Research article

A NEW BSMV-BASED VECTOR WITH MODIFIED β MOLECULE ALLOWS SIMULTANEOUS AND STABLE SILENCING OF TWO GENES

ADAM KAWALEK, MARTA DMOCHOWSKA-BOGUTA,
ANNA NADOLSKA-ORCZYK and WACLAW ORCZYK*
Plant Breeding and Acclimatization Institute – National Research Institute,
05-870 Błonie, Poland

Abstract: Virus-induced gene silencing is an important tool for functional gene analysis and the vector based on *Barley stripe mosaic virus* (BSMV) is widely used for the purpose in monocots. Of the tripartite BSMV genome, currently the BSMV:γMCS molecule is used to clone a fragment of a target gene. As an alternative, the BSMV:β molecule was engineered with a unique *Bam*HI site between the open reading frame of βc (ORF βc) and poly(A). The mixture of RNA particles α, β*Bam*HI and γMCS was fully infectious. Barley phytoene desaturase and wheat phospholipase Dα fragments were cloned to β*Bam*HI and γMCS. Delivery of the target gene fragment in γMCS induced stronger silencing, while delivery in β*Bam*HI yielded more stable transcript reduction. A quantitative analysis (qRT-PCR) of the transcripts showed that the silencing induced with a fragment carried in both particles was stronger and more stable than that from a fragment placed in one particle. The modification of β enables simultaneous silencing of two genes. Quantifying the β and γ particles in virus-inoculated plants revealed a 2.5-fold higher level of γ than β, while the stability of the insert was higher in β compared with γ. The possible influence of the relative quantity of β and γ particles in virus-inoculated plants on insert stability and gene silencing efficiency is discussed.

Key words: BSMV, Cereals, Functional analysis, Phospholipase D, Phytoene desaturase, Silencing, Wheat, Vector stability, VIGS

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* Author for correspondence. e-mail: w.orczyk@ihar.edu.pl, tel.: +4822 733 4621, fax: +4822 725 4714

INTRODUCTION

The performance of large sequencing projects and the increasing number of genes known only from their nucleotide sequence has created a growing demand for experimental methods for the verification of a gene's function. Transient gene silencing induced with virus-based vectors offers a relatively quick and straightforward method for assessing the function of a given gene. There are several vector systems of virus-induced gene silencing (VIGS). The systems designed for use in *Nicotiana benthamiana* include vectors based on *Tobacco mosaic virus* [1], *Potato virus X* [2] and *Tobacco rattle virus* [3, 4]. Grasses are more recalcitrant for this method of gene silencing. Out of the several VIGS vectors elaborated for these plants, the system based on *Brome mosaic virus* (BMV) has been relatively seldom used [5-7] while the system built on *Barley stripe mosaic virus* (BSMV) has been applied most often. BSMV has been used for virus-induced gene silencing in barley [8-12], wheat [13-15] and *Brachypodium distachyon* [12, 16]. The complete list of other viruses adapted for VIGS and used in grasses was presented and discussed in a recent review [17]. The genome of BSMV is composed of three positive-strand RNA molecules called α, β and γ. Their cDNAs were used to construct BSMV-based vectors [18] from which infectious RNA could be generated by *in vitro* transcription. In all BSMV-based silencing systems, only the γ molecule was modified to accept the fragment of the analyzed gene. Holzberg *et al.* [8] modified the γ molecule by inserting the unique *Pac*I and *Not*I cloning sites downstream of the STOP codon of the γb open reading frame. This system was the first one reported for gene silencing in barley. Constructs based on the original Holzberg vector were further used for gene silencing in barley [9, 11] and wheat [13]. A similar approach to constructing the BSMV vector was presented by Bruun-Rasmussen *et al.* [19], who engineered a cloning site in the γ molecule downstream of the γb region. Another strategy was applied by Tai *et al.* [14]. They cloned the silencing fragment in front of the γb coding sequence, which prevented the expression of the γb gene.

A consistent analysis of gene function requires efficient and relatively stable silencing. Virus-induced silencing does not fulfill these requirements well. It is generally transient and incomplete. Many results indicate that these drawbacks are the consequence of vector instability, which leads to the elimination of the insert. Avesani *et al.* [20] found that the stability of an insert cloned to the *Potato virus X* vector negatively correlated with the insert size and the number of virus passages. Zhong *et al.* [21] revealed that *GFP* (a gene of bacterial origin) was more stable in the *Tomato bushy stunt virus* than a DNA fragment derived from a plant. There are only a few published papers where the problem of insert stability in a BSMV-based vector was addressed by systematic investigations of various factors. Bruun-Rasmussen *et al.* [19] found that the size of the insert ranging from 128 to 584 bp did not affect the efficiency of silencing, but the largest insert (584 bp) was less stably retained in the vector.

Fourteen days after inoculation the shortest insert (128 bp) was retained intact while most of the 584 bp insert was lost from almost all of the inoculated plants. These observations indicated that the transient nature of the virus-induced silencing could be the result of insert elimination. This remained in agreement with the observation that the fraction of plants showing long-lasting silencing always contained a small portion of the insert. So far the effect of foreign fragment localization in the viral genome on silencing stability has not been tested. In this work, the β molecule of the BSMV-based vector was modified to facilitate the cloning of a plant gene fragment. It was subsequently used as a component of the BSMV-based vector to test the efficiency and the stability of silencing. Moreover, the possibility of parallel silencing of two genes with this system was assessed. The fragments of two wheat genes, i.e. phytoene desaturase (PDS), previously used as a model gene to study silencing efficiency in monocots [13, 19], and phospholipase D (PLD), were cloned into β and γ BSMV molecules. Wheat plants inoculated with infectious RNA containing different combinations of tested fragments in BSMV β and γ were assessed for intensity of silenced phenotype. The silencing with a gene fragment cloned in both β and γ was more stable than the silencing induced by a single fragment placed in either β or γ. The quantitative analysis of the β and γ molecules in the leaves inoculated with BSMV indicated that they were not equimolar. The possible dependence of the insert stability and the silencing efficiency on the β to γ ratio in the virus-inoculated plants is discussed. The modification of the BSMV β molecule combined with the earlier adopted γMCS [19] facilitates simultaneous and efficient silencing of two genes in a single VIGS experiment. The presented results indicate novel factors important in virus-induced gene silencing.

MATERIAL AND METHODS

Plant material
Wheat (*Triticum aestivum* L) cultivar Thatcher was germinated in pots (diameter 7.5 cm) filled with Kronen peat substrate (Lasland, Karnice, Poland) mixed with sand in a 10:3 v/v ratio. The seedlings were cultivated in a growth chamber with a 16 h photoperiod, at 22ºC in the day and 20ºC at night. The relative humidity was in the range 70-100%, and the light intensity was 130-230 $\mu M \cdot s^{-1} m^{-2}$. The first leaves of 7-day old seedlings were used for the BSMV inoculation.

Plasmids
The plasmids used in this study were donated by Dr Merete Albrechtsen (Aarhus University, Denmark). The plasmids contained the full-length cDNA of the α, β and γ molecules of BSMV with the promoter of T7 polymerase [18, 19]. The plasmid pBSMV-T7-γMCS contained a multiple cloning site (MCS) with unique *Pac*I, *Xma*I, *Bam*HI, enabling cloning of DNA fragments upstream of open reading frame γb. The plasmid pBSMV-T7-γ*PDS*$_{275}$ contained a 275-bp

fragment of barley phytoene desaturase, accession number AY062039 [19]. An empty γMCS molecule is described as γ(-).

Construction of the pT7-βBamHI plasmid

The β molecule in the pBSMV-T7-β plasmid was modified by the insertion of a DNA fragment containing a STOP codon and a *Bam*HI restriction site between ORF βc and poly(A). The construction started with two amplification reactions using unmodified pBSMV-T7-β plasmid as a template. The first reaction amplified the fragment from a unique *Sac*I site to the end of the open reading frame of βc (ORF βc) with the following primers: B_*Sac*I_Forward (GGAAATTCGTCAAGCATTCCTG) and B_*Bam*HI_Reverse (GTA GGATCCTTACCTTTTTGAAGAAAGTAAG). The second reaction amplified the fragment from the end of ORF βc to the unique site of *Spe*I with the primers B_*Bam*HI_Forward (CTGGGATCCTAAAAAAAAAAAAAAAAAAAAATGT TTG) and B_*Spe*I_Reverse (TATGACCATGATTACGCCA AGC). The primers B_*Bam*HI_Forward and B_*Bam*HI_Reverse contained the 9 nt fragment with the STOP codon and *Bam*HI site, allowing further religation. Amplification products were digested with *Sac*I and *Bam*HI, or *Bam*HI and *Spe*I, respectively, and gel purified. Both fragments and the 5087 bp *Sac*I-*Spe*I fragment of the pT7-β plasmid were combined in a three-component ligation reaction. The obtained plasmid pT7-β*Bam*HI contains the unique *Bam*HI site which can be used for cloning of the fragment derived from an analyzed plant gene. The modified β(*Bam*HI) molecule is described as β(-).

Cloning of a fragment of wheat phospholipase Dα (PLD)

The wheat phospholipase D (BT009262) fragment was amplified with the primers F3_132_F (ATGGGATCCACCACTCCTTGTTTAGG) and F3_132_R (TTAGGATCCGGGAAGCCAAAAGCAGC) using wheat cDNA as a template. The *Bam*HI recognition sequence is underlined. After digestion with *Bam*HI and purification, the 325-bp fragment was ligated to the *Bam*HI site of pBSMV-T7-β*Bam*HI and pBSMV-T7-γMCS.

Tab. 1. List of the plasmids containing cDNA from BSMV components, the restriction enzyme used for plasmid linearization prior to the *in vitro* reaction, and the list of the obtained transcripts.

Plasmid	Plasmid linearization	Transcript (fragment of the BSMV genome)
pBSMV-T7-α	*Mlu*I	α
pBSMV-T7-β*Bam*HI	*Bcu*I	β(-)
pBSMV-T7-βB-*PDS*$_{275anty}$	*Bcu*I	β*PDS*
pBSMV-T7-βB-*PLD*$_{325anty}$	*Bcu*I	β*PLD*
pBSMV-T7-γMCS	*Mlu*I	γ(-)
pBSMV-T7-γ*PDS*$_{275}$	*Mlu*I	γ*PDS*
pBSMV-T7-γ*PLD*$_{325anty}$	*Mlu*I	γ*PLD*

The plasmids containing the *PLD* fragment in the anti-sense orientation, pBSMV-T7-βB-*PLD*$_{325anty}$ and pBSMV-T7-γ*PLD*$_{325anty}$, were selected for further study. The *PDS* fragment originally cloned in the plasmid pBSMV-T7-γ*PDS*$_{275}$ [19] was amplified as a fragment flanked with *Bam*HI sites and cloned to the *Bam*HI site of pBSMV-T7-β*Bam*HI. The plasmid pBSMV-T7-βB-*PDS*$_{275anty}$, selected for further study, contained a 275-bp *PDS* fragment in the anti-sense orientation. Tab. 1 lists the plasmids and corresponding transcripts (viral RNAs). All of the restriction enzymes and T4 ligase were purchased from Fermentas (Vilnius, Lithuania).

In vitro transcription and plant inoculation
The plasmids used as the templates for the *in vitro* transcription were isolated using the Plasmid Mini Kit (Aabiot, Gdynia, Poland), linearized with *Mlu*I or *Bcu*I (Tab. 1) and purified by double phenol:chloroform (1:1 v/v) extraction followed by chloroform extraction and ammonium acetate-ethanol precipitation. Infectious RNA was synthesized using the *in vitro* transcription kit AmpliCap-Max T7 HighYield Message Maker Kit (Epicentre Biotechnologies, Madison, USA), according to the manufacturer's protocol. One *in vitro* reaction with a 1 μg template yielded 30-40 μg of RNA. One μl (approximately 2 μg) of each of the α, β and γ transcripts was mixed with 18 μl of FES buffer [22]. The resulting 21 μl of mixture was rubbed into the leaf surface of 7-day old wheat seedlings dusted with silicon carbide (Silicon carbide 400 mesh, Sigma-Aldrich, USA). The leaves of the control plants were powdered with silicon carbide and rubbed with FES buffer. Directly after the inoculation, the plants were transferred to a high humidity growth chamber (26ºC, 100% relative humidity, 16 h photoperiod and 130-230 μM photons $s^{-1}m^{-2}$ light intensity). After 4 days, the plants were transferred to standard growth conditions. In each experiment at least 5 plants were inoculated with the BSMV vector. Over 60% of them had symptoms of virus infection detectable on the second leaf 5 days post inoculation (dpi).

RNA isolation, reverse transcription and quantitative PCR
The third leaves collected 9 and 13 dpi were used for the analysis of the transcript level and virus RNA. From several BSMV inoculated plants only the plants showing virus infection and silencing symptoms (i.e. leaf photobleaching [*PDS*] or fracturing [*PLD*]) were chosen for the analyses. At least three plants for each vector / time point combination from two independent VIGS experiments were selected and analyzed. The leaf samples were ground in liquid nitrogen. RNA was isolated with TRI Reagent (Ambion, Austin, USA) according to the manufacturer's protocol. RNA concentration and A260/A280 ratio were measured using Nanodrop (NanoDrop Technologies, USA). A260/A280 ratio was always higher than 1.8 and agarose gel was used to further determine the quality of obtained RNA. Isolated total RNA was treated with 2U of DNase (DNase I recombinant RNase-free, Roche, USA) per 1 μg of RNA for 15 min at 37ºC. DNase was inactivated by addition of EDTA and heating in

75ºC for 10 minutes according to the manufacturer's protocol. Complete digestion was confirmed by PCR with primers specific for wheat 18S rRNA using 200 ng of DNase digested RNA as a template (Tab. 2). No product on agarose gel was detected after 40 cycles of amplification.

Tab. 2. Primers and reaction conditions used for quantitative PCR.

Gene	Primers	Conditions
Wheat 18S rRNA (M82356)	Forward: GTGACGGGTGACGGAGAATT Reverse: GACACTAATGCGCCCGGTAT [12]	95ºC 5 min, 30 cycles (95ºC 10 s, 58ºC 15 s, 72ºC 10 s)
Wheat *PDS* (FJ517553)	Forward: GGATGAAGATGGTGATTGGT Reverse: TGGCAAATATCATGGAGTGT	95ºC, 5 min, 40 cycles (95ºC 10 s, 60ºC 20 s, 72ºC 20 s)
Wheat *PLD* (BT009262)	Forward: CGGGCAAAGGACTTCATCTAC Reverse: TAAACCGTTCTCCGGCTTC	95ºC 5 min, 40 cycles (95ºC 10 s, 60ºC 15 s, 72ºC 15 s)
BSMV β	qBsmvB_F: CATCCCCTGGAATGTTGTTG qBsmvB_R: GAATTGTTTCCGCCTGAGTC	95ºC 5 min, 40 cycles (95ºC 10 s, 60ºC 15 s, 72ºC 15 s)
BSMV γ	qBSMV1_F:GGAGGTAGGTACTATCCCAGA qBSMV1_R: CTCCGTAGCAACAGTATAACC	95ºC 5 min, 40 cycles (95ºC 10 s, 60ºC 15 s, 72ºC 15 s)

Two micrograms of isolated total RNA after treatment with DNase (DNase I recombinant RNase-free, Roche, USA) were used for one reverse transcription reaction with random oligomers (RevertAid FirstStrand cDNA synthesis kit, Fermentas, Vilnius, Lithuania). The obtained cDNA was diluted 200x and directly used as a template for quantitative PCR. The standard qPCR reaction mix was composed of the following: 7.5 μl of the mastermix (2x Sso-Fast EvaGreen Supermix, Bio-Rad Laboratories, Hercules, USA), 0.5 μl primer F (10 μM), 0.5 μl primer R (10 μM), and cDNA and water to 6.5 μl. For the analysis of the 18S rRNA level, 1 μl of cDNA diluted 200x was used. For the analysis of the *PDS* and *PLD* transcripts, 2.5 μl of 200x diluted cDNA was used. The reactions were performed in triplicate in a Corbett Rotor-Gene 6000 5-plex thermocycler. PCR conditions are summarized in Tab. 2. In each run, for each primer pair, the standard curve composed of serial dilutions (10x, 30x, 90x, 270x, 810x) of the cDNA from control noninoculated plants was included. The samples were used to calculate reaction efficiency based on the slope value (M). Reaction efficiency (RE) was calculated according to RE = $[10^{-1/M}]$ – 1. The RE values for all primer pairs were in the range of 0.95 to 1.01 samples template. Threshold line, Ct values and standard curve were determined using the proprietary Rotor-Gene 6000 software v 1.7 supplied by the thermocycler manufacturer. Melting curve analysis (72°C to 95°C) was performed to ensure specificity in the amplification. Relative expression of the analyzed gene was calculated by means of the two standard curves method with 18S rRNA as a reference using Rotor-Gene 6000 software v 1.7.
Comparison of the relative transcript levels was done by unpaired Student's t-test using GraphPad software http://www.graphpad.com/quickcalcs/ttest1.cfm? Format=SD.

Quantitation of the relative amount of β and γ molecules in wheat leaves was performed on cDNA synthesized on the RNA isolated from wheat inoculated with the empty BSMV vector. The primers (BSMV β, BSMV γ) and the conditions of PCR reactions are presented in Tab. 2. The amplification efficiency was exactly the same for both primer pairs (used for quantitation of β and γ), so one standard curve could be used to quantify both molecules. The results were the same regardless of the standard curve used. Six plants from 2 biological repetitions were used per time point. To estimate the insert stability in β and γ molecules the following primers, flanking the insert ligation site, were used in a PCR reaction: the forward primer for β (BinsB_FW: ATGTGGGGAGGTTTAGTCAG) and the forward primer for γ (Gins_FW: GAAGAAGATGCAGGAGCTGAA). The sequence of the reverse primer for β and γ was common for both molecules (Bins_Re: CACTCCCAT CATATGGTTGAT). The fragments were amplified using Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, USA) in the following conditions: 95ºC 5 min, 35 cycles (95ºC 30 s, 58ºC 30 s, 72ºC 60 s), 72ºC 5 min. The products were separated and stained in agarose gel.

Staining and microscope observation
The samples were stained with Evans Blue to detect the presence of dead cells. Leaf samples were infiltrated for 10 min with 0.1% Evans Blue water solution. After 2-hour incubation, the samples were examined under a white light microscope (Nikon Diaphot). Staining with DAB (3,3'-diaminobenzidine tetrahydrochloride) allowed the detection of H_2O_2 [23]. Leaf samples were stained in a 1 mg/ml water solution of DAB (pH = 3.8) in darkness for 4 hours followed by overnight destaining in ethanol-chloroform (4:1 v/v) mixture containing 0.15% TCA. The samples were placed in 25% glycerol and examined under a white light microscope. A brown precipitate indicated the sites in the leaf tissues where H_2O_2 had been accumulated.

RESULTS

Construction of the pBSMV-T7-β*Bam*HI plasmid
Modification of the pBSMV-T7-β plasmid resulted in the integration of a unique *Bam*HI site at the end of the βc open reading frame. Inoculation of wheat seedlings with two mixtures of *in vitro* transcribed RNA, α, β and γMCS or α, β*Bam*HI and γMCS, confirmed the full infectability of the plants with both RNA combinations (data not included). The arrangement of the open reading frames, the position of the engineered *Bam*HI site, the location of the primers and the sizes of the amplicons used to test the insert stability in the β and γ molecules in the infected plants are shown in Fig. 1.
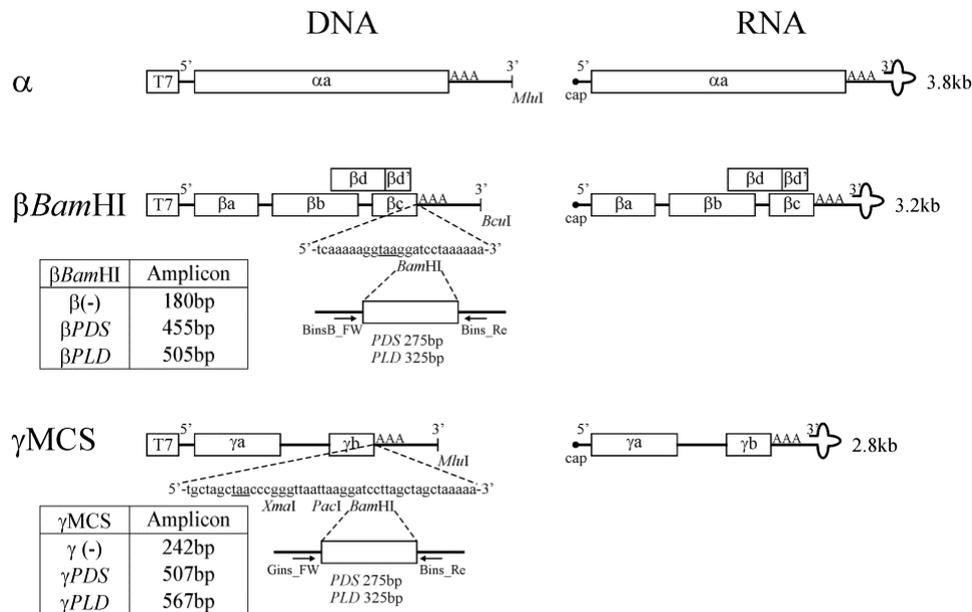
Fig. 1. The organization of the α, β*Bam*HI and γMCS molecules of the BSMV vector. The right panels indicate the open reading frames and the size of the infectious RNA. Panel α – unmodified α molecule with the indicated open reading frames. Panel β*Bam*HI – β molecule modified in this study. Indicated: the open reading frames, the integration of the *Bam*HI site at the end of ORF βc, the positions of the primers flanking the cloned DNA fragment, and the size of the amplified fragments. Panel γMCS – γ molecule obtained from Bruun-Rasmussen *et al*. [19]. Indicated: the open reading frames, the integration of MCS, the positions of the primers flanking the cloned DNA fragment and the size of the amplified fragments.

Phenotype analysis

Phytoene desaturase (PDS) is frequently used as a marker gene during the establishment of silencing methods in plants. VIGS of PDS in wheat resulted in the appearance of photobleached chlorophyll-free leaf regions [13]. In presented experiments, the photobleaching of wheat leaves was visible 8 to 9 days post inoculation with BSMV β(-):γ*PDS*. Phenotypic changes after inoculation with the remaining vector combinations were clearly seen 10 to 11 dpi. The estimation of the relative intensity of the leaf photobleaching is presented in Tab. 3.

Plants inoculated with BSMV β(-):γ*PDS* showed the strongest phenotypic effect of PDS silencing, visible as large stripes of photobleached tissue on the third leaf (Fig. 2). Plants inoculated with BSMV β*PDS*:γ(-) rarely showed chlorophyll-free stripes. In plants inoculated with vector containing one or two *PDS* fragments, β*PDS*:γ*PDS* and β*PLD*:γ*PDS*, similar amounts of photobleached tissue were observed. Photobleaching was observed only in plants inoculated with the vector containing the *PDS* fragment.

Tab. 3. The relative intensity of the phenotypic changes caused by the silencing of phytoene desaturase (*PDS*) or phospholipase D (*PLD*) after inoculation with different combinations of BSMV subunits. Data represent average phenotype intensity of at least 15 plants from 5 independent experiments.

BSMV combination	β*PDS* γ(-)	β(-) γ*PDS*	β*PDS* γ*PDS*	β*PDS* γ*PLD*	β*PLD* γ*PDS*	β*PLD* γ(-)	β(-) γ*PLD*	β*PLD* γ*PLD*	β(-) γ(-)	N I
Phenotype *PDS*	+	++++	+++	++	+++	-	-	-	-	-
Phenotype *PLD*	-	-	-	+	-	-	++	++++	-	-

- – no visible symptoms of silencing, + – very weak phenotype alterations, ++ – clearly visible phenotype alterations, +++ – strong phenotype alterations, ++++ – very strong phenotype alterations, NI – not inoculated.
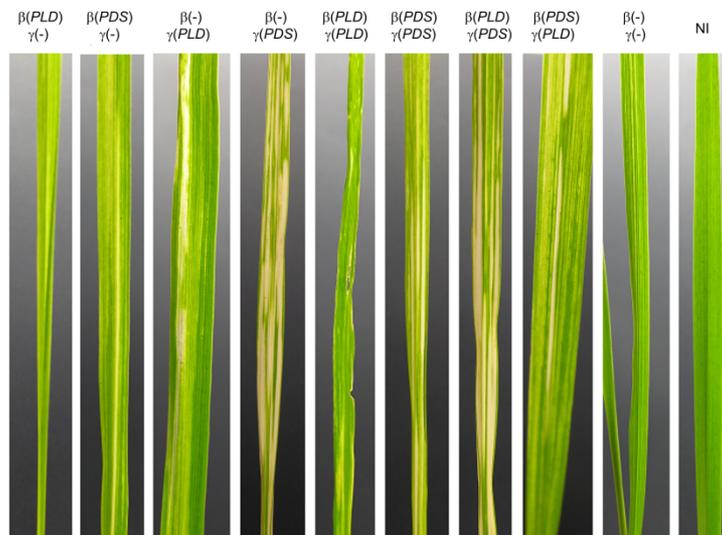
Fig. 2. Macroscopic phenotype alterations caused by the silencing of phytoene desaturase (*PDS*) or phospholipase D (*PLD*) 13 days after inoculation with different combinations of BSMV vectors. NI – not inoculated. Only representative pictures are presented.

Fig. 3. The microscopic effects of phospholipase D silencing in wheat leaves. The leaf blade splitting visible in the third leaf after inoculation with BSMV β*PLD*:γ*PLD* (A). Evans Blue indicating dead cells (B) and DAB staining indicating accumulation of H_2O_2 (C) in the tissues surrounding the splitting. Only representative pictures are presented.

The leaves inoculated with the BSMV vector carrying a fragment of phospholipase D gene showed longitudinal splits (Fig. 2 an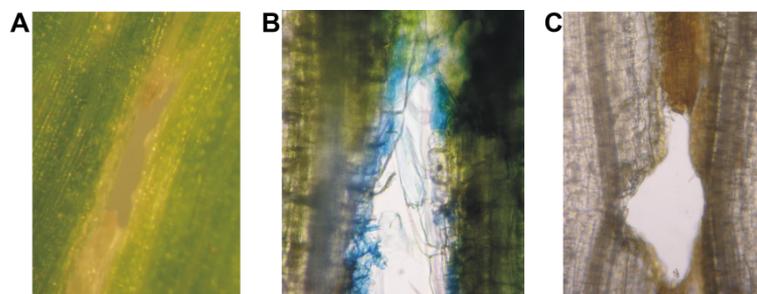d 3A). Some of the leaves showed leaf tip necrosis. The presence of the blue stain after Evans Blue staining indicated that the cells surrounding the leaf fractures were dead (Fig. 3B). Staining with DAB led to the formation of a brown precipitate in the tissues neighboring the leaf breaks (Fig. 3C), which indicated the presence of an elevated accumulation of H_2O_2 in this area. Representative pictures of phenotypes resulting from *PDS* and *PLD* silencing are presented in Fig. 2. The phenotype changes were visible in all plants inoculated with BSMV β*PLD*:γ*PLD* and in some plants inoculated with BSMV β(-):γ*PLD*.

Transcript levels of PDS and PLD
For all of the vector combinations, the transcript levels were measured using qRT-PCR on RNA from the third leaves collected 9 and 13 dpi. The results were normalized to the level of 18S rRNA (Fig. 4). The level of the *PDS* transcript was reduced to 48 and 46% (9 and 13 dpi respectively) when the *PDS* fragment was placed only in the β molecule, i.e. β*PDS*:γ(-). The silencing was slightly enhanced with the vector contained the *PDS* fragment in the β and γ molecules. The transcript level measured 9 and 13 dpi was 26 and 32% for β*PDS*:γ*PLD* and 22 and 27% for β*PDS*:γ*PDS*. The silencing fragment cloned in the γ molecule reduced the transcript level to 28 and 44% for β(-):γ*PDS* and to 48 and 42% for β*PLD*:γ*PDS* (Fig. 3A). The strongest silencing was induced with the vector carrying the *PDS* fragment placed in both the β and γ molecules (Fig. 4). A similar tendency of silencing was detected in plants inoculated with the vectors carrying *PLD* fragments. The level of the *PLD* transcript in plants inoculated with β*PLD* was reduced to 77% (9 dpi) and 70% (13 dpi) of the level in the control plants. Inoculation with vectors containing the silencing fragment in both particles β and γ enhanced silencing either 9 and 13 dpi. The transcript level was 62 and 46% for β*PLD*:γ*PDS* and 29 and 19% for β*PLD*:γ*PLD*. The results indicate that the strongest silencing was induced with the fragment delivered in both β and γ molecules (Fig. 4). This effect was consistent for the two analyzed genes (*PDS* and *PLD*). The lowest level of *PLD* transcript correlated with the highest number of leaf blade fractures and the highest level of leaf tip necrosis (Fig. 3). This observation is important for the experimental application of the VIGS system, and indicates that the strongest depression of the analyzed gene transcript correlates with the phenotypic changes. The suitability of the VIGS system for the functional analysis of a particular gene directly depends on: i) how quickly the gene is silenced; ii) whether the silencing is maintained for a sufficient period of time; and iii) how the silencing of the gene affects the phenotype. To check dynamics and stability of the silencing, wheat plants were inoculated with BSMV vectors carrying different combinations of single and double *PDS* and *PLD* gene fragments. Inoculation with the BSMV vector carrying a single *PDS* fragment in the β molecule reduced the level of the *PDS* transcript to 48% at 9 dpi and 46% at 13 dpi.

Inoculation with the vector carrying a single *PDS* fragment in γ induced 28% silencing at 9 dpi and 44% at 13 dpi. The silencing induced with a *PDS* fragment cloned in both the β and γ molecules reduced the transcript to 22 and 27% of the control level 9 and 13 dpi respectively (Fig. 4). Inoculation with BSMV vector carrying a silencing fragment in the two subgenomic particles β and γ (i.e. β*PDS*:γ*PDS*) led to stronger and more stable repression of the transcript. The differences between stability of silencing with single and double gene fragments were even bigger for *PLD*. For a single fragment in β, it was 77 and 70%, in γ it was 29 and 66%, while a double fragment reduced the transcription level to 29 and 19% (Fig. 4). The level of silencing of both of the analyzed genes varied depending on the time and the vector combination, but the general trends were common for all combinations. The single fragment silencing was stronger with the γ molecule but more stable with β. Cloning two fragments in β and γ led to both stronger and more stable silencing.
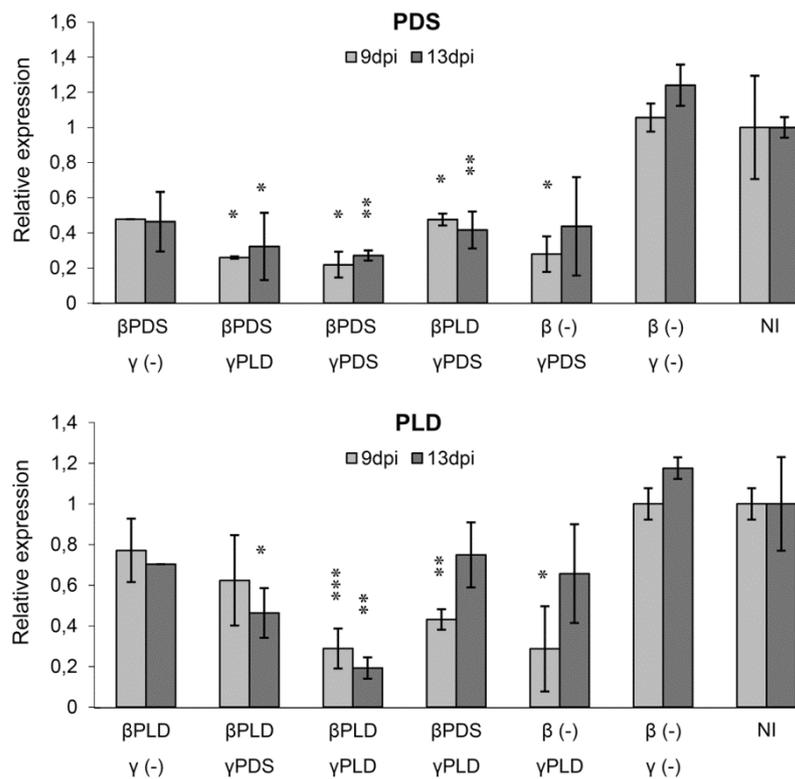
Fig. 4. The relative levels of *PDS* (upper panel) and *PLD* (lower panel) transcripts in the third wheat leaves collected 9 and 13 dpi with BSMV. The results represent the mean values and the standard deviations. Asterisks indicate the level of confidence (Student's *t* test) for the difference between the relative transcript level of the tested vector combination and the control (NI): * for $P < 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.0001$. NI – plants not inoculated.

Insert stability
The influence of the stability of foreign DNA in the BSMV vector on the strength and the durability of silencing was investigated. To analyze the stability of *PDS* and *PLD* fragments ligated into the β and γ molecule, the corresponding fragment of the β and γ molecule was amplified with primers flanking the cloning site. The template was the RNA isolated from plants 13 dpi, and the corresponding plasmids were used as controls. The length of the amplicon indicated the presence or the absence of the insert (Fig. 1).

The *PDS* and *PLD* inserts cloned in β or γ and introduced into the plant as part of the BSMV vector underwent rearrangements and were gradually eliminated (Fig. 5 and 6). In plants 13 dpi, various stages of insert elimination were detected. All of the β molecules retained the *PDS* insert, but it was always shorter than the original one. It is worth noting that none of them was completely empty (Fig. 5, lanes 4 and 9). However, in the same plants, the *PDS* fragment cloned into γ was either almost completely eliminated (Fig. 5, lane 5) or there were only very short remnants of the *PDS* fragment (Fig. 5, lane 10). A similar process was observed in plants inoculated with the *PLD* fragment (Fig. 6), which was gradually eliminated from β (Fig. 6, lanes 4 and 9) and was completely eliminated from γ (Fig. 6, lanes 5 and 10).

Fig. 5. Electrophoresis of the PCR amplification products. PCR primers flanked the insertion site in β and γ molecules. Plasmid templates were used as the control: β(-) lane 7, β*PDS* lanes 2 and 8, γ(-) lane 11, γ*PDS* lanes 3 and 12. cDNA was derived from plants 13 days post inoculation with the BSMV vector: BSMV:α, β*PDS*, γ*PDS* lanes 3 and 4, BSMV:α , β*PDS*, γ(-) lane 9, BSMV:α, β(-), γ *PDS* lane 10. 100-bp Marker (Fermentas, Vilnius, Lithuania) lanes 1 and 6. The analysis was done on the cDNA derived from 3 plants of a single experiment and the experiment was repeated twice. Only pictures representative for each combination are presented.
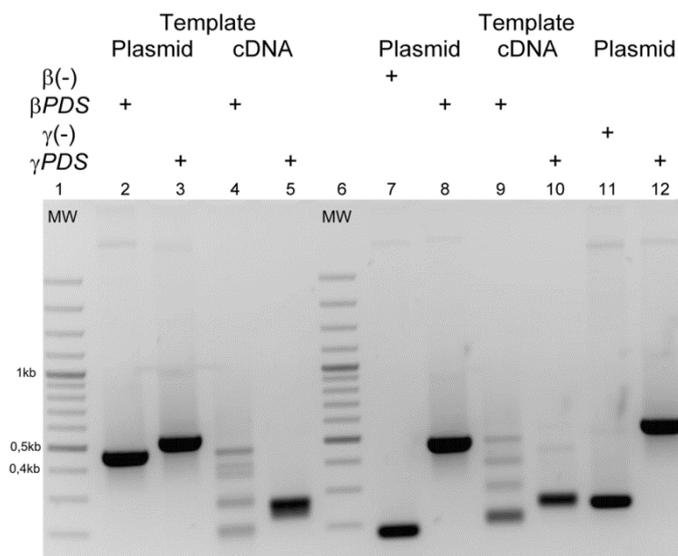
Fig. 6. Electrophoresis of the PCR amplification products. PCR primers flanked the insertion site in β and γ molecules. Plasmid templates were used as the control: β(-) lane 7, β*PLD* lanes 2 and 8, γ(-) lane 11, γ*PLD* lanes 3 and 12. cDNA was derived from plants 13 days after inoculation with the BSMV vector: BSMV:α, β*PLD*, γ*PLD* lanes 3 and 4, BSMV:α , β*PLD*, γ(-) lane 9, BSMV:α, β(-), γ *PLD* lane 10. 100-bp Marker (Fermentas, Vilnius, Lithuania) lanes 1 and 6. The analysis was done on the cDNA derived from 3 plants of a single experiment and the experiment was repeated twice. Only pictures representative for each combination are presented.
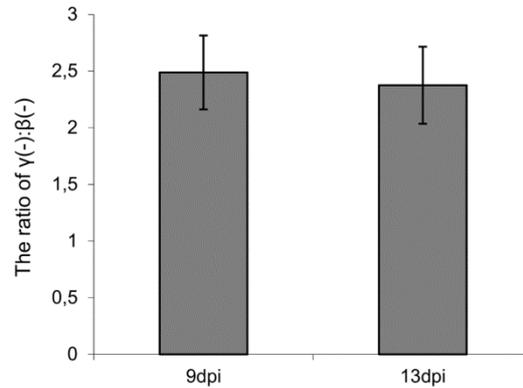
Relative amount of β and γ molecules

In an attempt to explain the variable stability of inserts cloned in the β and γ molecules, the relative quantities of both the β(-) and γ(-) molecules were analyzed in wheat 9 and 13 dpi with the BSMV vector. To estimate the γ(-) to β(-) ratio, quantitative PCR was performed using one sample of cDNA for the two pairs of primers specific to β(-) and γ(-). The efficiencies of amplification established for five cDNA dilutions were identical for the primers qBsmvB_F/qBsmvB_R (quantitation of BSMV β) and qBSMV1_F/qBSMV1_R (quantitation of BSMV γ molecule). The amplification efficiencies ($e_β$ and $e_γ$) and correlation coefficient for the standard curve (R) calculated for both reactions were identical: $e_β = 0.92$ with $R = 0.999$ and $e_γ = 0.92$ with $R = 0.999$. Since the corresponding values for both primer pairs and the amplification features of both molecules were the same, the quantitation of both β(-) and γ(-) was calculated using one standard curve. Based on this, the γ(-) to β(-) ratios in RNA isolated 9 and 13 days after inoculation with BSMV were 2.5 and 2.4, respectively (Fig. 7). This indicates that the quantity of the γ(-) molecule is about 2.5x higher than that of the β(-) molecule in plant RNA isolated at both time points.

Fig. 7. The ratio of quantities of γ(-) to β(-) molecules in wheat leaves inoculated with the BSMV β(-):γ(-) vector. The results show the mean values and the standard deviations of γ(-) to β(-) ratio found in the leaves of 6 plants obtained after two VIGS experiments.

DISCUSSION

In all BSMV-based vectors used to date, only the γ molecule was adapted for cloning a DNA fragment to induce gene silencing. The results of using both β and γ molecules to deliver a gene fragment and induce silencing are presented. The unique *Bam*HI site and the STOP codon were engineered between the ORF βc and the poly(A) tail to facilitate the cloning of a foreign gene fragment to the β molecule. The β*Bam*HI molecule combined with the unmodified α and γMCS was tested as a novel BSMV-based silencing system.

The fragments of two genes, *PDS* (the reporter gene for VIGS) and *PLD*, were cloned and used to analyze the efficiency and stability of single- and double-gene silencing and to associate the process with the stability of foreign DNA cloned in β and γ. The level of silencing with a fragment cloned only in γ was found to be higher but less stable than that found with cloning only in β. However, using both β and γ molecules to deliver a gene fragment led to either more efficient or more stable silencing. To explain these results, the stability of inserts cloned in the β and γ molecules and the relative quantity of both molecules in virus-inoculated plants were tested. The results indicated that both molecules underwent rearrangements that led to the gradual elimination of this insert. Two common features of the process were found: i) the fragments of both tested genes were noticeably more rapidly eliminated from γ than from β; and ii) the elimination of the insert, although varying in β and γ, was a gradual process. This was indicated by the ladder-like pattern of the amplicons in RT-PCR tests. This also indicated that certain elements of the insert were less prone to elimination. Insert instability in the BSMV γ molecule has been reported by Bruun-Rassmusen *et al.* [19] and Cakir and Tör [24]. The process could be detected in other virus-based gene silencing systems, although the intensity and the rate of the elimination might be different.

Earlier data indicated that the length of the insert and the time after virus inoculation were positively correlated with insert elimination [19, 24], and both factors correlated with the transient nature of virus-induced silencing. The presented results, consistent with the findings reported earlier, add other factors that are possibly involved, namely the type of the virus molecule and the relative quantity of this molecule in plant cells. Using the molecule β as well as γ as the carriers to deliver a silencing signal, it was found that foreign DNA was eliminated from both of them but the elimination rate was clearly lower from β.

The quantity ratio of γ(-) to β(-) measured in the inoculated plants was close to 2.5. This indicated a 2.5-fold higher quantity of γ(-) and indirectly a higher replication rate of γ(-) compared to β(-). This finding was in agreement with the higher rearrangement rate in γ than in β, and it was consistent with the finding of Bruun-Rassmusen *et al.* [19] that insert elimination was proportional to the time after inoculation, i.e. the number of virus molecule replication cycles. The use of both β and γ molecules, the approach presented in this manuscript, improved the efficacy of the BSMV-based vector and facilitated the efficient simultaneous silencing of two different genes. The simultaneous silencing of two genes by a single BSMV construct has been reported by Cakir and Scofield [25]. In plants, phospholipase D (PLD) is a key component of signal transduction in diverse pathways including programmed cell death, senescence and responses to biotic and environmental stresses [26]. The clone of *PLD* was isolated in a wheat subtractive hybridization cDNA library as one of the clones upregulated in leaves after pathogen infection (unpublished results). It was chosen for VIGS experiments as an example of a gene of interest to be silenced simultaneously with the *PDS* marker gene. *PLD* silencing led to leaf fracturing. As indicated by Evans blue staining, the cells directly adjacent to the punctures were dead while tissues surrounding the punctures were alive and etiolated. The phenotypic changes (i.e. the fractures surrounded with dead cells and photobleaching) occurred in similar leaf areas, which indicated the possibility of simultaneous silencing of both genes. This implies that the modified VIGS vector can be used for the simultaneous silencing of two genes including the combination of the *PDS* marker gene and a gene of interest. The results demonstrated varying, not equimolar quantities of β(-) to γ(-) molecules in plants inoculated with BSMV and correlation of the higher amount of γ molecule with the more pronounced insert elimination from this molecule. These results indicated the possible dependence of the insert stability and the silencing efficiency on the ratio of β to γ in the virus-inoculated plants.

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