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SILENCING OF G-PROTEIN α-SUBUNIT IN BARLEY (*Hordeum vulgare* L.) BY VIGS AND ITS EFFECT ON THE PLANT GROWTH

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ABSTRACT

G-proteins play important roles in a number of cellular and developmental processes in plants. In this study, we have cloned a 317-bp fragment encoding a part of barley G protein α-subunit into γ-genome of barley stripe mosaic virus (BSMV). Barley seedlings were inoculated with this construct denoted as pSL038-1/Gα at 3-leaf stage, and assayed for their growth, leaf area and membrane ion leakage. Silencing was achieved after 2 weeks of inoculation and confirmed by the decrease in Gα subunit mRNAs in barley leaves. There was a significant decrease in plant growth in terms of plant height and leaf area following the viral infection. Leaf area in silenced plants were decreased by >10% compared to control plants (wild-type or inoculated by BSMV-PDS constructs). In conclusion, silencing resulted in etiolated phenotypes and growth retardation. Our optimized VIGS protocol in barley could be adopted to evaluate extensive physiological parameters and molecular changes in Gα silenced plants.

Keywords: BSMV, G-proteins, Ion Leakage, Phytoene desaturase (PDS), Virus induced gene silencing (VIGS).

INTRODUCTION

The recent advances in functional genomics have provided abundant plant genome information. Traditional approaches for determining functions of genes, including random mutation, T-DNA insertion mutations and chemical mutagenesis have been extensively used for knockdown gene expression in plants. However, mutation approaches complicates gene function studies in non-model individuals due to complex genetic background, low transformation efficiency and their large genome size (Cakir et al., 2010; Huang et al., 2012). Virus-induced gene silencing (VIGS), on the other hand, is a useful and efficient approach for plant loss-of-function evaluation that can overcome the above-mentioned complications and permits knockdown of genes-of-interest and phenotypic observations within 3 to 4 weeks. VIGS allow stable RNA interference (RNAi) in plant cells and can also be performed in the species that are difficult to transform (Burch-Smith et al., 2004; Scofield and Nelson, 2009; Cakir et al., 2010). Recently, the plants in which VIGS has been used were increased considerably, and more than 30 viruses have been reported to have great prospective as VIGS vectors (Yuan et al., 2011). Barley stripe mosaic virus (BSMV) has been applied for cereal crops and is a positive-sense RNA virus with a wide-ranging hosts (Holzberg et al., 2002; Tai et al., 2005; Hu et al., 2009; Jackson et al., 2009; Meng et al., 2009). Silencing of PDS (Phytoene desaturase), an enzyme required for the biosynthesis of carotenoid pigments, can be observed as white streaks on plant leaves (Holzberg et al., 2002).

Heterotrimeric G proteins, also called as guanine nucleotide-binding proteins, are a family of proteins required for transduction of external signals into cellular responses (Assmann, 2002). G protein complexes consist of alpha (α), beta (β) and gamma (γ) subunits (Hurowitz et al., 2000; Assmann, 2002) and encoded by less numbers of genes in plants compared to mammals. In *Arabidopsis*, there is one gene (*GPA1*) encoding G-Protein α subunit, one gene (*AGB1*) encoding the G-Protein β subunit, and at least three genes (*AGG1, AGG2* and *AGG3*) encoding the G-Protein γ subunits (Chakravorty et al., 2011). Mutations in heterotrimeric G proteins are not lethal and proved that those were having roles in almost all developmental stages in *Arabidopsis* (Perfus-Barbeoch et al., 2004). Similarly, functional studies revealed that G proteins are associated with germination, development, stomatal opening, phytohormone and stress responses (Ullah et al., 2001; Pandey and Assmann, 2004; Joo et al., 2005; Pandey et al., 2006; Wang et al., 2007; Fan et al., 2008; Zhang et al.,

2008). For example, both Gα and Gβ subunits were found to be related to stomatal aperture that controls water status and photosynthesis (Zhang et al., 2008; Nadeau, 2009). Their associations with stomatal movements were determined by using electrophysiological and pharmacological methods (Wang et al., 2011). ABA prevents stomatal opening and stimulates stomatal closure, reducing water loss through transpiration. Therefore, the role of heterotrimeric G-proteins in the opening/closing of stomata may be through the ABA signaling (Wu and Assmann, 1994). The Gα subunit also regulates cell division and development: the Gα mutants caused reduction in cell division in the leaf-length (Ullah et al., 2001; Bommert et al., 2013). The Gα-null alleles produced stunted seedlings in maize, rice and *Arabidopsis* (Ullah et al., 2001; Bommert et al., 2013; Urano et al., 2014). Mutations in heterotrimeric G-protein had a significant effect on phenotypic plasticity for yield related traits in *Arabidopsis* (Nilson and Assmann, 2010).

In rice, single genes encode Gα and Gβ, while at least five genes encode Gγ subunits and one of them is involved in seed size (Huang et al., 2009). Gα and Gβ subunits are also associated with pathogenic response and tolerance to salinity (Colaneri et al., 2014). So far, efficiency of a VIGS-based silencing of G-proteins was not tested in barley which is an important model cereal from *Poaceae* family. Therefore, the objective of this study was to investigate the possibility of production of knockout barley plants for Gα subunit and analyze the effects of gene silencing on growth conditions and membrane ion leakage. For this purpose, we first cloned a part of barley G protein α-subunit and successfully cloned the fragment into the BSMV vector γ-genome. Silencing was followed by phenotypic changes and confirmed by RT-PCR analysis.

MATERIALS AND METHODS

Plant material

Hordeum vulgare L. cv. Tokak157/37 (Tokak) seeds were kindly provided by Aegean Agricultural Research Institute and re-propagated from field-grown plants. Seeds were planted in the pots with 190 mm diameter and grown under controlled growth chambers (Angeloantoni ECHL) at 20–25 °C with a 14/10 h light (~75 µmol / m^2 s) /dark photoperiod. Relative humidity of growth chamber was kept at 50-65%.

Construction of BSMV vectors for silencing experiments

Plasmids containing the tripartite BSMV genomes α, β and γ were kindly provided by Dr. Steven R. Scofield (Purdue University). Barley G-protein α subunit mRNA sequence (NCBI, AF267485.2) was used to design the primers by IDT PrimerQuest program (Table 1).

Table 1. Primer sequences used in the study

Gene	Study	Primer sequences 5'→3'	Amplicon size (bp)
Gα	Cloning to pSL038-1	AAAGGCGGCCGCGCACATCCACAAG CCTGTACGAGTTCTTAATTAAGGAG	317
Gα	RT-PCR	AGCTTGCCCAAGTGGAACTA CTCGCACACAGTCAAAGGAA	632
α-TUB	RT-PCR	GAAAGGCGTCTTCGTACTCG GAGTGGGTGGACAGGACACT	676

Newly designed primers consists of the *Not*I and *Pac*I restriction sites for further cloning to pSL038-1 vector which is γ genome of BSMV vector system. Total RNA was isolated from barley leaves with the TRIZOL® reagent (Invitrogen) and treated with DNase I (Fermentas) in a final volume of 100 µl. First strand cDNA synthesis was performed using 4 µg of total RNA, 100 U of SuperScriptII RT (Invitrogen) and 1 µg of Oligo $(dT)_{20}$ primer according to the manufacturer's instructions in a final volume of 40 µl. Using previously described primers, a specific fragment of 317-bp was amplified by PCR and cloned into pCR8/GW/TOPO vector (Invitrogen, K250020). BSMV-γ vector (pSL038-1) was isolated from *E. coli* DH5α cells which were grown in LB media including 50 mg/L ampicillin. After digestion of pSL038-1 with *Not*I/*Pac*I, fragment was ligated to the vector by T4 DNA ligase (Takara 20115). Vector-insert construct was confirmed by restriction enzyme analysis on agarose gel and denoted as pSL038-1/Gα (Figure 1). We have used BSMV-γ RNA with 185-bp fragment from barley PDS gene (denoted as BSMV-PDS) which cause a photo-bleaching phenotype as positive control in silencing experiments. Wild-type plants were kept as control.

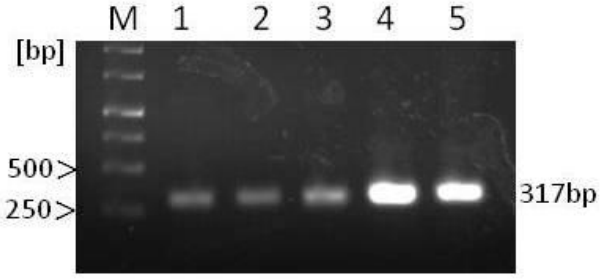

Figure 1. Cloning of a 317-bp insert corresponding to G-α barley subunit into BSMV-γ vector confirmed by PCR. M: DNA Ladder, 1-5: colonies carrying pSL038-1/Gα construct

In vitro transcription of BSMV vectors

The procedures for linearization of plasmids and *in vitro* transcription of viral RNAs were performed as described by Scofield et al. (2005). Complete linearization was checked via separation of 1 µl product on 1% w/v

agarose gel. Then linearized plasmid reaction was treated with RNase inhibitor before *in vitro* transcription step. Linearized DNAs of each vector (α, β, γ) were used as a template for the synthesis of RNAs. Ambion mMessage mMachine T7 (Cat No:1344) Kit was used for *in vitro* transcriptions with following procedure. From each genome a transcription reaction mix was prepared in volumes of 2.5 µl. Each reaction mix contained 0.75µl (80 ng) of template DNA, 0.25 µl Buffer (10x), 1.25 µl of NTP-mix (2x) and 0.25 µl of enzyme mix. Mixture was incubated at 37°C for 2 hours. 1 µl of DNase was added to tubes and kept at 37°C for 15 minutes. Then 30 µl nuclease-free water and 30 µl LiCl (7.5 M) were added to tubes. The tubes were placed at −20°C for 1h to precipitate the RNAs and then centrifuged at 4°C, 15,000 rpm for 15 minutes. Supernatant was discarded and pellet was washed with 70% ethanol. Pellets were dried at RT (room temperature) and dissolved by 2.5 µl nuclease-free water. The quality and quantity of RNAs were measured by 1% agarose gel and Nanodrop 2000 (Thermo Scientific, USA)

Inoculation of barley plants with BSMV construct

Plants were infected with BSMV construct using a modified protocol (Holzberg et al., 2002). One µl of each of the *in vitro* transcription reactions for α, β and γ RNAs were mixed and added to 20 µl of FES buffer (0.1 m glycine, 1% w/v sodium pyrophosphate, 0.06 m K_2HPO_4, 1% w/v celite, 1% w/v bentonite, pH 8.5). This solution was then applied to plants by rub inoculation method. The solution was pipetted on the leaf between the thumb and a first finger of the hand and the leaf was gently pressed with first finger and thumb. The whole upper part of leaf was coated with this solution by sliding the gently pinched fingers throughout whole leaf and this process was repeated twice.

Plant growth assessments and ion leakage

Plant growth was considered by plant height and leaf size. The measurement of plant height was performed daily after inoculation during 20 days using a ruler from the base of the plant to top of the leaf. Plant height was given as the average growth (mm). The leaf sizes of the 2nd leaf of barley plants were calculated according to the following formula as "leaf size = length × width × 0.7" (Jamaux et al., 1997). These calculations were performed continuously for 20 days after the inoculation of BSMV, using the fully expanded 2nd leaf from the top. Membrane ion leakage was measured by the following procedure: Five leaf pieces from each plant were sampled and placed in glass tubes. The pieces were then washed with distilled water for three times, capped and placed in a thermostatically controlled chamber maintained at 25°C in the dark. After 16 hours, 60 µl of the sample were used for conductivity measurement (E1) by HORIBA conductivity meter B-137. The tubes were then autoclaved at 121°C for 20 min and samples were again analyzed with the conductivity meter for E2 values. Ion leakage was calculated and presented as "E1 / E2 x 100".

RNA extraction and RT-PCR

Total RNA was extracted from leaves using the TRIZOL® reagent (Invitrogen) by a protocol as given by the manufacturer. The RNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer. Primer pairs for RT-PCR analyses were designed using NCBI Primer-BLAST program and given in Table 1. Alpha-tubuline (*α-TUB*) was used as a reference gene to amplify a 676-bp fragment. RT-PCR was performed in 50 µl volume, with 25 mM $MgCl_2$, 10 mM dNTP, 0.8 U RNase inhibitor, 5U AMV RTase XL, 5U AMV-Optimized Taq DNA polymerase (Promega) and 1 µM each of forward and reverse primers in 1×One Step RNA PCR Buffer (AccessQuick RT-PCR System, Promega). PCR profile was as follow: 50°C for 30 minutes for reverse transcription and 94°C for 2 minutes for initial denaturation then 30 cycles at 94°C for 30 s, annealing at 60°C for 60 s and 72°C for 45 s, final extension at 72°C for 10 minutes. The amplified samples were then analyzed on 1% agarose gel.

Statistical analyses

The data were analyzed by SPSS software version 21 (Chicago, IL, USA) using analysis of variance (ANOVA). Significance of differences between treatment means was calculated by Tukey's HSD test. P value less than 0.05 is considered as statistically significant.

RESULTS AND DISCUSSION

Gene silencing in barley seedlings

The plants were grown in pots under controlled growth conditions (See Materials and Methods) in three independent biological replicates and pSL038-1/Gα was applied to plants at 3-4 leaf stage for silencing of Gα subunit. Barley leaves depicted photo-bleaching symptoms typical for PDS silencing within 7 d after inoculation (Figure 2A). Inoculated plants showed stunted growth compared with control plants. Photobleaching started from the upper part of the leaf, especially in tips, and later on white narrow streaks spread along the veins of whole leaf in inoculated plants. Similar photobleaching symptoms were reported in previous studies (Holzberg et al., 2002; Ma et al., 2012). Etiolated leaves were also observed three weeks after inoculations by pSL038-1/Gα.

RT-PCR was carried out to confirm the visual virus induced gene silencing. No band was observed in silenced plants after 2 and 3 weeks, while control seedlings produced a clear band of 632-bp of Gα subunit (Figure 2B). BSMV-PDS inoculated plants also showed the presence of Gα subunit fragment (Figure 2B).

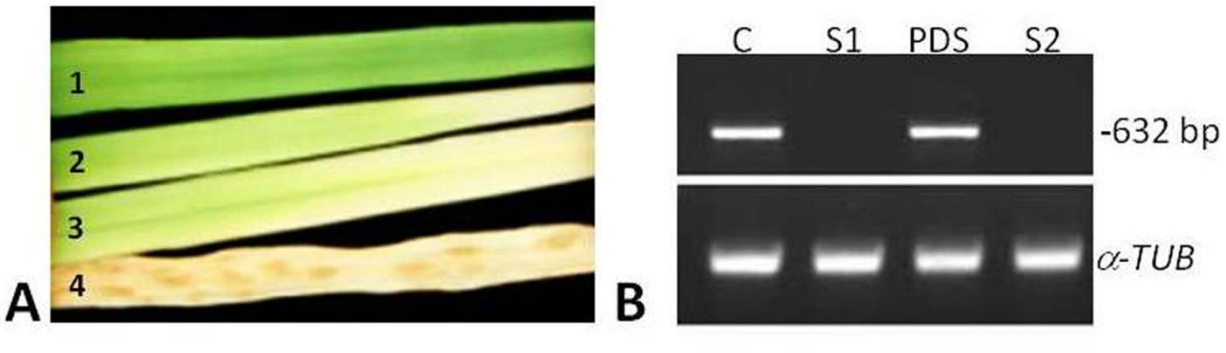

Figure 2. (A) Different phenotypes of barley leave from control (1); BSMV-PDS (2); pSL038-1/Gα inoculated leaf after 5 days (3) and after 3 weeks (4). (B) RT-PCR analysis of pSL038-1/Gα silenced plants. C: Control, S1: silenced plant after 2 weeks, PDS: plant inoculated by BSMV-PDS, S2: silenced plant after 3 weeks, lower panel is alpha-tubuline (*a-TUB)* as reference gene

Effect of silencing on plant growth and physiology

There was a decrease in the plant growth after the inoculation of pSL038-1/Gα construct. The plants inoculated with pSL038-1/Gα construct showed significantly lower plant heights than BSMV-PDS and control plants (Figure 3). Silenced plants also showed reduction in leaf area compared to control plants (Figure 4). Our results depicted that there was more than 10% decrease in leaf area of silenced plants with pSL038-1/Gα, 20 d after inoculation. Overall, the growth was not totally suppressed but decreased compared to control plants.

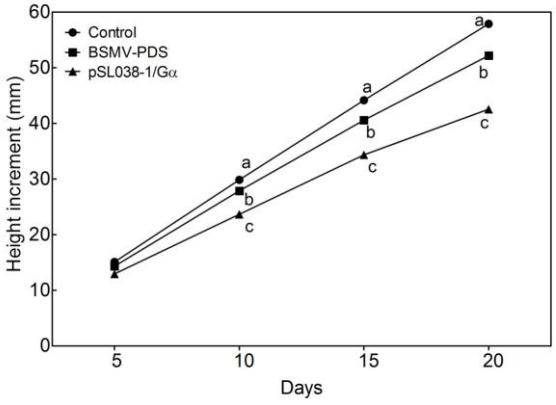

Figure 3. Effect of pSL038-1/Gα inoculation on plant height. Plant height of 12 plants were measured (*n=3*) each day after the inoculation. BSMV-PDS: plants inoculated with phytoene desaturase (PDS) construct. Different letters indicate significant differences ($P \leq 0.05$) between treatments according to Tukey's HSD test.

Ion leakage was between 25.27 and 26.47 in control and test plants at the beginning of inoculation (0 day) (Table 2). After 10 days of inoculation with BSMV constructs, all infected plants showed slightly higher ion leakage (Table 2) while control plants maintained their values after 10 or 20 days. Ion leakage values between control and Gα silenced plants were 43.20 and 79.73 after 10 and 20 days, respectively. Ion leakage in BSMV-PDS inoculated plants was also increased slightly. As ion leakage is an important indication of membrane injury and frequently occurred in the stressed cells (Babu et al., 2004; Kocheva et al., 2014; Faralli et al., 2015); this parameter should be studied more detailed. In addition, pleitropic effects in Gα silenced plants should be considered due to central roles of G-proteins in many cellular pathways in the cell.

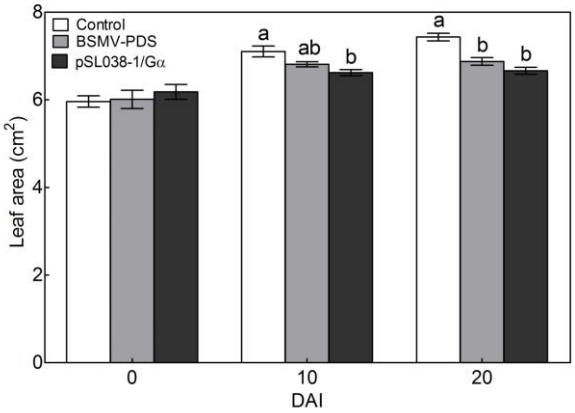

Figure 4. Effect of silencing of Gα subunit on leaf area. Leaf area was measured from the first day to 20th day after inoculation. C: Control; BSMV-PDS: inoculated with phytoene desaturase (PDS) construct; pSL038-1/Gα : Gα silenced plants. Different letters indicate significant differences ($P \leq 0.05$) between treatments according to Tukey's HSD test.

Table 2. Ion leakage in barley leaves of plants inoculated with BSMV-PDS and pSL038-1/Gα after 0, 10 and 20 days. Data represent mean values of three biological replicates with ± SD.

	0 day	10 Dai[a]	20 Dai
Control	25.27 ± 0.97	27.60 ± 0.59 c	26.52 ± 0.78 c
BSMV-PDS	26.43 ± 0.78	36.10 ± 0.65 b	51.87 ± 1.04 b
pSL038-1/Gα	26.47 ± 0.46	43.20 ± 1.38 a	79.73 ± 0.50 a

[a] Days after inoculation. Different letters indicate significant differences ($P \leq 0.05$) between treatments according to Tukey's HSD test.

BSMV-VIGS vectors provide an effective and reliable tool for plant functional genomics research as shown in various monocot species (Burch-Smith et al., 2004; Scofield and Nelson, 2009; Cakir et al., 2010; Pacak et al., 2010; Ma et al., 2012). Notably, we found complete silencing of pSL038-1/Gα two weeks after inoculation in barley plants. A similar study has shown that BSMV-VIGS can be used for down regulation of important genes in *B. distachyon* (Pacak et al., 2010). VIGS is a fast and reliable method for stable knock-down of a desired gene which would produce a phenotype; therefore it could be employed as a quick method for cereal species where stable genetic transformation is required but a very laborious choice (Wege et al., 2007; Ma et al., 2012). To our knowledge, this is the first report on the functional analysis of G-protein on barley plants through BSMV-based VIGS.

In the present study, results showed that silencing caused etiolated phenotypes and also reduced the plant growth in terms of height and leaf area which is important for photosynthetic capacity. Elimination of Gα subunit in cells suppressed the seedling development at 3-leaf stage that an active stage of vegetative growth. These results are consistent with Colaneri et al. (2014), who also reported reduction in shoot growth and early senescence of leaves under salt stress in *Arabidopsis* plants lacking with heterotrimeric protein. Gα subunit required for cell division under stress and the Gα–null alleles caused decline in cell division in the leaf-length in *Arabidopsis* and maize (Ullah et al., 2001; Bommert et al., 2013). Maintenance of higher leaf area index seems to be a crucial factor leading to an increase in sink potential in maize and pearl millet (Khanna-Chopra and Maheswari, 1998; Vijayalakshmi et al., 2012). These results revealed that G-protein is necessary for plant growth and are in agreement with the earlier studies which reported that heterotrimeric G-proteins played diverse roles in germination, stomatal aperture regulation, growth and development under normal and various stress conditions (Pandey and Assmann, 2004; Joo et al., 2005; Melotto et al., 2006; Pandey et al., 2006; Wang et al., 2007; Fan et al., 2008; Zhang et al., 2008).

CONCLUSION

We have established a construct carrying Gα subunit for obtaining knockout barley plants efficiently for further investigations. Silencing of Gα subunit in barley was shown by both phenotypic and genotypic analyses. Our results suggest that silencing of Gα subunit produces etiolated plants with reduced growth which is an indication of suppressed photosynthetic activity in barley.

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