved in nuclease-free water and incubated for 5 to 10 min at 55-60 ^{o}C in order to disrupt secondary structure of RNAs and stored in -20 ^{o}C.

2.2.2 Concentration determination of the isolated RNA samples

RNAs were dissolved in approximately 40-50 µL nuclease free water. A sample of 1 µL was used for concentration determination on NanoDrop ND-1000 spectrophotometer. Isolated RNAs were separated on 2 % agarose gel in order to check intactness of RNAs.

2.2.3 Clean up of total RNAs

Total RNAs of samples of the *B. distachyon* leaves were treated with RNAse free-DNAse and precipitated with Lithium chloride. DNAse treatment was performed using 1 U of Turbo DNAse (Ambion Inc. Lot# 095K81). Following components were combined in a 500 µL sterile PCR tube: 10-15 µg RNA was used for treatment. We incubated samples at 37 °C for 15 minutes and then reaction was inactivated by heating for 10 minutes at 65 °C. For removal of proteins and other remnants from mixture for further purification of RNAs; equal volume of (DNAse treated) mixture, 7.5 M lithium chloride (Ambion salt, 7.5M, #9480), and sterile water (1:1:1)

were added on sterile eppendorf tube. As a next step we incubated the tubes at -20 °C for minimum of 60 minutes; generally overnight incubation was applied. Samples were centrifuged at 15.300 rpm for 15 min at 4 ^{o}C. After centrifugation, supernatant was removed and pellets were washed with 70% ethanol prepared with nuclease free water. Then, samples were centrifuged at 10.000 rpm for 6 min at 4 ^{o}C. Supernatant was removed, RNAs were let to air dry and finally pellets were re-suspended in 15 µL of nuclease free water.

2.2.4 Synthesis of first strand cDNA

Following components were combined in a sterile PCR tube: 50 pmol oligo $dT_{(20)}$, total RNA (between 1 µg-5 µg, we used nearly 1,5 µg), 0.1 mM dNTPs (Fermentas) mix, sterile distilled water up to 12 µl. This mixture was incubated at 65 ^{o}C for 5 min and quickly chilled on ice for 2 min. As a next step, following components were added: 4 µL 5X First Strand Buffer, 1 µL 0.1M DTT, 1 µL 40 U RNase inhibitor (Invitrogen CA, USA). The content of the tube was spinned briefly and incubated at 50 ^{o}C for 2 min. Finally 200 U of SuperScript III^{TM} (Invitrogen CA, USA) Reverse Transcriptase enzyme was added. Reaction was carried out at 50 ^{o}C for 90 min and stopped by incubating at 70 ^{o}C for 15 min.

2.2.5 PDS gene amplification from *B. distachyon* cDNA

PDS gene was cloned as partial coding sequence (CDS) from *B. distachyon* ABR-1 ecotype plant cDNA. The amplification was performed by using PDS gene specific HvPDS195F 5'-ata**ttaattaa**ctggatgaaaaagcagggtgttcc-3' forward primer having *Pac*I site (bold letters) and HvPDS196R 5'-tat**gcggccgc**ctactttcaggaggattaccatcc-3' reverse primer having *Not*I site (bold letters) designed for barley PDS gene (Holzberg et al. 2002). This primer set targeted 185 bp of PDS gene in barley. Annealing temperature for PCR

amplification of those primers was 64 ^{o}C. The reaction components were as following in 30 µL of final volume: 2 µL *B. distachyon* cDNA, Gentaq 1X PCR Buffer (75 mM Tris-HCl with pH 8.8 at 25 ^{o}C, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20), 0.25 mM dNTP mix (Invitrogen), 1,5 mM $MgCl_2$ (MBI Fermentas) 1 U of *Taq* DNA polymerase (Gentaq), 10 pmol HvPDS195F primer and 10 pmol HvPDS196R primer and nuclease free water. PCR cycling conditions were 94°C for 3 min as an initial denaturation step, 35 cycles of three steps of denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min and extension at 72 °C for 1 min. A second set of primers targeted to amplify longer partial PDS fragment, 546 bp in length, and for its amplification HvPDS193F 5'-ata**ttaattaa**ctaaacccatattgcttgaggcaa-3' forward primer having having *Pac*I site (bold letters) and HvPDS196R 5'-tat**gcggccgc**ctactttcaggaggattaccatcc-3' reverse primer having *Not*I site (bold letters) primers were used.

cgcgaattcactagtgattatattaattaactggatgaaaaagcagggtgttcctgatcgagtcaacgatg aggtttttattgcaatgtccaaggcgctcaatttcataaaccctgatgagttatctatgcagtgcattctgatt gctctaaaccgatttctccaggagaagcatggttccaaaatggcatttttggatggtaatcctcctgaaagt ag

Figure 2.1. Partial sequence of barley PDS. $PDS_{(185)}$ sequence amplified using HvPDS195F forward and HvPDS196R reverse primers.

Amplified PCR products were loaded on 1% agarose gel and analyzed. PCR products were purified and then ligated with pGEM-T Easy vector (Promega) for amplification and sequencing analysis.

2.3 Cloning and transformation of *B. distachyon* PDS fragments

2.3.1 Ligation of amplified PDS gene fragments and pGEM-T Easy vector

Amplified PCR product was cleaned with PCR purification kit (Qiagen Cat no: 2810), according to the instruction of the manufacturer. Purified PCR products ligated with pGEM-T Easy vector (Promega) with a final volume of 10 µL in an Eppendorf tube. Following components were combined; 6 µl PCR product, 5 ng pGEM-T Easy vector (Promega), 1 x Ligase Buffer (Promega) and 2 U T_4 DNA Ligase enzyme (Promega). Reaction was incubated at 4°C overnight, for ~18 hours.

2.3.2 Transformation of ligation products

Prior to transformation of plasmid into the cells, *E.coli* Dh5α cells were made competent by following this procedure: A single colony of *E.coli* cells was inoculated into 2 mL LB medium without any antibiotic. Cell growth was let at 37 °C with shaking (~250 rpm) overnight. 1 mL of overnight grown culture was taken and inoculated into 100 mL of LB medium in a sterile 500 mililiter flask. Cells were grown at 37 °C, with shaking (250 rpm) until A_{590nm} of 0.375. Then culture was aliquated into two 50-mL tubes and put on ice for 10 min. Cells were centrifuged for 10 min at 4000 rpm. Supernatant was poured and each pellet was re-suspended in 5 mL ice-cold sterilized $CaCl_2$ (4mM) solution. Centrifugation of cells for 5 min at 2500 rpm was carried out and supernatant was discarded. Each pellet was re-suspended again in 2 mL of ice-cold $CaCl_2$ (4mM) solution. Centrifugation step was repeated again. Cells were suspended in fresh 2 mL 4mM $CaCl_2$ dispensed into sterile Eppendorf tubes; their ability for efficient transformation continues up to two weeks at 4°C. *E.coli* Dh5α competent cells were transformed with ligation products as following. In a sterile 2

mL tube following components were combined; 5 µL ligation product (pGEM-TEasy-BdPDS$_{(185)}$PN and pGEM-TEasy-BdPDS$_{(546)}$PN), and 50 µL *E.coli* Dh5α competent cells. Mixture was put on ice for 30 min, and then was heat shocked at 42 °C for 45 sec. LB liquid medium was added up to 200 µL. The cells were incubated 37 °C for 45 minutes and quickly chilled on ice for 2 minutes. Cells were spread as 50 µL/plate on LB agar plates which contain ampicilin (50 µg/mL) for selection, X-Gal (80 µg/mL) for blue/white screening and IPTG 100 µg/mL for induction of cell growth. Plates were incubated at 37 °C overnight. After the incubation, white colonies were chosen by picking them among grown colonies and these selected colonies were transferred to sterile flask that has 10 mL of LB medium with ampicillin for overnight incubation. For further verification; selected colonies were PCR amplified using M13F 5´-cgccagggttttcccagtcacgac-3' forward and M13R 5´-tcacacaggaaacagctatgac-3' reverse primers. Cells can be stored in 25 % glycerol stock after growth.

2.3.3 Plasmid isolation of the selected colonies

Plasmids were isolated using Qiagen QIAprep Spin Miniprep Kit (Cat no: 27104) according to the kit protocol as following: Bacterial culture of 2 mL was harvested by centrifugation for 2 min at 14.000 rpm. Supernatant was decanted; tube was inverted and blotted to on a paper towel to remove excess media. 250 µL of Buffer P1 was added on to the collected cells which were suspended by vortexing up to no visible cell clumps seen. 250 µL of Buffer P2 was added, then the tube content was mixed gently inverting four times, incubated at RT for approximately 5 minutes. 350 µL of N3 solution was added and mixed by inverting 4-5 times. Sample tubes were centrifuged at 14.000 rpm for 10 minutes. A spin column, provided by manufacturer, inserted in to collection tube. Cleared lysate, the upper phase was transferred to the spin column, centrifuged at 14.000 rpm for 1 minute. After the centrifugation, filtrate in the collection tube was discarded

and collection tube reinserted, and well washed 750 µL of column washing solution Buffer PE was added to spin column, centrifuged at 14.000 rpm for 1 minute, flow-through discarded and the collection tube reinserted and to remove residual wash buffer completely additional 1 min centrifugation was carried out. The spin column was transferred to a new sterile tube, the plasmid DNA was eluted by adding 50 µL of Elution Buffer and centrifugated at 14.000 rpm for 1 min. Spin column assembly was removed and plasmid DNA was stored at –20 °C. All the centrifugations were applied at room temperature.

2.3.4 Excision of BdPDS insert and restriction digestion of pγ sense and antisense vectors

*Not*I and *Pac*I extended *B. distachyon* PDS gene (185bp) insert in pGEM-T Easy vector was removed by double digestion using *NotI* and *PacI* restriction enzymes. On the other hand the gama vector was prepared by excision of barley PDS fragment (HvPDS) from pγ.HvPDS sense and antisense plasmids in order to create sticky ends in pγ plasmids later to clone *B. distachyon* $PDS_{(185)}$ fragment in it. Following components were combined in Eppendorf tubes for digestion. *Not*I and *Pac*I (NEB) 10 U each, 1X NEBuffer 2 (NEB), templates (purified pGEM-T Easy and pγ plasmids). The mixture was incubated at 37 °C for 4 hours. After restriction digestion reaction samples were loaded onto 1 % agarose gel to monitor double digestion and later to extract bands from the gel.

2.3.5 Gel extraction of BdPDS fragment and pγ sense and antisense vectors

Gel extraction was made according to Qiagen, Qiaquick Gel Extraction Protocol. The excised fragments in correct length (migrated at 185 bp) were excised with a clean scalpel and weighed. 3 volumes of Buffer QG were

added per one volume gel and the content was incubated at 50 ºC for 10 min, until all the agarose was dissolved completely. 1 gel volume isopropanol was added to the dissolved agarose and content was mixed and the solution was transferred to QIAquick columns. 1 min centrifugation at 10000 g was carried out. Flow-through was discarded and 0.75 mL Buffer PE was added. Flow-through was discarded again and columns were centrifuged one more time for 1 min at 10000 g. For elution of DNA step, 50 µL Buffer EB was added in the tube, and tubes were centrifuged at 10000 g for 1 min, after 3 min of incubation at room temperature.

2.3.6 Ligation of amplified *B. distachyon* $PDS_{(185)}$ fragment with pγ sense and antisense vectors and transformation of ligation products

After gel extraction step ligation of *B. distachyon* PDS *Not*I and *Pac*I engineered fragment and restriction digested pγ sense and antisense plasmids were ligated. $BdPDS_{(185)}$ was inserted into pγ sense and antisense vectors by following same procedure in section 2.3.1 for ligation reaction.

As a next step, the transformation of ligation products into competent *E.coli* Dh5α cells was performed. Method applied for transformation and colony selection is presented in section 2.3.2. Plasmids were isolated by following steps in section 2.3.3.

2.4 Barley Stripe Mosaic Virus (BSMV) vectors for silencing

BSMV vectors pα, pβΔβa, pγ, pγ.GFP, barley PDS fragment containing pγ.HvPDS (sense) and pγ.HvPDSas (anti-sense orientation) (Figure 2.1) were obtained from Large Scale Biology Corporation (CA, USA). The pγ.HvPDS sense and antisense vectors were used to construct pγ.BdPDS sense and antisense by replacing barley PDS insert with 185 bp *B. distachyon* PDS fragment by restriction digestion as presented in section 2.3.4. All the vectors were transformed into competent *E.coli* DH5α strains

as described in section 2.3.2. Amplified plasmids were isolated and purified as described in section 2.3.3.

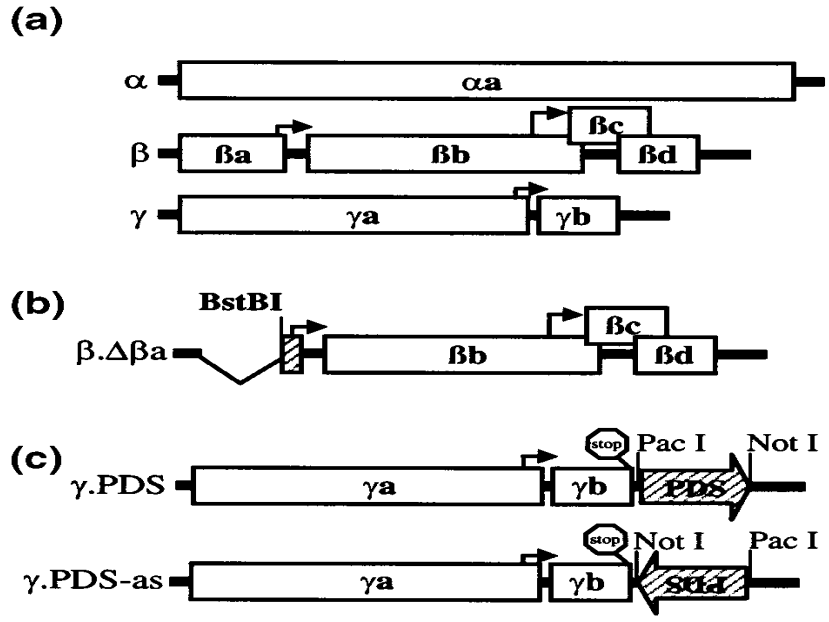


Figure 2.2. BSMV RNA derived vectors. **(a)** pα, pβ and pγ genomes of BSMV. **(b)** pβΔβa representing the deleted version of pβ genome. **(c)** pγ.PDSs and pγ.PDSas orientations The picture was taken from Holzberg et al. 2002.

pγ.bpds4As

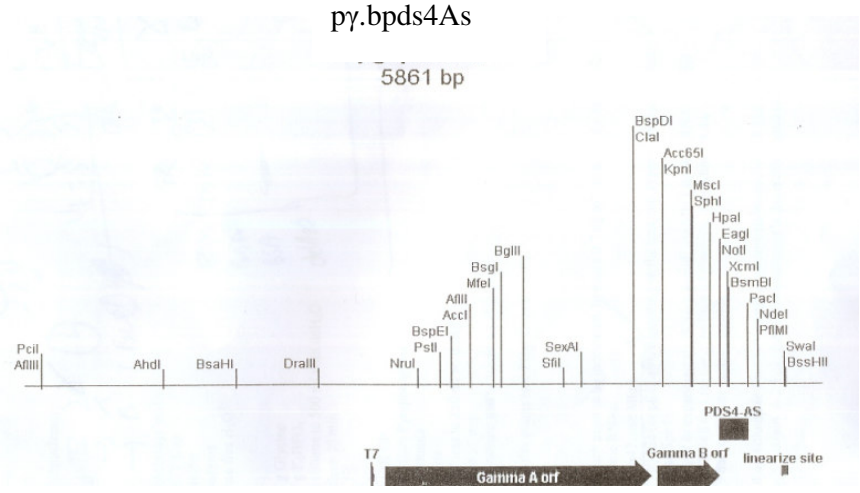


Figure 2.3. pγ.HvPDSas map representing restriction cleavage sites.

2.4.1 Linearization of plasmids

All plasmids were digested with restriction enzymes in order to be linearized. pα plasmid DNA was digested with *Mlu*I enzyme (MBI Fermentas) as combining components with following amounts. 10 µg purified pα plasmid DNA, 1X RE Buffer (MBI Fermentas), 10 U *Mlu*I enzyme (MBI Fermentas) and PCR water were combined in a PCR tube to a final volume of 50 µl. Mixture was incubated at 37 °C for 4 or 5 hrs. pβΔβa plasmid DNA was digested with *Bcu*I or *Spe*I enzyme (MBI Fermentas). 10 µg purified pβΔβa plasmid DNA, 1X yellow enzyme buffer (MBI Fermentas), 10 U *Bcu*I (MBI Fermentas) and PCR water were combined in a PCR tube to a final volume of 50 µL. Mixture was incubated at 37 °C for 4 or 5 hrs. All pγ plasmid (pγ, pγ.HvPDS, pγ.HvPDSas, pγ.BdPDS and pγ.BdPDSas) DNAs were digested using *BssH*II enzyme (New England Biolabs). 10 µg pγ plasmid DNA, 1X NEB3 Buffer (New England Biolabs), 10 U *BssH*II enzyme (New England Biolabs) and PCR water were combined in a PCR tube to a final volume of 50 µl. Mixture was incubated at 50 °C for 4 hrs. After restriction digestion, samples were separated in 1 % agarose gel by electrophoresis. Linearized plasmids were purified by gel extraction kit of QIAGEN (Cat no: 28104) as described in section 2.3.5.

2.4.2 *In vitro* transcription of linearized vectors

All of the α, βΔβa and γ linearized genomes were transcribed *in vitro* according to manufacturer's procedure of the mMessage mMachine T7 *in vitro* transcription kit (cat no: 1340, Ambion, Austin, TX). Following components were combined in a 500 µL sterile PCR tube: separately for each plasmids per one silencing reaction 80 ng template was used (linearized plasmid DNA), 1X Buffer (Ambion), 1X nucleotide mix with NTP Cap (Ambion), 0.15 µl of T7 RNA polymerase mix (Ambion) and sterile distilled water up to 1.5 µl. Mixture was incubated at 37 ^{o}C for 2 hrs and stored at -80 °C until use. Before inoculation, the transcripts were purified as described in section 2.2.3.

2.4.3 Inoculation of plants with BSMV transcripts

B. distachyon ABR-1 ecotype and barley (cv. Bulbul) plants were used for BSMV mediated VIGS. The second leaves (approximately 7-10 days upon germination) were inoculated with BSMV for silencing. For *B. distachyon* silencing we used BSMV:BdPDS and BSMV:BdPDSas transcripts meaning that *B. distachyon* PDS was used to silence PDS gene in this organism. For positive control barley samples were infected with BSMV:HvPDS and BSMV:HvPDSas constructs in order to silence barley PDS gene. For both plant samples BSMV:00 infection was carried out as a negative control and FES as mock. Transcripts of each of the BSMV transcripts were mixed in a 1:1:1 ratio as shown in table 4 (1.5 µg of each transcript concentration determined on NanoDrop ND-1000 spectrophotometer). Transcription mix was combined with 45 µL FES (50 mL 10X GP (18.77 g glycine, 26.13 g K_2HPO_4, dd$H_2$0 upto 500 mL, sterilized by 20 min autoclaving, 2.5 g sodium pyrophosphate, 2.5 g bentonite, 2.5 g celite and dd$H_2$0 up to 250 mL (Pogue et al. 1998) for Barley leaves and 15 µL FES for *B. distachyon* and

directly applied, with two light strokes to the second leaf (when it is 5-7 cm long) from the bottom of leaf to the tip. The systemic spread of virus was observed by the appearance of mosaic symptoms on leaves after 7-10 days post inoculation (dpi). 3rd leaves from inoculated plants were collected after 10 dpi in order to quantify PDS gene expression level by qRT-PCR.

Table 4. Transcripts of each of the BSMV inoculation were mixed in a 1:1:1 ratio using 1.5 µg of each of them.

	pα	pβΔβa	pγ	Pγ.Bd PDS	Pγ.Bd PDSas	Pγ.Hv PDS	Pγ.Hv PDSas	FES
BSMV:00	1.5 µg	1.5 µg	1.5 µg	-	-	-	-	45 µL
BSMV:BdPDS	1.5 µg	1.5 µg	-	1.5 µg	-	-	-	15 µL
BSMV:BdPDSas	1.5 µg	1.5 µg	-	-	1.5 µg	-	-	15 µL
BSMV:HvPDS	1.5 µg	1.5 µg	-	-	-	1.5 µg	-	45 µL
BSMV:HvPDSas	1.5 µg	1.5 µg	-	-	-	-	1.5 µg	45 µL
FES	-	-	-	-	-	-	-	45 µL

2.5 PDS gene silencing of *B. distachyon* and Barley and quantification of silencing levels

2.5.1 RNA isolation from plant leaf tissues and purification

In this thesis total RNAs of all the plant materials (barley, *Brachypodium distachyon*) were extracted from the collected leaf samples using Trizol® reagent (Invitrogen, CA, USA) according to suggested procedure by the manufacturer as presented in section 2.2.1. BSMV:00 treated *B. distachyon* and barley leaves, and BSMV:BdPDS and BSMV:BdPDSas inoculated *B. distachyon* leaves and BSMV:HvPDS and BSMV:HvPDSas inoculated barley leaves were isolated with the same procedure. After RNA isolation of plant tissue samples we determined concentration and integrity of RNAs by NanoDrop (ND-1000) spectrophotometer and agarose gel

electrophoresis. As a next step isolated samples were cleaned up as in section 2.2.3 to use them in first strand cDNA synthesis. Procedure in section 2.2.4 was followed to prepare first strand cDNA from purified RNA samples. These cDNAs were diluted in $1/10^{th}$ and used in quantification of PDS gene expression level *via* qRT-PCR method.

2.5.2 qRT-PCR and measurement of PDS gene expression levels

The measurements of PDS transcripts of inoculated and mock inoculated barley and *B. distachyon* plants were performed on Stratagene Mx3005p qPCR System using the Brilliant SYBR Green qPCR Master mix (Stratagene, Cat no: 600548). Total RNAs of the 10 dpi leaf samples of wild type and modified BSMV and mock inoculated *B. distachyon* and barley silenced plants have been used for qRT-PCR experiments. qRT-PCR was performed in triple replicates for each RNA sample per primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for *18S rRNA* (GenBank accession number: X16077.1) 18s rRNA forward 5'-tttgactcaacacggggaaa-3' and 18S rRNA reverse 5'-cagacaaatcgctccaccaa-3' primer pairs for real-time PCR normalization. PDS gene qPCR analyses were performed by the TaPDS gene 5'-ccctgacgagttatccatgcag-3' forward and 5'-ggacctcaccacccaaagact-3' reverse primers designed using Primer3 software (Rozen and Skaletsky, 2000). Threshold (Ct) values and gene transcript numbers were used to determine the gene expression levels from different cDNA samples. Real-time PCR data were analyzed according to Pfaffl's model (Pfaffl, 2001). For expression analysis the BSMV:00 inoculated and modified BSMV inoculated plant PDS gene transcript levels were compared. Prior to performing the qRT-PCR for the gene expression level change the standard curve has been calculated as follows; The Standard Curve is a plot of the initial template quantity in the Standard wells (X-axis), versus the Ct (threshold cycle). A standard curve consists of 3 to 4 points, and each

concentration should be run at least in triplicate. A linear standard curve provides the efficiency of qRT-PCR amplification. Indeed standard curve analysis was conducted previously by members of Akkaya lab; it might be useful to give principle. To perform the standard curve analysis, initial template was assumed to be 8X concentration and then the serial dilutions have been applied to the sample as using template 4X, 2X and 1X concentrations. Templates with these concentrations were used for 18S rRNA reactions and at the end of reactions it is expected to obtain 2 fold, 4 fold, and 8 fold differences comparing to each other. After gaining the results, the amplification plots were calculated. One hundred percent efficiency implies perfect doubling of amplicon each cycle.

2.6 GFP expression by BSMV in wheat

For testing BSMV mediated VIGS in *B. distachyon* GFP was used as a reporter gene. For this purpose the BSMV-GFP recombinant vector was used constructed by Holzberg et al. (2002). This recombinant vector was transferred into *E.coli* Dh5α cells as described in section 2.3.2. Purification of pγ.GFP plasmids and linearization of plasmid procedures were stated in section 2.3.3 and 2.3.5, respectively. For linearization of pγ.GFP *Bssh*II enzyme was used as in section 2.4.1. In order to obtain transcripts, mMessage mMachine T7 Ambion kit was used in *in vitro* transcription reactions (IVT) for α, βΔβa, and pγ.GFP linearized genomes as described in section 2.4.2. All the transcripts were applied on *B. distachyon* ABR-1 ecotype with 15 μL FES as described in section 2.4.3. After inoculation of GFP bearing BSMV, the GFP expression was monitored by Leica flourescent microscope DFC 280 digital camera system at 2 and 3 dpi under 10x and 40x magnifications.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 PDS gene silencing in *B. distachyon via* BSMV mediated VIGS

In previous studies BSMV mediated VIGS was applied successfully in monocots such as Barley (Holzberg et al. 2002) and wheat (Scofield et al, 2005). In this study, the experiments were conducted to provide VIGS as a new tool that can be used in functional analysis of genes in *B. distachyon*, a new model organism for crops.

3.1.2 RNA isolation from *B. distachyon* leaf tissue

Total RNA was isolated from *B. distachyon* leaf tissue using TRIzol reagent as described in section 2.2.1. Integrity of these RNA samples were tested by agarose gel electrophoresis (Fig. 3.1).

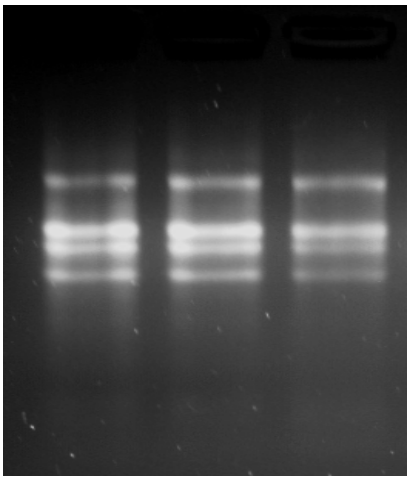


Figure 3.1. Total RNA samples of *B. distachyon* leaf tissues on the 2 % RNA agarose gel (wells correspond to 3 different technical repeats).

As seen in Fig. 3.1 all isolated total RNA samples have four ribosomal RNA bands, namely; 23 S rRNA, 18 S rRNA, 16 S rRNA, and 5 S rRNA. The bands appeared intact due to absence of smear in the background, therefore they can be used in further experiments.

3.1.3 Synthesis of first strand cDNA

After total RNA isolation of *B. distachyon*, first strand cDNA was synthesized as in section 2.2.4. Quality of cDNA was tested by PCR using actin primers (Fig 3.2). Since actin is produced constitutively in the cells, it is suitable for internal control.

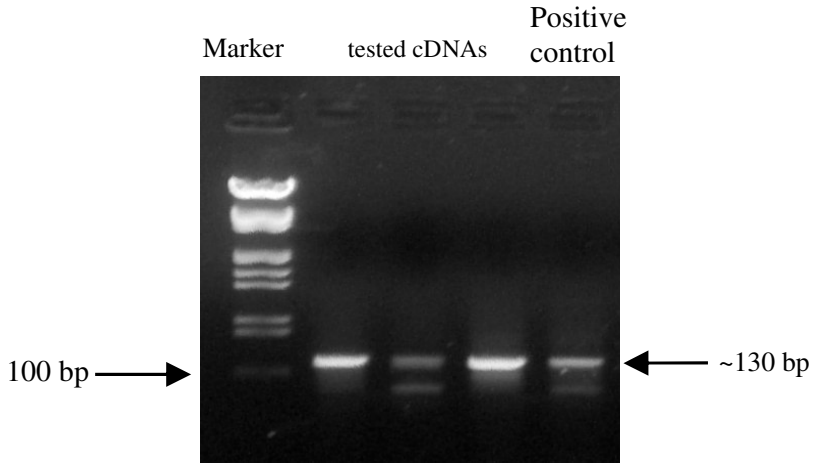


Figure 3.2. Control PCR using cDNA as template and actin primers. Amplified actin fragments on the 1 % agarose gel. Marker used is 100 bp marker.

Sizes of PCR products on the agarose gel were as expected sizes (133bp) based on the primers used. Therefore quality of tested *B. distachyon* cDNA was high enough to use them in further experiments. As a positive control barley cDNA was used.

3.1.4 Amplification of *B. distachyon* PDS fragments

After checking for cDNA quality, two different fragments of *B. distachyon* PDS were amplified by PCR (Fig 3.3). First one is 185 bp in length and second one is longer (546 bp in length) using primer sets presented in section 2.2.5. $PDS_{(185)}$ was used in silencing experiments and on the other hand $PDS_{(546)}$ was used in sequence similarity analysis comparing with PDS sequences of other species.

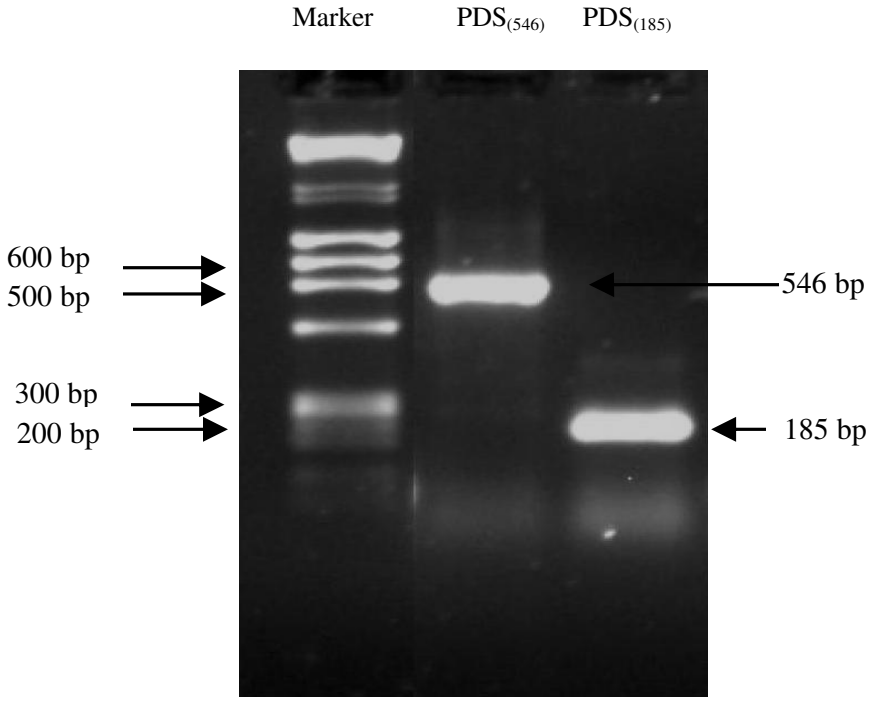


Figure 3.3. *B. distachyon* PDS fragments on the 1 % agarose gel. Marker used is 100 bp marker.

Sizes of PCR products on the agarose gel were as expected sizes based on the primers used.

3.1.5 Ligation of PDS fragments into pGEM-T Easy vector, transformation of *E.coli* Dh5α cells with ligation product (pGEM-TEasy-BdPDS$_{(185)}$PN) and colony PCR

Amplified PDS fragments, PDS$_{(185)}$ and PDS$_{(546)}$, were cloned into pGEM-T Easy vector (Promega) applying T/A cloning according to the procedure in section 2.3.1. After ligation, *E.coli* DH5α cells were transformed by the recombinant vectors: pGEM-TEasy-BdPDS$_{(185)}$PN and pGEM-TEasy-

$BdPDS_{(546)}PN$ vectors as in section 2.3.2. After propagation of cells, insert containing colonies were selected by X-Gal screening. Presence of inserts in selected colonies verified by colony PCR for $BdPDS_{(185)}$ using M13 primers given at section 2.3.2. Numbers 1-9 in Fig. 3.4 indicates randomly selected colonies.

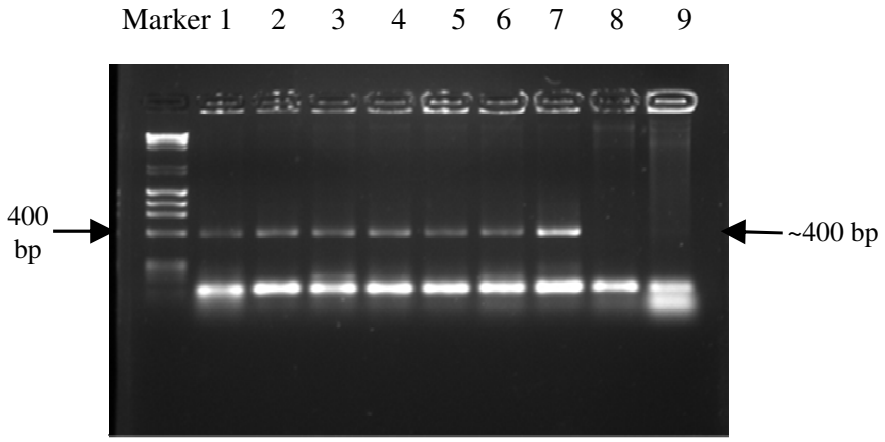


Figure 3.4. Colony PCR of *B. distachyon* $PDS_{(185)}$ fragment. 1 % agarose gel was used to monitor the PCR product. Marker is 100 bp marker.

The first seven colonies possessed insert containing vectors. Sizes of bands in first seven colonies were as expected sizes; approximately 400 bp that covers insert size and M13 primer targeting sites sizes, meaning that first seven colonies contain inserts in them. Therefore, they were used in further experiments. The other bands observed at bottom of the gel figure correspond to primer dimers. Plasmids isolated from the colony # 3 and 4 were the ones that were selected for sequencing.

3.1.6 Sequencing and similarity analysis

Both PDS fragments ($PDS_{(185)}$ and $PDS_{(546)}$) were sequenced using either of the M13 5´-gttttcccagtcacgac-3' forward or 5´-caggaaacagctatgac-3' reverse primers by RefGen Company (ODTU-Teknopark Ank, TURKEY).

Most conserved region of PDS gene in grass family which corresponds to $PDS_{(185)}$ fragment of *B. distachyon* shows 94 % nucleotide sequence homology with Barley PDS. $PDS_{(546)}$ fragment of *B. distachyon*, shares 91 % homology with Barley PDS at DNA level. Similarity analysis of *B. distachyon* PDS sequence with other species were given in Fig. 3.5a, obtained using ClustalX (Thompson et al. 1997) and Boxshade softwares. Phylogenetic tree of these plants was drawn using Phylogeny fr (Dereeper et al. 2007) as shown in Fig 3.5b.

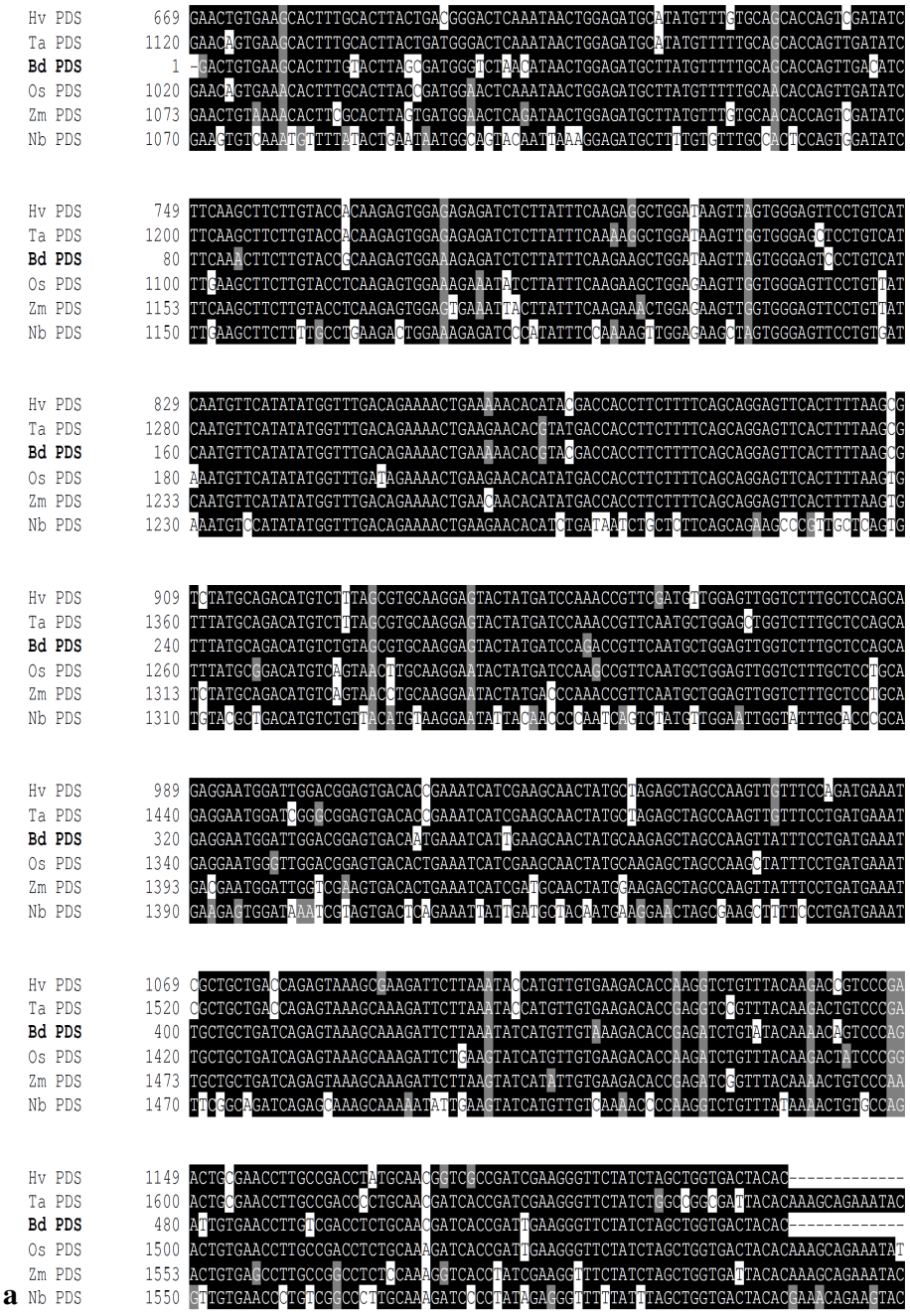
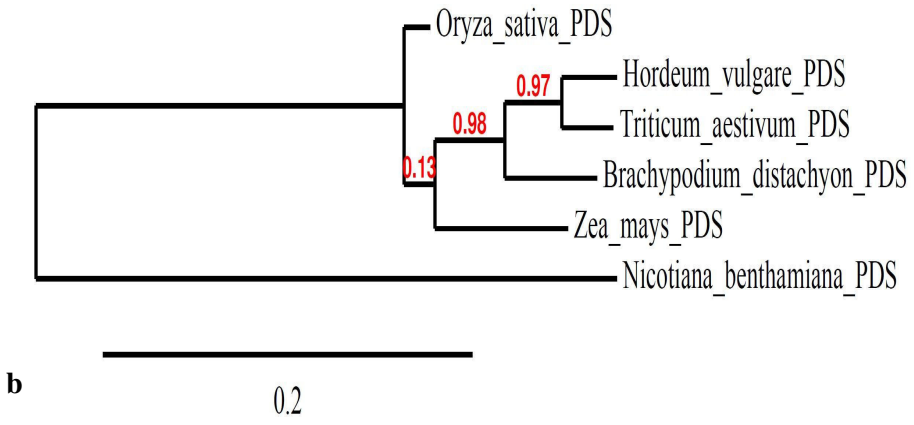


Figure 3.5(a). PDS sequence similarity analysis of *Hordeum vulgare* (AY062039), *Triticum aestivum* (DQ270236), *Brachypodium distachyon* (FJ913272), *Oryza sativa* (AF049356), *Zea mays* (NM_001111911), *Nicotiana benthamiana* (DQ469932). Clustalx (Thompson et al. 1997) and boxshade softwares were used.



b

Figure 3.5(b). Phylogenetic tree of *Brachypodium distachyon*, *Hordeum vulgare*, *Triticum aestivum*, *Nicotiana benthamiana, Oryza sativa*, *Zea mays*. Phylogeny fr (Dereeper et al. 2007) was used to draw Phylogenetic tree.

3.1.7 Cloning into Pγ sense and antisense vectors for silencing

Double restriction digestion of pGEM-TEasy-BdPDS$_{(185)}$PN was conducted to generate sticky ends using *Not*I and *Pac*I restriction enzymes (Fig. 3.6) as in section 2.3.4. Same procedure was followed for Pγ-HvPDS-PN vector to excise barley PDS fragment from Pγ sense and Pγ antisense vector (Fig. 3.7).

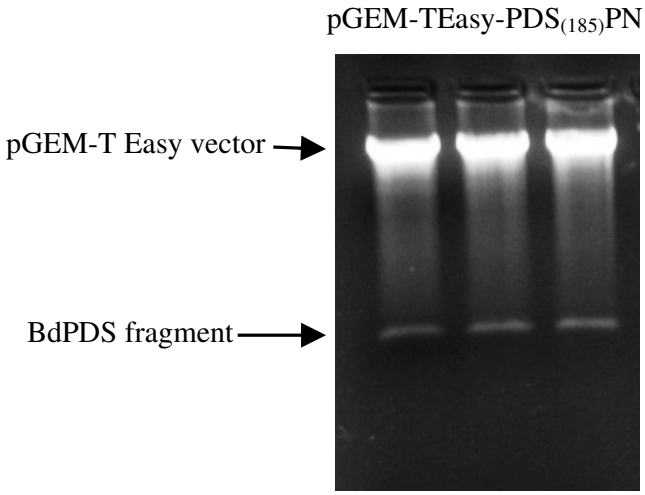


Figure 3.6. Double digestion of pGEM-TEasy-$PDS_{(185)}$PN. Plasmids were cut with *Not*I and *Pac*I restriction enzymes, which released the cloned fragment of $BdPDS_{(185)}$. Samples were seperated on 1 % agarose gel by electrophoresis.

As it can be seen from the Fig. 3.6 $BdPDS_{(185)}$ was excised from pGEM-TEasy-$PDS_{(185)}$PN successfully and used in cloning into Pγ sense and antisense vectors.

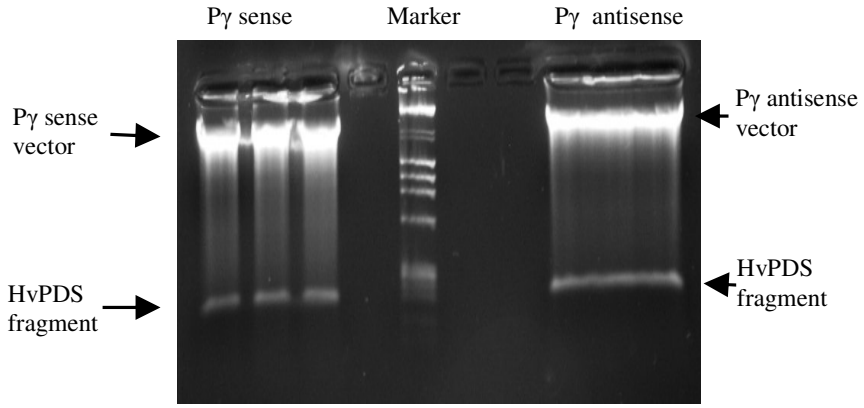


Figure 3.7. Excision of HvPDS from Pγ sense and Pγ antisense vectors. Both vectors were cut with *Not*I and *Pac*I restriction enzymes. Samples were separated on 1 % agarose gel by electrophoresis.

After excision of HvPDS from Pγ sense and antisense vectors, ligation of $BdPDS_{(185)}$ into free Pγ sense and antisense vectors was conducted in order to use these vectors in PDS gene silencing of *B. distachyon.* Cloning procedure was followed as in section 2.3.1. As a next step transformation of Pγ-$BdPDS_{(185)}$-PN sense and Pγ-$BdPDS_{(185)}$-PN antisense vectors to *E.coli* DH5α cells were carried out.

3.1.8 Isolation of plasmids

Plasmids (pα, pβ, pγ, pγ-GFP, pγ-HvPDS, pγ-HvPDSas, pγ-BdPDS, pγ-BdPDSas containing plasmids) were isolated from the *E.coli* cells containing either as naked (unmodified) or recombinant genome of virus as in section 2.3.3 (Fig 3.8). Plasmids were collected in 40 µL volume and concentrations of plasmids were measured by NanoDrop ND-1000 spectrophotometer (Fig. 3.9 and Table 5).

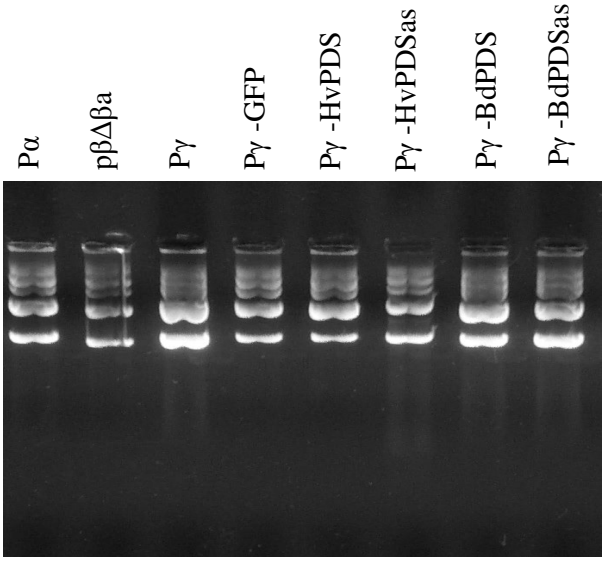
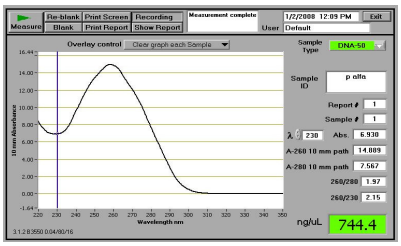
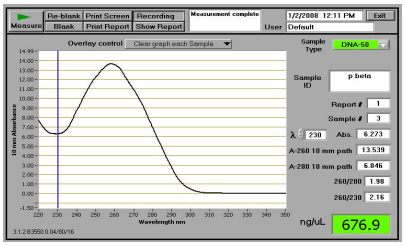


Figure 3.8. Isolated plasmids were separated on 1 % agarose gel. The bands at the bottom are the ones that we cut from the gel.

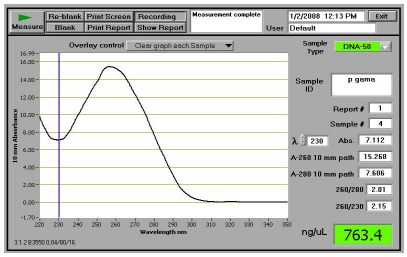
BSMV is an RNA virus that has tripartite genome as α, β, γ genomes. For VIGS studies with this virus, γ genome of virus is modified by insertion of fragment into γ genome. As can be seen in Fig 3.8 both naked and recombinant genome of virus containing plasmids were isolated from *E. coli* cells successfully. Plasmids were extracted from the gel and linearized in order to prepare them as appropriate templates for in vitro transcription (IVT) reactions for VIGS experiments.



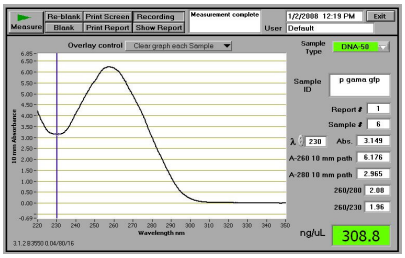
Pα



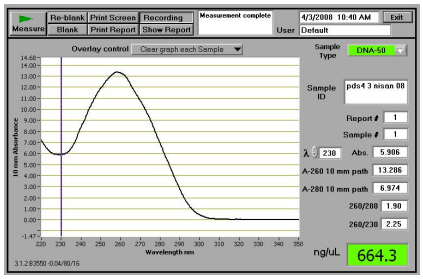
PβΔβa



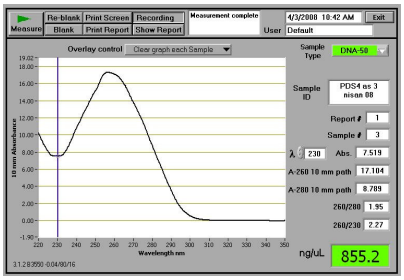
Pγ



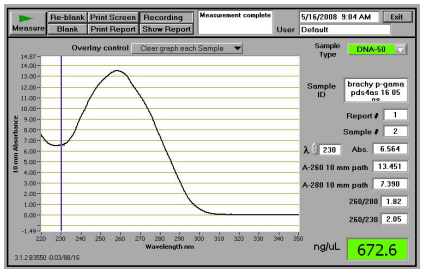
Pγ -GFP



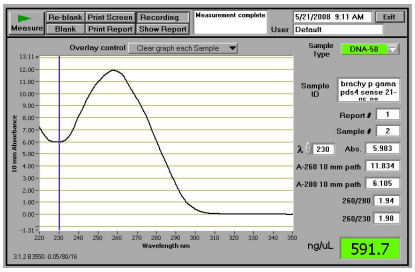
pγ –HvPDS sense



pγ –HvPDS as



pγ –BdPDS sense



pγ –BdPDS as

Figure 3.9. Concentration measurements of plasmids using NanoDrop (ND-1000)

Table 5. Concentration of plasmids determined using NanoDrop ND-1000 spectrophotometer.

Sample name	Concentration (µg/µL)
pα	744
pβΔβa	676
pγ	763
pγ -GFP	308
pγ –HvPDS sense	664
pγ –HvPDS antisense	855
pγ –BdPDS sense	591
pγ –BdPDS antisense	672

3.1.9 Linearization of plasmids

Plasmids that contain proviral DNAs were used for *in vitro* transcription reactions. Since RNA production from circular template may bring about concatemeric nonfunctional RNAs, vectors have to be linearized to provide efficient transcription before *in vitro* transcription reaction. Thus, pα, pβ, and naked or recombinant pγ genome containing plasmids were digested with restriction enzymes to make them linear template for IVT. Pα plasmid DNA was digested with *Ml*uI enzyme (MBI Fermentas), pβΔβa plasmid DNA with *Bcu*I or *Spe*I enzyme (MBI Fermentas) and all pγ plasmids were digested using *BssH*II enzyme (New England Biolabs) as presented in section 2.4.1. Efficiency of restriction digestion was monitored using 1 % agarose gel to compare circular and linearized plasmids (Fig. 3.10).

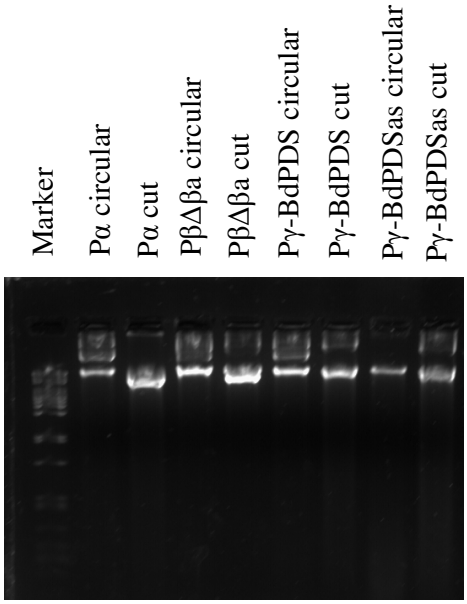


Figure 3.10. Digested plasmids and their circular pairs. Samples were separated on 1 % agarose gel by electrophoresis.

Linearized plasmids move faster than circular ones. The bands which corresponded to linearized plasmids were cut from the gel and purified. The purification was made according to gel purification kit of QIAGEN as in section 2.3.5.

3.1.10 *In vitro* transcription

For efficient BSMV mediated VIGS, viral genome has to be in RNA form. Therefore, linear plasmids containing α, βΔβa and γ (both naked and recombinant ones) genomes of BSMV were transcribed *in vitro* according to manufacturer's procedure of the mMessage mMachine T7 *in vitro* transcription kit (Ambion, Austin, TX) as presented in 2.4.2. After *in vitro* transcription reaction, RNA transcripts were cleaned following the procedure at section 2.3.3 to remove DNA, proteins and incorporated

nucleotides to increase efficiency of infection. Intactness and integrity of RNA samples were controlled by running them on agarose gel (Fig. 3.11)

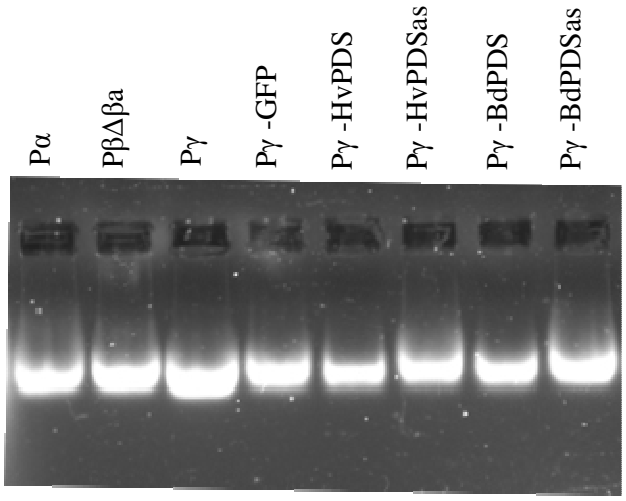
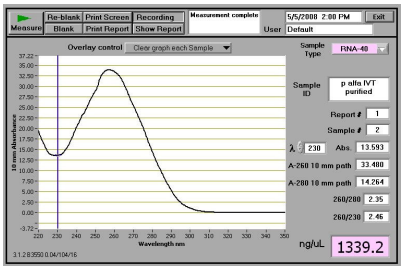


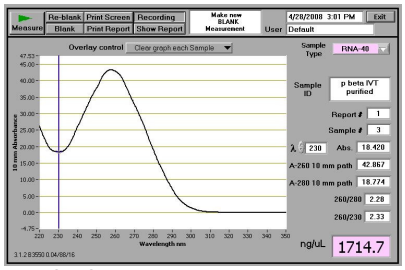
Figure 3.11. RNA gel photo of transcribed BSMV genomes. Samples were seperated on 2 % agarose gel.

As seen in Fig. 3.11 all purified RNA samples have one sharp band and there was not any smear on gel meaning that RNA transcripts were intact and can be used in BSMV mediated VIGS in *B. distachyon* and barley.

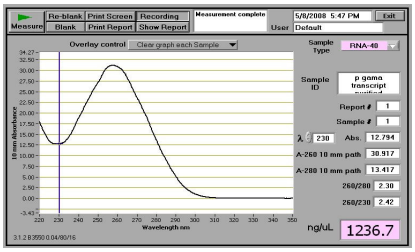
As a next step, concentration of transcripts were measured using NanoDrop ND-1000 spectrophotometer (Fig. 3.12 and Table 6).



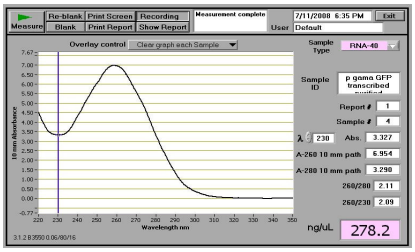
Pα transcript



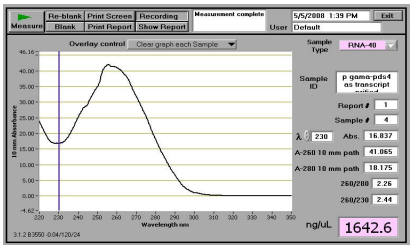
PβΔβa transcript



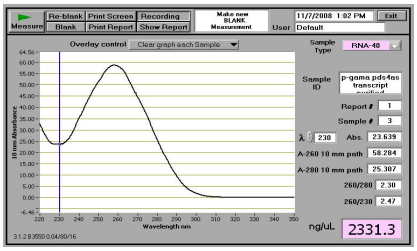
Pγ transcript



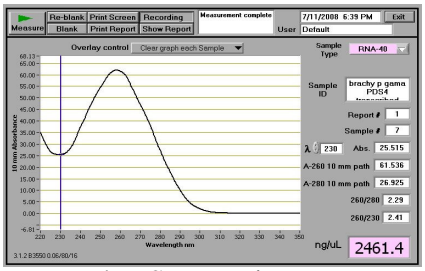
Pγ –GFP transcript



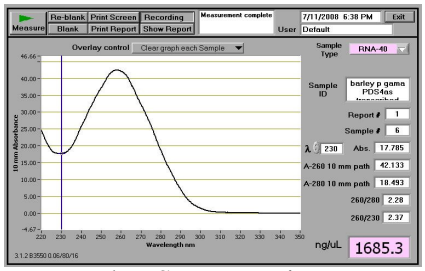
Pγ –HvPDS transcript



Pγ –HvPDSas transcript



Pγ –BdPDS transcript



Pγ –BdPDSas transcript

Figure 3.12. Concentration measurements of transcripts using NanoDrop (ND-1000)

Table 6. Concentration of transcripts determined using nanodrop ND-1000 spectrophotometer

Sample name	Concentration (μg/μL)
pα transcript	1340
pβΔβa transcript	1714
Pγ transcript	1236
pγ –GFP transcript	280
pγ –HvPDSs transcript	1640
pγ –HvPDSas transcript	2330
pγ –BdPDSs transcript	2641
pγ –BdPDSas transcript	1685

Concentrations of transcripts were high enough to use them in silencing experiments. Based on information from previous studies by Holzberg et al. (2002), concentrations of all transcripts was adjusted to 1.5 μg per plant before inoculation to leaves.

3.1.11 Virus induced gene silencing (VIGS) studies

3.1.11.1 GFP expression *via* BSMV

In molecular biology studies, reporter genes are commonly used for assessment of success and efficiency of an implementation. In this thesis, Green Flourescent Protein (GFP) was used as a reporter gene in order to show infection ability of BSMV to *B. distachyon.* For that purpose, pγ – GFP transcript was mixed with pα and pβΔβa transcripts and recombinant virus (BSMV-GFP) was delivered to *B. distachyon* 2nd leaves with the help of wounding agent FES as described in section 2.4.3. Spreading of virus

was monitored under fluorescence microscope (Leica, DFC 280) due to GFP expression.

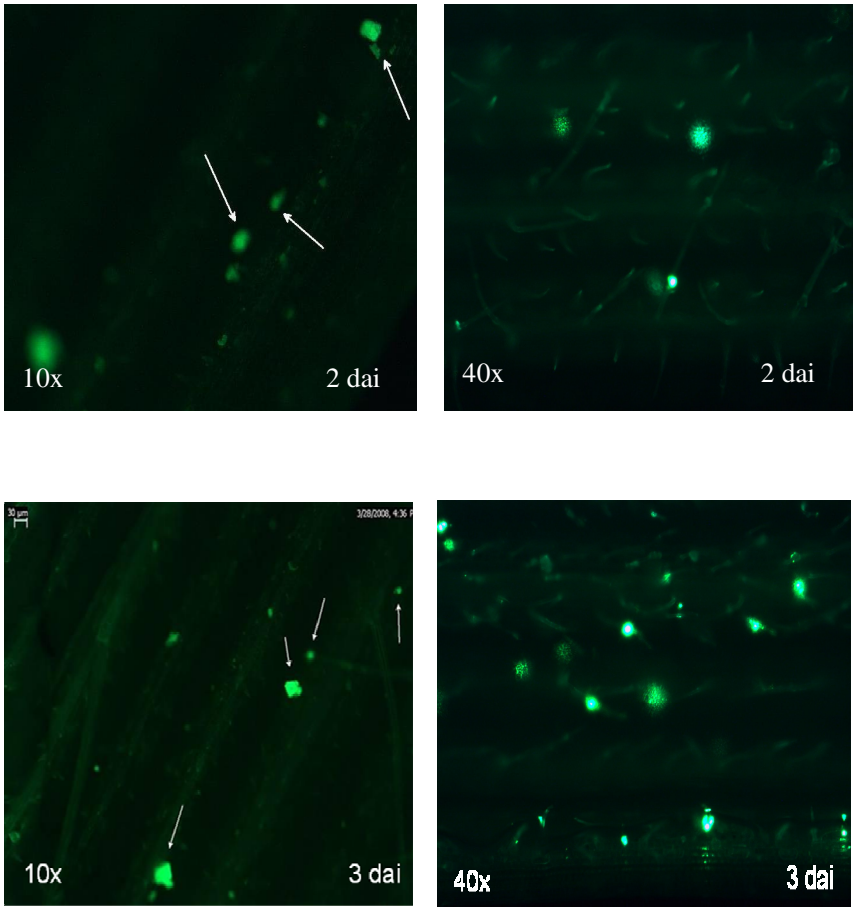


Figure 3.13. GFP expression in *B. distachyon* after infection with GFP gene bearing BSMV. Fluorescence microscope (Leica, DFC 280) was used to monitor GFPs under 10x and 40x magnifications at 2 and 3 dai.

As it is seen in Fig. 3.13 GFP expression was observed with spreading of virus at 2-3 days after inoculation with GFP bearing BSMV. This considered as indication of BSMV infection capability in *B. distachyon.*

3.1.11.2 *B. distachyon* and Barley PDS silencing *via* BSMV

Since GFP expression in *B. distachyon* can be considered as a proof of BSMV infection capability in this organism, BSMV mediated gene silencing in *B. distachyon* through silencing of *Phytoene desaturase* (PDS) gene was carried out as a complementary experiment. In this part of study, pγ-BdPDS$_{(185)}$ sense and antisense vectors including BSMV were transferred to the 2nd leaves of *B. distachyon*. As a positive control, pγ-HvPDS sense and antisense transcripts including viruses were delivered to the barley 2nd leaves. For negative control, BSMV:00 (virus alone) was used for both *B. distachyon* and barley. Effect of PDS gene silencing is used as a phenotypic marker in this study. Color change of leaves from green to white (or formation of white patches on leaves) is the sign of silencing of PDS gene in the plants. Color changes of the leaves for both of plants at 10 days post infection (dpi) were shown in Fig. 3.14.

Figure 3.14. PDS silencing phenotypes on the leaves of *B. distachyon* and barley at 10 dpi. * mark corresponds to BSMV:HvPDSs.

Expected color changes can be observed for barley and *B. distcahyon* using pγ-HvPDS and pγ-BdPDS$_{(185)}$ bearing BSMV, respectively. No color change was observed in both barley and *B. distcahyon* leaves after infection with BSMV:00.

3.1.12 Quantification of PDS gene level *via* qRT-PCR

3.1.12.1 RNA isolation from Barlay and *B. distachyon* leaves

After detection of color changes on the leaves of *B. distachyon* and barley, quantification of PDS gene level decreases by qRT-PCR was performed to show efficiency of BSMV mediated VIGS in *B. distachyon.* For that purpose total RNA of 3rd leaves of both Barley and *B. distachyon* were isolated using procedure described as in the section 2.2.1. The 3rd leaves of plants infected with BSMV:00 or PDS fragment carrying BSMV were isolated and then purified as in the section 2.3.3. To test the quality of RNA before cDNA preparation, RNA samples were separated on 2 % agarose gel (Fig. 3.15a & b).

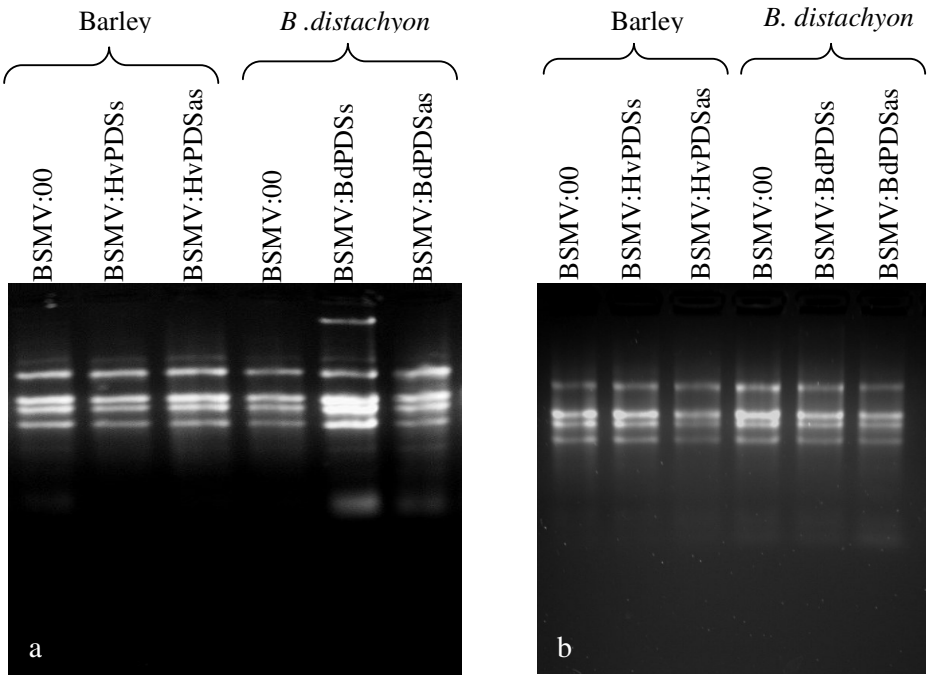


Figure 3.15(a). Isolated RNAs of infected 3rd leaves of Barley and *B. distachyon* with either BSMV:00 or PDS fragment carrying BSMV **(b)** DNase treated and LiCl purified samples. Samples were seperated on 2 % agarose gel by electrophoresis.

As seen in Fig. 3.15 all isolated total RNA samples have four sharp and intact ribosomal RNA bands, that are 23 S rRNA, 18 S rRNA, 16 S rRNA, and 5 S rRNA. In Fig. 3.15a there is an extra band for BSMV:BdPDSs lane that corresponds to DNA and after purification it was eliminated as seen in Fig. 3.15b. The bands appeared intact due to absence of smear in the background, thus they were used in first strand cDNA synthesis.

3.1.12.2 cDNA synthesis

After total RNA isolation of barley and *B. distachyon*, first strand cDNA was synthesized as in section 2.2.4. For testing the quality of the synthesized cDNAs, PCR was conducted using barley 18S rRNA primers (Fig. 3.16).

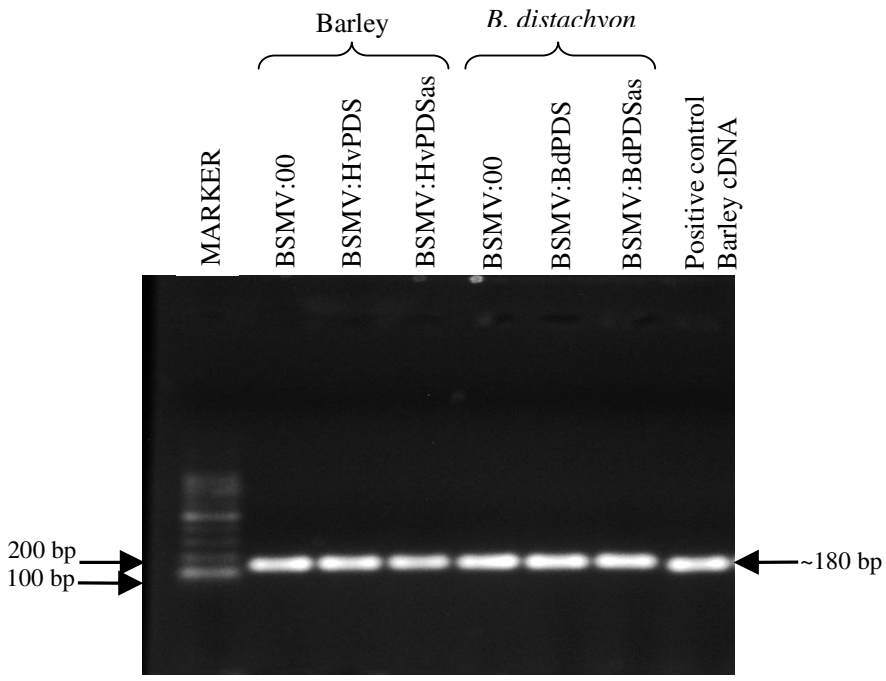
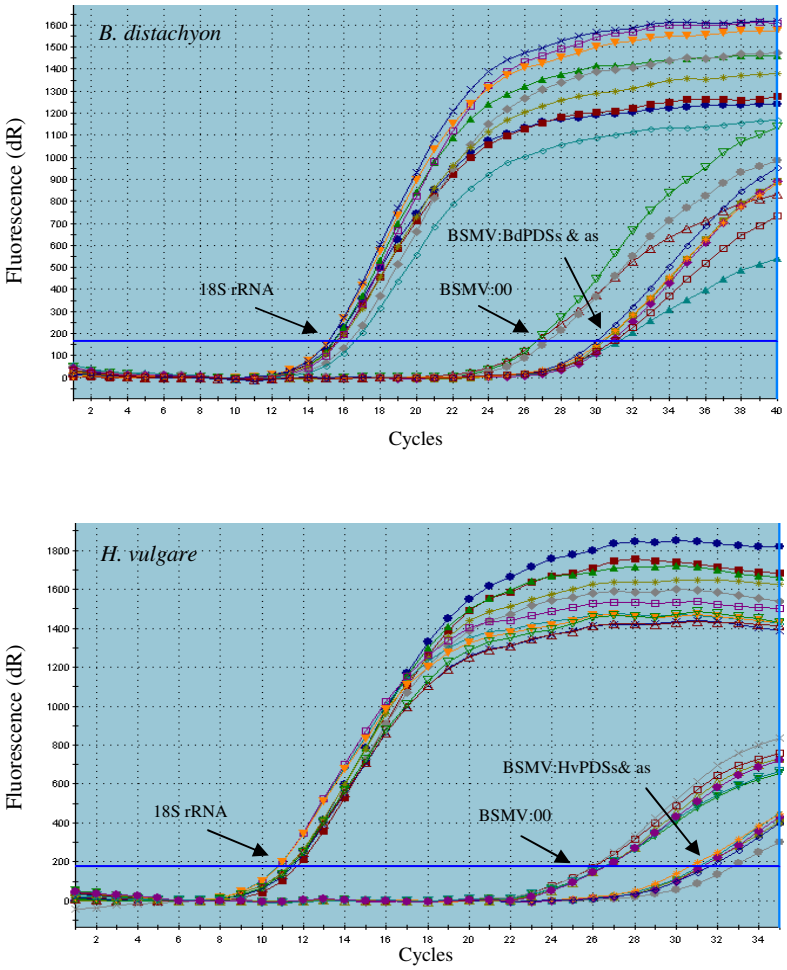


Figure 3.16. Control PCRs using cDNA samples as template and 18S rRNA primers. Amplified 18S rRNA fragments separated on the 1 % agarose gel by electrophoresis. Marker used is 100 bp marker.

Sizes of PCR products on the agarose gel were as expected (~180 bp) based on the designed primers. Thus, quality of checked barley and *B. distachyon* cDNA was high enough to use them in qRT-PCR. A barley cDNA, previously confirmed as a template, was used as positive control.

3.1.12.3 Expression level determination of PDS

qRT-PCR was carried out as a tool for quantification of PDS gene expression level in both infected and control samples. First step in quantification was the normalization of these cDNAs using 18S rRNA primers. 18S rRNA gene is constitutively expressed in plants and used as a suitable internal control for qRT-PCR experiments in plants (Bozkurt et al. 2007). cDNA normalizations were followed by quantification of PDS expression levels in both barley and *B. distachyon* (Fig. 3.17), to be able to perform expression level comparisons.



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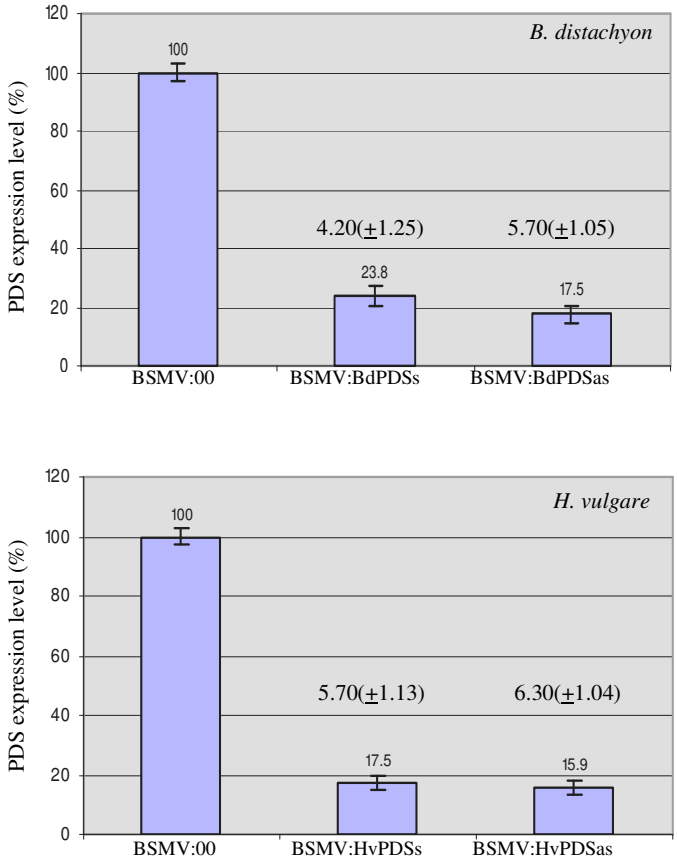

Figure 3.17. Quantification of decrease in PDS gene expression level in *B. distachyon* and *H. vulgare* on the leaves after 10 dpi with the transcripts of BSMV:00 (virus alone), and BSMV transcripts carrying PDS fragments in sense and antisense directions. The fold (X) of silencing levels are also presented in the figure. Normalizations were conducted using 18S rRNA. Three biological samples of each treatment and three qRT-PCR technical repeats were performed (Stratagene Mx3500P).

The decreased expression levels of PDS gene in infected plants were compared with control infections using BSMV:00 (virus alone). In both plants, there were apparent decreases in expression levels of PDS gene, more than four fold for *B. distachyon* and more than five fold for barley (Fig. 3.17).

3.2 DISCUSSION

3.2.1 BSMV mediated virus induced gene silencing in *B. distachyon,* an efficient tool to explore functions of genes in crops

Since *B. distachyon* has emerged as a new model organism for crops (Devos et al. 1999; Tikhonov et al. 1999), researches on this promising organism have been carried out intensively. Studies on grass family to arm them against pathogens need exploring the functions of genes especially with ones that have roles in defense mechanism. However, complex genome organization of barley and wheat makes difficult to dissect the functions of genes in these plants. This problem can be overcome by considering *B. distachyon* as potential gate to understand nature of grass family. BSMV mediated VIGS implementation was done successfully for barley (Holzberg et al. 2002) and wheat (Scofield et al, 2005) and it is an effective and fast method to use it as a reverse genetic tool. Power of this method over creating mutant library is that it doesn't need time and labor to create and screen the libraries and on the other hand it offers quite high efficiency of gene silencing. For *B. distachyon* there is not any reported study about functional analysis *via* VIGS in the literature. In this study, BSMV mediated VIGS was applied in *B. distachyon* by silencing PDS gene of it. To achieve sequence specific gene silencing, *B. distachyon* PDS partial coding sequence was amplified to increase specificity of silencing, rather than using barley PDS fragment.

3.2.2 PDS sequence similarity analysis

Sequenced *B. distachyon* PDS fragments were used in sequence similarity analysis (Fig. 3.5a). $BdPDS_{(185)}$ shared 94 % DNA sequence homology

with HvPDS. A longer partial cDNA clone (FJ913272) $BdPDS_{(546)}$ showed 91 % homology with barley PDS at the nucleotide level. Phylogenetic tree in Fig. 3.5b is based on PDS fragment of different plant species and according to PDS gene homology. *B. distachyon* is evolutionary closest plant to barley and wheat which is consistent with previous data that support close evolutionary relation of barley and *B. distachyon* (Vogel et al. 2006).

3.2.3 GFP expression

GFP was used as a reporter gene in this study to test infection ability of BSMV in *B. distachyon*. Recombinant virus (BSMV-GFP) was delivered to *B. distachyon* leaves and GFP expression pattern was visualized under fluorescent microscope at 2 and 3 dai. As shown in Fig. 3.13 GFP expression was observed in the virus spreaded part of the leaves, meaning that BSMV infected the *B. distachyon* effectively and has ability to spread in this plant. Intensity of GFPs was higher at 3 dai. This data was regarded as a proof of BSMV infection to *B. distachyon*.

3.2.4 PDS silencing

PDS gene product has indispensable roles in carotenoid synthesis (Qin et al., 2007). Carotenoids quench the free radicals in the organism (Bendcih et al. 1994), thus it protects chlorophyll from free electron attack. This photo-protection mode of carotenoids can be inhibited by silencing PDS gene, once this happens leaves loose their shield against photobleaching. Phenotypic effect of silencing of this gene is formation of white colored leaves instead of green ones due to loss of chlorophylls. White patches formation on the leaves and observation of white-green leaves is considered as success of implementation. As shown in Fig. 3.14, expected color change was achieved for the leaves of *B. distachyon* and barley which infected with

recombinant virus that contains PDS fragment in gama genome. Silencing of PDS genes started at the 3rd leaf of the plants and can be observed also in 4th leaves meaning that spreading of virus caused systemic silencing. In order to exclude possible effect of empty virus BSMV:00 in silencing of PDS gene, infection with this virus was performed for both barley and *B. distachyon* as negative control manner. As expected, there wasn't any color change on the leaves infected with empty virus as shown in Fig. 3.14. Therefore, this meant that silencing of PDS gene was due to recombinant virus that contains PDS fragment, not due to virus alone. Another point that is worth mentioning is level differences in white patch formation in barley and *B. distachyon* leaves. Since color change on barley leaves was more drastic than *B. distachyon* leaves, it can be concluded as either efficiency of silencing in barley was higher than in *B. distachyon* or the mode of viral spread may be different in *B. distachyon* than that in barley.

For quantification of expression level changes of PDS gene, qRT-PCR was conducted. In order to prevent the effect of different cDNA levels in reactions, cDNAs normalization was done with 18S rRNA primers to bring all cDNAs to equal concentration. As shown in Fig. 3.17 PDS gene level was decreased slightly higher in barley while comparing with *B. distachyon*. BSMV mediated VIGS in *B. distachyon* provided PDS gene silencing with 4.20-5.70 fold decreases. For barley 5.70-6.30 fold decreases was achieved for PDS gene silencing *via* BSMV mediated VIGS. This result was also consistent with the difference levels of color changes of leaves for these two plants. This is not unacceptable given the fact that barley is the natural host of BSMV. Another significant data obtained from qRT-PCR experiments, silencing level was a little higher for the leaves infected with the virus bearing PDS fragment in antisense direction. Although both directions of inserts brought about efficient silencing, it can be speculated that antisense direction of fragment might cause faster and more efficient silencing due to matching up the target rapidly.

CHAPTER 4

CONCLUSION

Researches on grass family including analysis of gene functions, has been maintaining its importance due to significance of grass family members in the agricultural production for both human food and animal feed. In this aspect, studies on gene functions are invaluable especially for discovering the molecular mechanism of the plant-pathogen interactions. Using new plant model organisms and development of new genetic tools to explore functions of the genes has overcome limitations due to difficulties of manipulation of plants and for grass family *B. distachyon* has been considered as a new model organism.

In this thesis, it was aimed to show that BSMV mediated VIGS implementation can be used efficiently as a reverse genetic tool for loss of function studies to analyze gene functions in *B. distachyon*. Phenotypic changes observed in plants after infection with recombinant virus informed us that silencing of *B. distachyon* PDS gene using BSMV was conducted successfully and this was the first application of VIGS in *B. distachyon*. This study can be considered as a well standing foundation for further researches in *B. distachyon* to understand functions of genes in grass family especially to study genes that have roles in plant-pathogen interactions.

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APPENDIX A

Sequences of BSMV vectors

1) BSMV *α* genome sequence:

GTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCACATAATCGTAATCGATTCTTCTTGATCTCTAAACAA
CACTTTCCCGTTAGCATGGCTAGCGATGAGATTGTCCGCAATCTGATCTCCCGTGAGGAGGTGATGGGTAATTTGA
TTAGCACAGCTTCTAGCTCAGTAAGGTCACCCTTACATGACGTACTGTGCTCGCACGTAAGGACCATCGTCGATTC
CGTGGATAAGAAAGCGGTCAGTCGCAAGCATGTTGATGTACGGCGCAACATCTCCTCTGAAGAGTTACAGATGTTG
ATAAATGCATATCCTGAATATGCCGTTTCATCCTCAGCTTGTGAATCTGGTACTCATAGCATGGCGGCTTGTTTTC
GATTTCTGGAGACAGAATACCTCTTAGATATGGTTCCAATGAAAGAGACTTTTGTTTATGACATTGGTGGTAACTG
GTTTTCTCATATGAAGTTTCGTGCTGATAGAGAAATTCATTGTTGCTGTCCGATCTTATCTATGAGAGATTCTGAA
AGACTGGAAACACGCATGATGGCAATGCAAAAATATATGCGTGGATCGAAAGACAAACCGTTACGCTTGTTAAGCC
GTTATCAAAATATCCTGCGTGAACAAGCGGCGAGAACAACTGCCTTTATGGCAGGTGAGGTGAATGCGGGTGTTCT
CGATGGAGATGTGTTTTGTGAGAACACTTTTCAAGACTGTGTGAGACAGGTGCCCGAAGGTTTTTTGAAGACAGCT
ATAGCAGTTCATAGCATCTACGATATCAAAGTGGAAGAATTTGCGTCTGCATTGAAAAGAAAAGGTATAACACAGG
CTTATGGGTGCTTCCTGTTTCCTCCTGCTGTATTGATAGGTCAGAAGGAAGGTATTTTACCTTCCGTGGACGGTCA
TTACTTGGTGGAGAATGGCAGGATTAAGTTCTTCTTTGCGAATGATCCGAATGCCGGTTACTCTCATGACCTTAAG
GATTATCTGAAGTATGTGGAAAAAACCTACGTGGATATAAAGGATGGAGTGTTTGCTATTGAGCTGATGCAAATGC
GAGGTGATACCATGTTCTTTAAGATCACGGATGTCACCGCAGCAATGTATCATATGAAATACAGAGGTATGAAACG
TGATGAAACATTCAAATGCATTCCGTTGCTAAAAAATTCATCCGTTGTCGTACCTCTATTTTCGTGGGACAACCGT
TCTTTAAAGATCACAAGTGGTTTATTACCACGAACTTTGGTCGAGCAAGGTGCGGCGTTTATTATGAAAAACAAGG
AGAAGGACTTGAACGTTGCTGTGTTGAAGAACTATCTTTCCGCTGTGAACAACTCATACATTTTCAACGGATCCCA
GGTTAGAGATGGTGTGAAAATTGCCCCGGATTTAATCTCCAAATTGGCAGTGACTCTGTACCTGAGAGAAAAGGTC
TATCGACAAAGAGAAAATTCAATCATAAGTTATTTCGAGCAAGAAATGCTTCACGATCCCAACTTGAAAGCCATGT
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GTTTGGCTACGCAGAATTTGACTTGACTACTTTTGATATTTGCGATCCCGTTCTCTACGTAGAGATAGTGGATCGG
TATAAGATCATTCAAAAAGGGCGAATTCCACTTGGTGAGTTTTTTGATTGTCATGAAGAATGCGAGAATTACGAAC
TGCGTGAGAAGGAGAAAAATGACCTAGCGGTGAAAATGGCCCAGAAGGTAACAGGGACGGTGACCGAATGCGAGAA
GGACCTGGGACCTCTTGTTCAACCGATAAAACAGATATTGGTTCAACTTGTGATGCCCAATTTGGTCAGAGCGCTG
TGTAGACCTCGTAGCCCAACGTCTCCTTTGGACTTAAATATCCCAGGGTCAACTCCATCACACTCAAGTTCAGATT
CTGAACAATCTATGACTGAAGAAGCGAGCTGCGCCATTGCGGGTAGCGTACCAACATGGGAAATTGCGACTAAGAA
AGATCTAACCTTTCAGCGAATTGATGAAGATATGTCTCGACGAACTGGTATGCCTCCAAGACCAAAAGTAACTTCT
AGTTACAACATGAATGCCAGAGCTGAGTTTCTCTACTATCAACTGTGTAGCGTGATTTGTGAAAGGGCTCAGATTT
TGAGTGTCATCGAAGACTTTCGTCAGAATTTGATATTCTCAGATAAAGTGGCCGTTCCATTGAACGCTAGATTCTA
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CGGGTGGACCTAGGTCCGCCCCTTACATTGTGGTTGGGACTTCAAGGCATACAAAAACTTTCACTTATTGTAGTGT
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GGAAACCATGAATCTTAACGGATTCTGGAGAGAAAATTTAGGAATTGGTATGTAAGCTACAACTTCCGGTAGCTGC
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2) BSMV β genome sequence:

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
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TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGC
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGC
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CT

3) BSMV γ genome sequence:

CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
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TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGC
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGC
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TTGGGCGCT

4) pγ.BdPDS$_{(185)}$as sequence:

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GTAGACTTAAGTTCGGGAAGACTTTTCGACCTTATGAATTTAAGGAATCACTGAAACCAGTACTGAGGACAGCAAT
GCCAGAAAAACGACAGGGTAGTTTGATTGAAAGTGTGCTGGCCTTTCGTAAAAGAAATTTGGCTGCGCCCAGATTA
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GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTA
TTGGGCGCT

APPENDIX B

Ct values of qRT-PCR experiments

Table 7. Ct values for *B. distachyon* samples

Well	Well Name	Threshold (dR)	Ct (dR)
A1	brachy BSMV00 18s	248,102	16,97
A2	brachy BSMV00 18s	248,102	16,76
A3	brachy BSMV00 18s	248,102	16,84
A4	brachy PDS4 18s	248,102	17,41
A5	brachy PDS4 18s	248,102	17,37
A6	brachy PDS4 18s	248,102	17,43
A7	brachy PDS4as 18s	248,102	16,58
A8	brachy PDS4as 18s	248,102	16,61
A9	brachy PDS4as 18s	248,102	16,57
A10	18s NTC	248,102	32,76
B1	brachy BSMV00 PDS	248,102	30,29
B2	brachy BSMV00 PDS	248,102	30,27
B3	brachy BSMV00 PDS	248,102	30,57
B4	brachy PDS4 PDS	248,102	31,70
B5	brachy PDS4 PDS	248,102	31,77
B6	brachy PDS4 PDS	248,102	31,62
B7	brachy PDS4as PDS	248,102	32,07
B8	brachy PDS4as PDS	248,102	32,13
B9	brachy PDS4as PDS	248,102	31,92
B10	PDS NTC	248,102	33,79

Table 8. Ct values for barley samples

Well	Well Name	Threshold (dR)	Ct (dR)
A1	barley leaf BSMV 18s	173,273	10,73
A2	barley leaf BSMV 18s	173,273	10,74
A3	barley leaf BSMV 18s	173,273	10,76
A4	barley leaf PDS4 18s	173,273	11,26
A5	barley leaf PDS4 18s	173,273	11,29
A6	barley leaf PDS4 18s	173,273	11,23
A7	barley leaf PDS4as 18s	173,273	11,66
A8	barley leaf PDS4as 18s	173,273	11,61
A9	barley leaf PDS4as 18s	173,273	11,64
A1O	18s NTC	173,273	31,51
C1	barley leaf BSMV PDS	173,273	26,02
C2	barley leaf BSMV PDS	173,273	26,47
C3	barley leaf BSMV PDS	173,273	26,12
C4	barley leaf PDS4 PDS	173,273	29,24
C5	barley leaf PDS4 PDS	173,273	29,57
C6	barley leaf PDS4 PDS	173,273	29,51
C7	barley leaf PDS4as PDS	173,273	29,96
C8	barley leaf PDS4as PDS	173,273	29,88
C9	barley leaf PDS4as PDS	173,273	30,02
C10	PDS NTC	173,273	No Ct

APPENDIX C

PDS sequences

Brachypodium distachyon partial PDS (Bd, FJ913272)

GCCTAGTGTAGTCACCAGCTAGATAGAACCCTTCAATCGGTGATCGTTGCAGAGGTCGACAAGGTTCACAATCTGG
GACTGTTTTGTATACAGATCTCGGTGTCTTTACAACATGATATTTAAGAATCTTTGCTTTACTCTGATCAGCAGCA
ATTTCATCAGGAAATAACTTGGCTAGCTCTTGCATAGTTGCTTCAATGATTTCATTGTCACTCCGTCCAATCCATT
CCTCTGCTGGAGCAAAGACCAACTCCAGCATTGAACGGTCTGGATCATAGTACTCCTTGCACGCTACAGACATGTC
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TACAAAGTGCTTCACAGTCNNNCAGGATT

Hordeum vulgare (Hv, AY062039), [barley]

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Triticum aestium (Ta, DQ270236), [wheat]

AGCCCCTCCCCTCGCGACTCCCTCCTCCCTCTTCCCATCCGCCTCGCCGCTCGGTCCATCTCCTCCGCCCTCCTCC
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Zea mays (Zm, NM_001111911), [maize]

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CTCAGAT

Oryza sativa (Os, AF049356), [rice]

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Nicotiana benthamiana (Nb, DQ469932)

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