EFFICACY OF *BARLEY STRIPE MOSAIC VIRUS* TO INDUCE GENE SILENCING OF A GENE REPRESSING FLOWERING IN SUGAR BEET

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Introduction and Objectives:

Sugar beet (*Beta vulgaris*) is a biennial plant that requires a cold period in order to induce flowering, a process called vernalization. The period of vernalization required to induce flowering can vary by genotype (between 90-120 days). Holding beets in cold storage (4^{o}C) at high humidity (95%) is expensive and labor intensive, requiring specialized equipment and multiple fungicide applications. Therefore it would be advantageous to both breeders and seed producers to develop a system that would require less than the 90 day minimum vernalization period in order to initiate uniform flowering of sugar beet plants for seed production.

The sugar beet gene, *BvFL1*, which is a homologue of FLC in Arabidopsis, has been proposed to act as a repressor of flowering in sugarbeet (Reeves et al., 2007). In that study, it was shown that four mRNA variants are created from a single genomic locus via alternate splicing. Virus-induced gene silencing (VIGS) can be used to study the effect of turning off or down regulating gene expression of a specific gene (Meister and Tuschl, 2004). A viral vector is used to deliver dsRNA corresponding to a gene of interest into the cells, which triggers the plant's natural ability to destroy that sequence of RNA (Baulcombe, 2004). This technique has been shown to be effective in diverse plant species such as wheat (*Triticum aestivum*), California poppy (*Eschscholzia californica*), and Arabidopsis (Burch-Smith et al., 2006; Scofield et al., 2005; Wege et al., 2007). Delivery of a specific region of the *BvFL1* sequence and subsequent silencing of this gene could potentially induce flowering without vernalization. Such a procedure could expedite breeding efforts without alteration of the genotype.

Barley stripe mosaic virus (BSMV) has been used previously to affect VIGS in sugar beet (Larson and Weiland, 2005). BSMV is a tripartite RNA virus with all three subunits (alpha, beta, and gamma) required for infection. BSMV does not naturally infect sugar beet. The objectives of this research were to develop dsRNA sequences that would suppress *BvFL1* expression in sugar beet and potentially induce floral development without vernalization. The VIGS silencing system has an approximate 21-28 day window of effectiveness (Tai et al., 2005). Induction of flowering within this time frame would be a dramatic improvement over the current 90–120 day vernalization requirement.

Materials and Methods:

Silencing Target Sequence:

Four oligonucleotide pairs were designed from *BvFL1* to create 4 inverted repeat DNA sequences including a hairpin loop (GCGC) that would result in dsRNA necessary for VIGS (Table 1.). These oligonucleotides were designed to target either 2 or 4 *BvFL1* mRNA variants. All 4 oligonucleotide pairs were ligated first into pBluescript SK series. *Spe* and *Not*I restriction sites flanked the first oligonucleotide pair and *XbaI* was used for the remaining three

oligonucleotide pairs. Multiple transformants (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) were selected and the cloned sequence verified to insure the stability of the inserts that ranged from 84 to 110bp (Table 2). Once the target sequence was confirmed, each of the four target sequences were digested out of pBluescript SK series, treated with alkaline phosphatase and ligated into the BSMV gamma construct D4MCSb (Tai et al., 2005, Larson and Weiland 2005).

Table1. Paired oligonucleotides used to create double stranded inverted repeat for silencing

	Paired Oligonucleotide Sequences (5'-3')
1F	CTA GGC TGT TGA AAC AGA GAG TTC ACC TTC TAG TT
1R	**GCG C**AA CTA GAA GGT GAA CTC TCT GTT TCA ACA GC
2F	CTA GAA AGA CTC AAC TTA TGC TAG AAT CTA TCG GAA CAC TAA GTG AAC AG
2R	**GCG C**CT GTT CAC TTA GTG TTC CGA TAG ATT CTA GCA TAA GTT GAG TCT TT
3F	CTA GAA GCT GTT GAA ACA AGA GAA TGA ACA GTT GAA GGA TGA GGT AGC AAA TCT GAT A
3R	**GCG C**TA TCA GAT TTG CTA CCT CAT CCT TCA ACT GTT CAT TCT CTT GTT TCA ACA GCT T
4F	CTA GAA TAA GCT AGA TTC AGA GTA ATA AAG CAG ACA AGT GAA GTA CTC GGA GAA GGT T
4R	**GCG C**AA CCT TCT CCG AGT ACT TCA CTT GTC TGC TTT ATT ACT CGT AAT CTA GCT TAT T

Table 2. Four Silencing Sequences, the *BvFL1* variant targeted for silencing, and resulting insert size (bp).

Target Sequence	Primer name: Variant targeted	insert (bp)
Seq1	BvFL1.ex2/3vigs: A3,A4	84
Seq2	BvFL1.ex5vigs: A1,A4	104
Seq3	BvFL1.ex6/7vigs: A1-A4	110
Seq4	BvFL1.3'UTRvigs: A1-A4	110

***In-vitro* Transcription to create components for BSMV RNA:**

In-vitro reactions used the MEGAscript High Yield Transcription Kit (Ambion, Austin TX) with 1µg of *Mlu*I linearized template for each alpha, beta, gamma subunit (BSMV, gamma+Seq1, gamma+Seq3, or gamma+Seq4). *In-vitro* amplification of RNA required the presence of m^7G(5')ppp(5')GCap Analog (Ambion, Austin TX) to reassemble the virus to cause a successful infection in plants (Tai et al., 2005).

Plant Materials:

Chenopodium quinoa (*C. quinoa)* is a local lesion host of BSMV, and was used to confirm infection of the BSMV constructs prior to inoculation of sugar beet. Six to ten week old *C. quinoa* plants were grown in a glasshouse (16h/day 24°C). For sugar beet inoculations, FC606 plants (PI 590843) (Smith and Ruppel, 1980) were grown in Conviron growth chambers (CMP 4030, Winnipeg, Manitoba Canada). FC606 was the source of the *BvFL1* sequence (Reeves et al., 2007). A 24 hour day length was used to satisfy photoperiodic requirements for flowering. Growth chambers were maintained at 70% relative humidity and 20°C. Plants were fertilized weekly throughout the VIGS experiment (35 days post inoculation).

Inoculation of BSMV and RT-PCR Procedures for *Chenopodium quinoa*:

Leaves on the upper half of *C. quinoa* plants were inoculated with BSMV. Equal volumes of each *in vitro* product (2 µl each: alpha, beta and gamma subunits or

gamma+seq1,3,or 4) were added to 250µL of GKP buffer + 1% bentonite + 1% Celite (Larson and Weiland, 2005) and immediately mixed and transferred to multiple leaves of a single plant and rubbed lightly with gloved finger onto adaxial and abaxial surfaces. At 7 days post inoculation, 1 leaf punch (16mg) for each infected leaf was collected to extract RNA (RNeasy Mini Kit, Qiagen) (Valencia, CA). Total RNA was quantified with a Nanodrop Spectrophotometer (Nanodrop Corporation, Wilmington, DE) then 5 ng template was amplified with a One Step RT-PCR kit (Qiagen, Valencia, CA) using BSMV gamma-specific primers flanking the insertion site (Table 3). Optimization for amount of target template was performed using 27 cycles of amplification with an annealing temperature of 55°C.

Table 3. PCR Primers for RT-PCR detection of BSMV.

Primer	Primer Sequence (5'-3')
BSMV gamma F	GAA GAT GCA GGA GCT GAA ACT TTC
BSMV gamma R	TGG TCT TCC CTT GGG GGA CCG AGG

Inoculation Procedures for sugar beet:

Immediately prior to inoculation of sugar beet, infected *C. quinoa* leaves were ground in a mortar to a slurry consistency with GKP buffer in liquid nitrogen. The youngest fully expanded sugar beet leaf was inoculated by rubbing the *C. quinoa* GKP slurry onto labeled leaves. Mock inoculated (1ml GKP Buffer) plants were also inoculated as a control.

RT-PCR for sugar beet

One hundred mg leaf tissue was collected from the edge of the leaf to be inoculated on the day prior (time zero) and at 14 days post- inoculation for confirmation of virus in inoculated leaf tissue. RNA extraction and RT-PCR confirmation protocols were as described for *C. quinoa* except that 100 fold more RNA template (500 ng) was needed for sugar beet due to lower efficiency of infection in sugar beet. While the typical duration of a VIGS experiment is 28 days post-inoculation, our plants were held until 35 days post treatment to insure that any change in phenotype would be observed.

Results

Silencing Target Sequence

All four target sequences were successfully ligated into the pBluescript SK series plasmid vector (FERMENTAS INC., Glen Burnie, MD) and sequence confirmation verified this event. Subsequent insertion into D4MCSb containing gamma BSMV was also verified with *Hind*III digestion for Seq1, Seq3 and Seq4. Multiple attempts for Seq 2 were made, but were unsuccessful. (Figure 1.)

Inoculation and RT-PCR in *C. quinoa*

C. quinoa infection was successful as indicated by RT-PCR. The virus was able to replicate *in vivo* and exhibit local lesions as would be expected for both BSMV and BSMV+Seq4 (Figure 2). The quinoa maintained the target sequence in most inoculation events. Of 6 events, 4 had at least one quinoa plant that maintained the desired insert. To confirm that the virus was not systemic, adjacent non-infected leaves were also screened for virus and none was detected (data

not shown). Infected *C. quinoa* leaves at approximately 10-14 days post inoculation were used to infect sugar beet leaves.

Figure1. Example of Target Seq1 *Hind*III digestion to confirm presence of insert in D4MCSb. Lane1 and 20–100bp marker, Lane2 is DM4Sb; Lanes 3, 8, 9, 13, 15, 17 and 19 were unsuccessful transformants, Lanes 4-7,10-12,16 and 18 were successful with inserts of the expected size. Lane 14 is a contaminant.

Figure 2. Lane 1 and 6, 100bp marker; Lane 2, quinoa mock with no product; Lane 3, quinoa inoculated with BSMV showing lesions (400bp); Lane 4, quinoa inoculated with BSMV+Seq4 (485bp); Lane 5 negative control (H20 only template).

Inoculation of mature sugar beet plants did not cause flowering

Preliminary VIGS experiments were conducted using mature plants (15-40 leaves). Eighty plants were inoculated with BSMV+Seq1 and 9 plants were inoculated with BSMV+Seq3. Mock-treated and BSMV-only treated plants were included as negative controls. No plants flowered in any treatment category thus development of an RT-PCR assay was necessary to confirm the presence of the virus and the status of the target insert within the virus in a second round of experiments conducted using young plants.

VIGS target sequences were lost following inoculation of young sugar beet plants

Fifteen of the 56 plants inoculated at the 4 leaf stage were assayed for the presence of BSMV+Seq4 using RT-PCR. None of these plants flowered within 35 days after inoculation. Using the RT-PCR assay, the 15 plants were shown to have virus present, but none appeared to maintain the viral target sequence (Figure 3, middle gel). Moreover, RT-PCR products varied in

intensity, which may indicate non-uniform levels of infection among plants inoculated with the same construct and procedure.

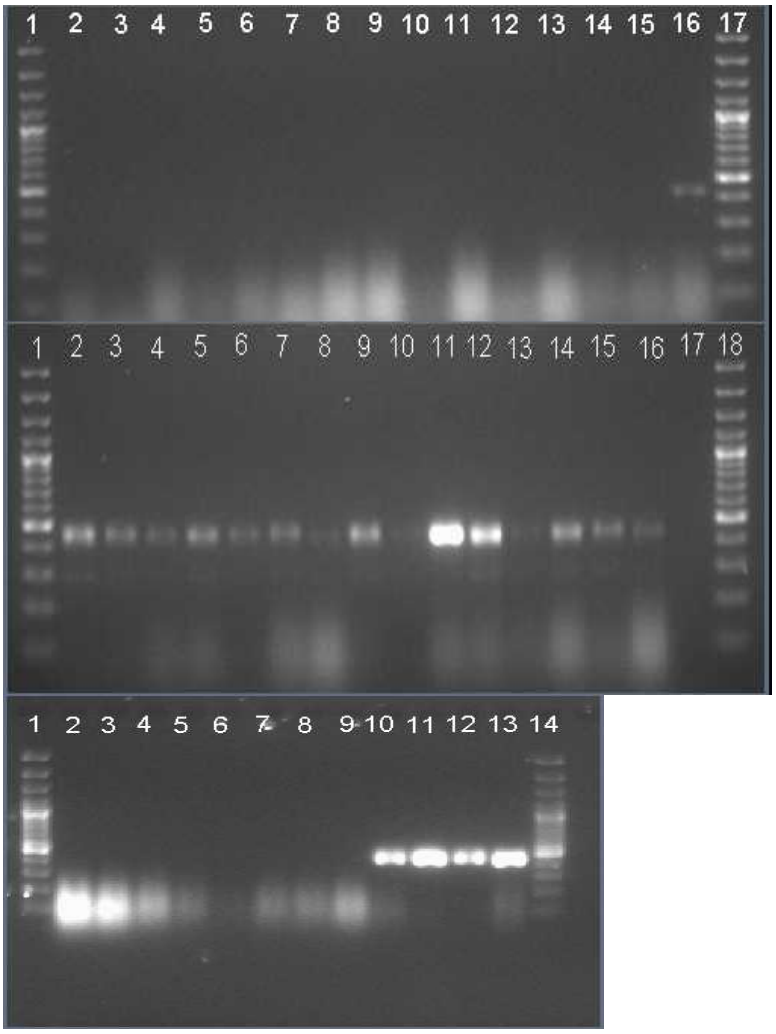

Figure 3. **Top gel:** Lanes 2-16, mock-inoculated plants 14days post inoculation with buffer only. Positive result in lane16 is presumably contamination. **Middle gel:** Lanes 2-16, presence of virus in plants 14day post inoculation with BSMV+Seq4. Note that positive bands are the same size as those found in plants inoculated with BSMV only (bottom gel), suggesting that the target sequence insert was lost following inoculation. Lane 17, H_20 only control. **Bottom gel:** Lanes 2-9, 8 plants time zero, Lanes 10-13, 14 day post inoculation with BSMV.

Discussion:

In vitro Transcription and RT-PCR in quinoa

I*n vitro* transcription of RNA, and subsequent inoculation in *C. quinoa,* was technically demanding because of the fragility of RNA. Once appropriate decontamination practices and handling of samples and inoculations were made, the successful inoculation rate increased. RT-PCR results showed that most (about 66%) of *C. quinoa* infections maintained the desired insert.

Efficacy of BSMV for VIGS of *BvFL1* in sugar beet

No plants flowered within 35 days post inoculation, regardless of the age of the plant at time of inoculation. Although RT-PCR confirmed infection of sugar beet plants inoculated with BSMV silencing constructs, the target sequence inserts, which direct the suppression of a desired gene, appeared to be lost during the infection process. RT-PCR products for BSMV+Seq4 inoculated plants were the same size as products from plants inoculated with BSMV only. (Figure 3 middle and lower gels). Thus BSMV may not maintain the extra target sequences efficiently following inoculation.

The VIGS system described here, which used BSMV to deliver a cue to silence the *BvFL1* gene in sugar beet, is not likely to be an effective breeding tool, as this system is technically difficult and labor intensive because it requires the progress of infections to be monitored using RT-PCR. Apparent variability in the severity of infection and loss of the inserted target sequence suggests that BSMV is not optimal for VIGS in sugar beet. VIGS shows promise as a tool for research, but a more effective vector is needed if it is to become routinely implemented in sugar beet research and improvement programs.

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