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Virus Induced Gene Silencing in wheat

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Factors influencing Barley Stripe Mosaic Virus-mediated gene silencing in wheat

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1. Introduction

There is a large demand for improved crop production through enhanced resistance to both biotic and abiotic stresses. Traditional breeding technologies have been effective at providing improved crop varieties but have been limited by several biological and economic factors. Some of these limitations have, in recent times, been overcome by the use of modern recombinant and molecular technologies to directly modify plant genomes. Efficient application of these new technologies, however, requires a more complete understanding of the genetic basis of plant stress resistance and enhanced growth. A new and a promising approach for identifying genes that contributes to desirable crop traits involves directed inactivation of target genes using RNA-based gene silencing, or RNA interference (RNAi). One of the most effective mechanisms for RNAi-based gene inactivation uses a plants natural response to virus infection, virus induced gene silencing (VIGS) (Baulcombe 1996, Ruiz *et al*., 1998).

In the VIGS system, a segment of DNA from the target gene (ranging from 21bp to 1000bp) is placed into a viral genome and the plant host is inoculated. The virus replicates within the host cell, producing double-stranded RNA (dsRNA) molecules that are recognized by the plant's RNAi defense system. Ribonuclease (RNase) III-like enzymes (Dicers) recognize and degrade the viral dsRNA into 19-29 nucleotide small RNA segments. One strand of the small dsRNA molecules is incorporated into a plant encoded RNA induced silencing complex (RISC) leading to the development of a systemic silencing, targeting the invading viral genome and any insert gene segments contained therein.

RNA-based post-transcriptional gene silencing was first discovered in plants as silencing, or 'co-suppression' of chalcone synthase in petunia (Van der Krol *et al*., 1990) and since then, VIGS (Baulcombe, 1996, Ruiz *et al*., 1998) has been developed and become a powerful tool to study gene function. The VIGS system has been widely used in dicotyledonous plants such as *Nicotiana benthemiana*, *Arabidopsis thaliana*, tomato, and potato and

tobacco by employing Tobacco Rattle Virus (TRV), Tobacco Mosaic Virus (TMV) and Potato Virus X (PVX) systems (Ratcliff *et al*., 1997, Ruiz *et al*., 1998, Baulcombe 1999, Dinesh-Kumar *et al*., 2003,).

Recently, Brome Mosaic Virus (BMV) and Barley Stripe Mosaic virus (BSMV) have been adapted as VIGS systems for monocotyledonous plants, especially for rice, barley and wheat (Holzberg *et al*., 2002, Scofield *et al*., 2005, Ding *et al*., 2006, Oikawa *et al*., 2007). BSMV is a rod shaped positive-sense RNA virus with a tripartite genome consisting of α, β, and γ RNA segments. The γ genome of the virus has a significant effect on viral virulence, long distance movement, symptom severity (Lawrance 2001; Petty, 1990a b; Donald, 1994; Brag *et al*., 2002a), and RNA silencing suppression (Yelina, *et al*,. 2002). BSMV was first tested as an expression vector in plant cells (Joshi *et al*., 1990), and subsequently was tested as a system for induction of VIGS in monocotyledonous plants. Genes involved in pathogen resistance of barley and wheat were successfully silenced using BSMV-IGS- based systems (Holzberg *et al*., 2002, Tai *et al*., 2005, Bruun-Rasmussen *et al*., 2007) and pathogen related resistance loci against *Puccinia triticina* (Scofield *et al*., 2005), *Blumeria graminis* f. sp. *hordei* (Hein *et al*., 2005), *Pyrenophora tritici-repentis* (Tai *et al*., 2007) were also identified using BSMV-IGS. In two studies, *RAR1*, *SGT1*, and *HSP90* were silenced in hexaploid wheat and barley plants to explore the genetic components of *Lr21*- and *Mla13*-mediated race specific resistance against *Blumeria graminis* f. sp. *hordei* (Scofield *et al*., 2005 and Hein *et al*., 2005).

Although BSMV-IGS gave efficient silencing, the extension of silencing was restricted to one or two leaves. For example, when inoculating the first leaf of wheat, primarily the third leaves were found to display target gene silencing. Although silencing was observed in other leaves, it was found to be less efficient then that displayed within the third leaf (Scofield *et al*., 2005). In barley, if the second leaf was inoculated by BSMV-IGS, the most extensive silencing was observed within the fifth leaf (Holzberg *et al*., 2002).

The level of silencing during viral infection depends on a balance between the plant defense mechanism and viral encoded suppressors of silencing. For VIGS, the stability of the insert in BSMV-IGS system also plays an important role in maintaining silencing of targeted plant genes. In a recent study, silencing of the endogenous *phytoene desaturase* (*PDS*) gene using a BSMV-IGS system occasionally persisted and was transmitted through the seed (Bruun-Rasmussen *et al*., 2007). However, Bruun-Rasmussen *et al*., (2007) reported that the DNA insert used in this VIGS system could be lost during infection. In many studies of VIGS and viral-based transient over-expression, genetic material introduced into the viral vector systems were found to be unstable (Bruun-Rasmussen *et al*., 2007; Rajamäki *et al*., 2005; Haviv *et al*., 2005). The observed instability of inserts in VIGS systems was found to be markedly impacted by the size and nature of the inserted target sequence (Szittya *et al*., 2003; Rajamäki *et al*., 2005; Bruun-Rasmussen *et al*., 2007; Avesani *et al*., 2007. There is a positive correlation between insert length and insert elimination rate, making shorter inserts more stable (Qu *et al*., 2005).

In the previous studies, silencing of a single gene using a BSMV-IGS system in wheat has been reported (Scofield, *et al*., 2005). However simultaneous silencing of two genes has not been tested in this vector system. If such a goal can be accomplished, one gene could be used as indicator of silencing of the second target gene or two genes in the same pathway could be simultaneously silenced and investigated. Here it was described the use of BSMV-based VIGS system in two different wheat varieties. It has been shown that two different genes can be silenced at the same time, but efficiency was found to be less than that occurring with a single target gene. Therefore, it was investigated the stability of differing target insert sizes using this VIGS system. In addition, viral RNA silencing appears to be affected by altered temperature. For this reason, it was compared gene silencing under three different temperatures.

2. Material and Methods

2.1. Construction of BSMV-Derived Vectors

The plasmids utilized in these experiments are based on the constructs described by Scofield *et al*., (2005).

2.2. Gene silencing constructs

Structure of the BSMV α, β, and γ RNAs and the recombinant γ RNA constructs used in this study were as described (Cakir and Scofield, 2008). *PDS* (BT009315) and *SGT1* (EF546432) genes were used in the silencing experiment (Scofield *et al*., 2005).

2.3. In vitro Transcription of Viral RNAs and Plant Inoculations

Capped *in vitro* transcripts were prepared from three linearized plasmids that contain the tripartite BSMV genome using the mMessage mMachine T7 *in vitro* transcription kit (Ambion, Austin, TX), following the manufacturer's protocol. This *in vitro* transcription reaction typically results in 1 to 1.5 mg/mL final concentration of RNA. Plants were infected with BSMV using a modified protocol (Scofield *et al*., 2005). One microliter of each of the *in vitro* transcription reactions for α, β, and γ RNAs (a 1:1:1 molar ratio or a 1:1:1:1 in the mix inoculation (γ:PDS and γ:SGT1)) were combined and added to 22 or 21 mL of FES buffer (Pogue *et al*., 1998). This mixture was then applied to plants by rub inoculation.

2.4. Plant Materials

In the experiments, two different wheat varieties, *Triticum aestivum*, were used; a winter wheat variety, Clark and common wheat cv Bobwhite, WI. Three seeds were sown in a 4-inch pot containing potting soil and placed in a Conviron PGR15 growth chamber (Winnipeg, Canada) equipped with high-intensity discharge lamps, with light intensity ranging between 450 and 700 mmol m22 s21, programmed for 16-h days with 22 °C during the day and 20

°C at night adjusted to the constant 22 °C with supplemental light to provide day lengths 16 h and watered as needed. Two genes silencing experiment were repeated four times; first two experiments were carried out in greenhouse condition using 6 plants in each experiment and the last two experiments were carried out in the growth chamber using 9 plants in each experiment. In the temperature experiment, three different constant temperatures were used; 18, 22 and 26 °C and repeated twice using 9 plants in each experiment. Phenotype for silencing the *PDS* gene was observed and recorded in the plants based on the relative photobleaching ratio that was calculated by dividing approximately photobleached area by whole leaf area and then multiplied by100 for percentage ratio.

2.5. Total and small RNA Extraction and cDNA Synthesis

Total RNA from the mock (water) and BSMV-infected plants was isolated using the Trizol reagent (Sigma-Aldrich, Milwaukee, WI) as described by the manufacturer. All RNA samples were digested with DNase I prior to synthesizing cDNA, using the TURBO DNA-Free kit as recommended by the manufacturer (Ambion). First-strand cDNA was synthesized using the I-SCRIPT kit (Bio-Rad, Hercules, CA), following the manufacturer's protocol. For the smRNA extraction, the same protocol for total RNA above was followed, but to enrich the smRNAs, total RNA was precipitated with ethanol instead of isopropanol detailed in manufacturer's protocols after storing at-80ºC for overnight.

2.6. Measurements of mRNAs by QRT-PCR

Expression of the genes targeted for silencing was quantified by comparative QRT-PCR. The measurements were performed in a Stratagene MX3000P QRT-PCR machine using the I-TAQ SYBR reagent kit (Bio-Rad). The third leaves of each treated plants were harvested 10 days after infection, unless described otherwise. Leaves from each treatment were individually evaluated depending on the experiments and total RNA was prepared as described above. QRT-PCR was performed in quadruplicate for each RNA

sample/primer combination. The amounts of RNA in each reaction were normalized using primers specific for GAPDH. The primer sequences used to detect each gene were as follows: GAPDH forward, TGG CAT TGT TGA GGG TTT GAT; GAPDH reverse, CAG TGC TGC TTG GAA TGA TGT T; *PDS* forward, TGT CTT TAG CGT GCA AG; *PDS* reverse, GAT GAT TTC GGT GTC ACT; *SGT1* forward, CAA GCT GGG CAG TTA C; and *SGT1* reverse, GCT TTA TGC ATC GAA GGA. In all cases, expression of the targeted gene is presented as the expression level in the silenced plant relative to expression of the same gene in plants infected with γ:00.

2.7. RNA Gel Blotting, Probe and Hybridization

Total RNAs (20 µg) extracted from samples were run on the polyacrylamide gel (7M urea/15% PAGE gel; 1.5 mm thickness, 16 cm length) for the small RNAs' accumulation of virus (γa probe 178 bp) and endogenous genes (*PDS* and *SGT1* probe, 178 bp). Then, they were transferred onto nylon membrane (0.45 µl Nytran SPC, Waltman, Cat# 10416296) and hybridized with PCR products amplified with γa primers (forward, GAAATTCGCCGTCATGTC and reverse, GCTCTCAGAACCACCTGGG). The 25 ng PCR products cleaned from gel were used for hybridization reaction using DECAprime II labeling kit (Ambion, cat # AM1455) and labeled with [α-^{32}P]ATP (Perkin, Elmer), then unincorporated ^{32}P-label was removed using Sephadex G-50. Probe was heated at 95 °C for 10 min and then cooled in ice for 2 min. The membrane was pre-hybridized with Perfect Hyb Plus Hybridization Buffer (Sigma, Cat#H7033). The labeled probe was added into reaction and hybridized at 37°C for overnight. Then, it was washed 2xSSC once and exposed for the plate of phosphoimaging. PCR product of 5S rRNA was hybridized as loading control using primers as follows: forward, ATG CGA TCA TAC CAG CAC TAA AG and reverse, GGG AAT GCA ACA CGA GGA CTT C. Micro RNA maker (17, 21 and 25nt; New England Biolabs Inc.) was hybridized to determine the size of viral and endogenous smRNA with probe (A 21-mer DNA oligonucleotide

complementary to the marker sequences supplied from same company) end labeled by [γ-^{32}P]ATP according to manufacturer's protocols.

2.8. Determination of γ viral RNAs and fragment stability of BSMV-IGS system in the infected plants

Total RNAs (5 µg) extracted from samples were run under denaturing condition in agarose formaldehyde gel (1.2% agarose, 10X MOPS (10X MOPS: 0.4 M 4-morpholinopropanesulfonic acid; 0.1 M Na-acetate and pH adjusted to 7.2 with NaOH) and 0.66 M formaldehyde) for determining γ viral RNA in the infected plants during infection. Then the RNA was transferred onto nylon membrane and was hybridized with γa probe, *PDS*, *SGT1* and 5S rRNA probes.

Determining the lost fragment in the infected plants by; **Hybridization:** To determine the lost fragment in the infected plant during infection, first the RNA samples were run under denaturing condition in agarose formaldehyde gel mentioned above and were transferred onto nylon membrane. Then, the membrane hybridized with γa probe, *PDS*, *SGT1* and 5S rRNA probes to observe the different size of viral γ RNAs. **PCR:** cDNAs were used as PCR template to amplify the outside region of cloning viral γgenome with two primers: For: TGA TGA TTC TTC TTC CGT TGC and Rev: TGG TTT CCA ATT CAG GCA TCG. Then, PCR samples were run on the agorese gel (2%) and observed under the UV light after staining ethidium bromide. Furthermore, after observing the UV light, PCR products were transferred onto nylon membrane by using semi dry blotting system and hybridized with the probe of the same PCR products amplified from empty γ genome (γ:00) to get clear signal and to eliminate the false signal. **Sequencing:** The cloning regions of γ genome γb:PDS-infected Bobwhite and Clark wheat plants were amplified by PCR with the same primers above and were run in agarose gel (1%). Then, the band around 20 bp were removed from agrose gel and cloned into PGEM vectors. The selected 10 colonies were sequenced and checked for *PDS* insert in the cloning area of genome.

3. Results:

3.1. Silencing SGT1 and PDS concurrently using BSMV-IGS

In our preliminary assessment, we reported that BSMV-IGS could be used to silence two genes simultaneously (Cakir and Scofield, 2008). To analyze this in detail, the growth chamber-grown Bobwhite wheat cultivar was inoculated with a 1:1:1 mixture of *in vitro* transcripts synthesized from plasmids containing the wild-type BSMV ND18 α, β, and γ RNAs and derivatives of the γ RNA (Table 1) that carried either no insert (γ:00) or fragment of wheat *PDS* (γ:PDS) and *SGT* (γ:SGT) individually (Scofield, *et al*., 2005) or both together (γ:PDS+SGT and γ:SGT+PDS) and/or mixture (γ:PDS+ γ:SGT). We choose SGT1, a key regulatoryprotein in centromere function, ubiquitin-mediated proteolysis and disease resistance conferred by many Resistance (R) proteins, as a positive control. Then, silencing of the *PDS* gene was visually examined as photobleaching phenotype in the third leaves 21 days after inoculation (Fig.1). The plants inoculated with a construct carrying both target showed photobleaching phenotype less frequently (35%) than a construct containing only *PDS* (66%) or a mixture of constructs (57%; Fig. 1A and B). These results indicate that although silencing of two genes with BSMV constructs is possible, it should be noted that photobleaching was less with simultaneous gene silencing than that silencing focused on a single *PDS* gene. Interestingly, the position of the inserted two genes within the viral construct also has had some effect on photobleaching.

In order to better understand the basis of the observed photobleaching phenotypes, quantitative RT-PCR was carried out to determine the expression levels of *PDS* and *SGT1* in the silenced plants. All experiments that were carried out under the greenhouse or the growth chamber condition support the observation that carried either no insert on that simultaneous silencing of two genes was possible but was less efficient than silencing a

single gene (Fig. 2 and Table 2). Interestingly, the two gene constructs, γ:PDS+SGT1, silenced both *PDS* and *SGT1* more consistently than a construct containing the two gene fragments in the opposite order (γ:SGT1+PDS). Also, mixed inoculation using single gene constructs (γ:PDS and γ:SGT1) showed better silencing than when both gene fragments were contained in the same constructs. The mixed inoculations did not, however, show consistency in silencing both genes, displaying a large variation between the experiments. In the mixed inoculation, one target gene was generally silenced more efficiently than other (Fig. 2 and Table 2).

3.2. Gene silencing generates small RNAs

A definitive product of any RNAi-based silencing is the smRNAs that are generated often as a result of some of gene regulator and the natural defense mechanisms against any foreign genetic materials like viroids, viruses, and transposable elements (Mlotshwa *et al*., 2002 and 2008). Any dsRNAs present within the cell cytoplasm are targeted by dicer and generally end up 21-29 nt long smRNAs. To determine the quantity and size of any smRNAs generated during the BSMV-IGS, total RNA samples were transferred onto nylon membrane and were hybridized with 3 different probes (γ*a* region of BSMV vector, *PDS* and *SGT1*). All the hybridizations indicated that 22 nt smRNAs were the main products of silenced viral and endogenous genes, *PDS* and *SGT1* (Fig. 3) based on the micro (mi)RNA marker (New England Biolabs Inc.). Interestingly, one gene containing constructs (γ:PDS and γ:SGT1) generated more smRNAs against *PDS* and *SGT1* genes than the two genes containing constructs (γ:PDS+SGT1 and γ:SGT1+PDS). The correlation between smRNA accumulation and the expression level of endogenous genes (*PDS* and *SGT1*) were consistent with constructs, as found in the quantitative PCR studies. When the two target genes contained within a single viral construct, silencing is reduced and less smRNAs were generated compared to each gene individually inserted into separate viral vectors (Fig. 3).

3.3. Temperature has an effect on BSMV-IGS

In previous studies with wheat cv. Clark, the photobleaching phenotype on *PDS* silenced wheat plants had been extended to the forth leaf and tilling leaves when the plants were grown at a single lower controlled temperature. It was not clear whether the observed extension of bleaching was due to the controlled temperature or specific to plant varieties used. For this reason, it was tested the affect of three different temperatures (18, 22 and 26 °C) on the efficiency of gene silencing using a BSMV-IGS system with two different wheat varieties (cvs. Bobwhite, and Clark).

When plants were scored for photobleaching, those grown at 22 °C showed more bleaching in the third leaves (Fig. 4A and 4B1). The cv. Bobwhite also developed more photobleaching symptoms at this temperature than cv. Clark. Interestingly, more photobleaching in the second leaf of both cultivars was observed at a very earlier stage (4 days after inoculation) of plant development when they were kept at the higher temperatures, especially at 26 °C. The appearance of the photobleaching in upper leaves of the viral infected plant was reduced at higher temperatures and enhanced at lower temperatures (Fig. 4B1, C1 and D1). Also the bleaching extended further in the forth leaves and the tilling leaves of plants treated at the lower temperatures (Supplementary Table S1, 2 and 3).

Quantitative RT-PCR was used to investigate the expression levels of *PDS* on both wheat varieties infected with the BSMV-IGS system at the three different temperatures. Although the *PDS* gene silencing on the individual wheat plants was mainly observed on the third leaves, silencing in the second and forth leaves was also evaluated. The best *PDS* silencing in the third leaves was observed at low temperatures (18 °C and 22 °C for cv. Bobwhite, and 22 °C for cv. Clark, Fig. 4 B2). For the second and forth leaves, the best silencing was observed at 22 °C for both cultivars (Fig. 4 C2 and D2) (Supplementary Table S1, 2 and 3). At the higher temperatures, photobleaching was generally seen a short time after inoculation however,

the photobleaching was more extensive in the forth and tilling leaves at the lower temperature (data not shown).

To confirm PCR analysis and to further evaluate the effect of temperature on gene silencing, it was analyzed the generation of smRNAs. Total RNA samples were hybridized with *PDS* and γa probes to detect smRNAs targeting these sequences. Using the *PDS* probe, 22 nt smRNAs were detected in samples from all leaves and at all temperatures tested in both cultivars. However, the best correlation between abundance of small RNA and degree of silencing was found mainly to be in third leaf samples grown at low temperature (Fig. 5A). Small RNAs generated from the endogenous *PDS* gene were generally found to be more abundant at the low temperatures especially in the second leaves (Fig. 5B).

3.4. Fragment size affects insert stability in the recombinant virus

Since simultaneous silencing of two genes by using BSMV-IGS system was possible, but the efficiency of silencing was less than that by a single gene, one possibility could be that the plant gene fragment was unstable or lost form the viral genome during the infection. To check the stability of the plant gene fragments inside the BSMV vector, the fragment outside the cloning region from cDNA template was amplified by using two different primers (Gamma Forward and Reverse) and PCR products were separated on an agarose gel. Two genes constructs should give a 532 bp fragments, however, no fragment in this size was observed. After hybridization with a γ probe from the empty viral vector, a weak signal of 532 bp fragment was detected, but only from the γ:PDS+SGT1 construct (Fig. 6A). The other construct, γ:SGT1+PDS did not show any band at the predicted size, but band were observed between 200 and 300 bp indicating a partial fragment loss in this viral construct. Surprisingly, we also detected loss of insert fragment in recombinant virus carrying only one gene. However, they appeared more stable than constructs containing either two genes or longer inserts. Agarose gel stained with ethidium bromide and associated Southern

blots showed the main PCR band obtained from the different constructs was around 200 bp, close to the predicted empty vector size (182 bp). These results clearly showed that the larger DNA inserts within the BSMV vector were not stable and all the constructs lost some or all of their inserted DNA during infection.

To understand the nature of DNA loss within the viral constructs during infection, cDNA from γ:PDS infected plants was amplified using γ primers and PCR products were separated on % 2 agarose gels. Southern hybridization using probe against the viral γ sequence showed that the viral RNA lost most of their inserted fragments, although some retained their intact plant gene fragment (Fig. 6B and 6C). It was further verified these results by sequencing the PCR products generated from cvs. Bobwhite and Clark. There was no clear correlation between the loss of the plant gene sequence and the level of silencing of the targeted gene. Interestingly, the size and amount of the lost fragments was changed from plant to plant.

4. Discussion

Silencing of two genes at the same time has certain advantages in pathway analysis and in understanding the interaction between genes. These results show that simultaneous gene silencing is possible with the BSMV-IGS system. Many viral vectors including TRV, PVX, BMV (Brome Mosaic Virus) and BSMV were successfully used to silence genes in dicotyledonous and monocotyledonous plants such as tomato, potato, *Nicotiana benthamiana*, pepper, tobacco, *Arabidopsis*, rice, oat, barley and wheat (Liu *et al*., 2002; Faivre-Rampant *et al*., 2004; Ruiz *et al*., 1998; Chung *et al*., 2004; Valentine et al 2004; Turnage *et al*., 2002; Ding *et al*., 2006; Holzberg *et al*., 2002; Scofield *et al*., 2005). Simultaneous silencing of two genes has been shown in some other viral systems (Ding *et al*., 2006; Chen *et al*., 2004). Here, it was attempted to determine whether BSMV-IGS system could also be employed to silence two genes simultaneously. For this reason, it was

placed two different genes in γ (gamma) vector as antisense in two different orders. All results were compared against single gene silencing and silencing induced by a mixture of two single gene-containing constructs. All experiments showed that simultaneous silencing of two genes was possible, but the efficiency of gene silencing was found to be less than that induced by a single gene. Turnage *et al*., (2002) silenced multiple genes using the Cabbage Leaf Curl Virus (CbLCV) in *Arabidopsis* and reported two different responses. These results indicated that the generation of silencing for the two genes occurred independently or that the initiation of silencing of the *PDS* gene was variable. The mixture of two single gene-containing constructs was also effective at silencing both targets, but silencing of a single gene remained more effective (Jiang *et al*., 2008).

One drawback of simultaneous gene silencing using BSM-IGS could be that it does not produce a uniform silencing in the whole plant. Single gene silencing appears on the second and mainly on the third leaves. The different silencing phenotypes in two different varieties of wheat were also observed. Bobwhite gave more silencing than Clark as measured in their third leaves. Also the temperature plays a role in the silencing and the most effective was detected in the third leaves at lower temperatures (18 and 22 °C) (with the bleaching phenotype extending to the forth leaf). At 26 °C, the second leaf showed more silencing than later leaves and the bleaching phenotype appeared very early (4^{th} day). Jiang *et al*., (2008) reported that silencing was more effective when plants were grown at 22 °C and suggested that movement and/or replication of the virus is affected by relatively small temperature changes. The data is in agreement with others (Fu *et al*., 2006) where silencing at low temperatures was found to be more efficient than higher temperatures. Fu *et al*., (2006) reported that a silencing-induced bleaching phenotype was delayed but enhanced 8 weeks after inoculation at lower temperature in tomato. It is known that at higher temperatures, many viruses become latent (Szittya *et al*., 2002 and 2003; Bruun-Rasmussen *et al*., 2007). Szittya *et al*., (2002 and 2003) showed in their study, that low temperature inhibits smRNAs, compared to higher

temperatures. In the study, at high temperature, the second but not the third leaf showed an early and more intense bleaching phenotype. Although there were smRNAs accumulation at all temperatures used, any clear correlation between the accumulation of smRNAs and the strength of the silencing could not been found. However, the amount of small RNAs generated from endogenous *PDS* gradually decreased in second leaves at the higher temperature compared with that obtained at the lower temperature (Fig. 5B). This is in agreement with the findings reported by others (Szittya *et al*., 2002 and 2003 and Chellappan *et al*., 2005). Although Chellappan *et al*., (2005) reported that the temperature has a major effect on virus derived siRNA generation especially at higher temperature (30 °C), it was also found that the small RNA accumulation in *PDS* silencing increased only in the second leaf samples at low temperature (18 °C, Fig. 5B). The observed different experimental outcomes may result different virus type, Cassava geminiviruses, and the temperature (30 °C) used or may reflect different smRNA pathways within the different plant (Deleris *et al*., 2006).

One of the problems in VIGS is the stability of the insert in the viral genomes during infection. The PCR amplification of the viral sequences flanking the plant gene insert followed by hybridization or sequence analyses confirmed the instability of the insert in the viral genome. It was found that the larger insert, especially in the two gene-containing constructs, was lost easily, conforming results reported by others (Avesani *et al*., 2007). In the expression study using BSMV vector system, Lawrance and Jackson (2001) showed that BSMV constructs lost GFP and GUS inserts in during their protoplasts-based expression studies. Bruun-Rasmussen *et al*., (2007) used fragments of *PDS* ranging from 128 to 584 nucleotides in BSMV, and reported that the insert length influenced stability, but not efficiency of VIGS.

In many studies, virus targeting smRNAs have been found to be generally 22nt size. In this study, not only 22nt small RNAs have been identified as

the final products of silencing targeting BSMV RNA, but also for siRNAs targeting the endogenous plant genes.

The efficiency of VIGS systems depends primarily upon the plant defense mechanisms that respond to any exogenous genetic materials inside the cytoplasm and viral suppressors that inhibit the silencing. Ultimately the interaction between the viral pathogen and the host plant determines the gene silencing efficiency. However, here it was showed that the temperature, and insert stability have a significant effect on this interaction.

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Figures

Fig. 1. Gene silencing in wheat using BSMV system. A) Cultivar Bobwhite was infected with *in vitro* transcribed RNAs of BSMV in which genomic RNA were engineered to carry different gene fragments from wheat genome to silence simultaneously or as a single gene in antisense orientation. B) Ratio (%) of photobleaching in *PDS*-silenced wheat cv Bobwhite in the third leaves using BSMV-IGS system

*Relative Bleaching Ratio was calculated by dividing approximately photo-bleached area to whole leaf area and then multiplied by 100.

Fig. 2. Relative gene expression of the silenced *PDS* and *SGT1* genes in the third leaves suppressed by BSMV-IGS system.

Fig. 3. Northern blot analysis of the small RNAs with γa orf region of BSMV vector, *PDS* and *SGT1* probes. Degradation of different recombinant BSMV constructs into the small RNA is shown as the result of silencing during BSMV infection and small RNA samples on polyacrylamide gel (15%) stained with ethidium bromide.

Fig. 4. The effect of different temperature on photobleaching and the relative expression level of *PDS* on two wheat varieties, Bobwhite and Clark after using BSMV **A)** Photo-bleaching phenotypes on third leaves, **B1)** The photo-bleaching percentages on third leaves, **B2)** The relative expression ratio on third leaves, **C1)** The photo-bleaching percentages on the second leaves, **C2)** The relative expression ratio on second leaves, **D1)** The photobleaching

percentages on forth leaves, **D2)** The relative expression ratio on forth leaves.

Fig. 5. Northern blot analysis of small RNAs with γa orf region of BSMV vector and *PDS* probes. The degradation of different recombinant BSMV constructs into small RNAs as a result of silencing during BSMV infection was investigated under different temperatures. **A)** Accumulation of small RNAs and relative *PDS* gene expression values in third leaves, **B)** accumulation of small RNAs and relative *PDS* gene expression values in second and fourth leaves.

Fig. 6. PCR and virtual Northern analysis to determine fragment loss in BSMV carrying inserts of plant genes. The same PCR amplification regions from empty γ genome of BSMV were used as a probe in Northern blotting. **A)** Two gene experiment results, **B)** The lost fragment confirming in PDS carrying BSMV constructs' results on third leaf individually, **C)** The lost fragment confirming in PDS carrying BSMV constructs' results on second and forth leaves.