Virus Induced Gene Silencing

a) Background

Virus-Induced Gene Silencing (VIGS) is a rapid method for gene function analysis that involves transient specific knock-down of expression (i.e. "silencing") of target genes followed by assessment of silencing phenotypes (Lee et al., 2012).

The most widely used vectors for VIGS in wheat are those derived from Barley stripe mosaic virus (BSMV), a plant virus with tripartite RNA genome that readily spreads throughout various organs and tissues following mechanical rub-inoculation onto the leaves. All three genomic RNAs, RNAα, RNAβ, and RNAγ, are required to cause infection. RNAγ has been modified to allow insertion of short (up to 350-nt) plant mRNA derived sequences. Infection of plants with the resulting recombinant virus induces a natural post-transcriptional gene silencing defence mechanism that targets the viral RNA but also the endogenous plant mRNA, having high level (> 70%) nucleotide identity with the plant sequence inserted into RNAγ, for degradation. Analysis of the phenotypic consequences of silencing helps to infer the function of the target plant gene.

BSMV-mediated VIGS could be applied to any wheat genotype of interest, and also likely to almost any gene of interest that is known to be expressed at least under certain conditions during the lifetime of a plant. This functional genomics tool is particularly useful in projects involving analysis of multiple candidate genes, for example those arising from map-based cloning projects (i.e. when identified physical intervals spanning genes or QTLs of interests contain more than one expressed gene) or those selected from RNA-Seq datasets based on specific patterns of expression suggestive of a role in phenotypic expression of a particular trait of interest. VIGS is also useful in wheat genotypes that are difficult or impossible to transform for stable gene overexpression or RNAi, and in those for which mutant/TILLING populations are as yet to be developed. Moreover, VIGS could accelerate gene function analysis even in model wheat genotypes, such as Chinese Spring, Cadenza, or Kronos, as this tool allows simultaneous silencing of all homoeologous copies of each gene followed directly by phenotyping without the need for any further manipulation e.g. establishment of genetic crosses. As VIGS exploits plant natural homology-dependent RNA degradation pathways, this tool, in principle, could also be used for simultaneous silencing and attributing a function to the entire small gene families. In some cases, it may also be possible to silence (1) individual homoeologous gene copies by targeting mRNA 5'- or 3' untranslated regions (UTR) as the nucleotide sequence identity across these regions between the homoeologous gene transcripts is often much lower than between the coding gene sequences (CDS), or even (2) specific alternative transcripts of the same gene by targeting the sequences originating from alternative exons.

b) Protocol for VIGs

VIGS Constructs Design

As gene silencing is homology-based, the VIGS constructs should be carefully designed to maximise silencing of target gene(s) and minimize potential off-target gene silencing. To select sequence regions suitable for designing RNAi constructs that fit the above criteria we recommend an open source downloadable software si-Fi (siRNA Finder). This easy to use software also predicts silencing efficiency for selected RNAi constructs.

1. Download the latest version of si-Fi (currently siFi21) from http://labtools.ipk-gatersleben.de/ and install it to your computer (Note: this software is only available for Windows)
2. Download the IWGSC RefSeq v1.1 cDNA sequence for your gene of interest in FASTA format from Ensembl Plants.
3. Build a local wheat cDNA sequence database in si-Fi using the ''Create new Database'' option in the "Database management" tab.
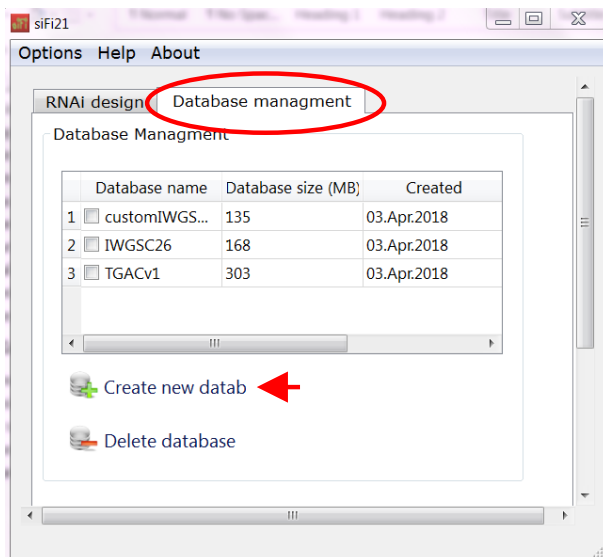

Figure 1: Building a local wheat cDNA sequence database on si-Fi for RNAi design and Off-target prediction

4. In "Options" select an "RNAi design" mode, paste the cDNA or CDS of your gene into the sequence box, and click the "Start" button. We recommend using the software's default settings.
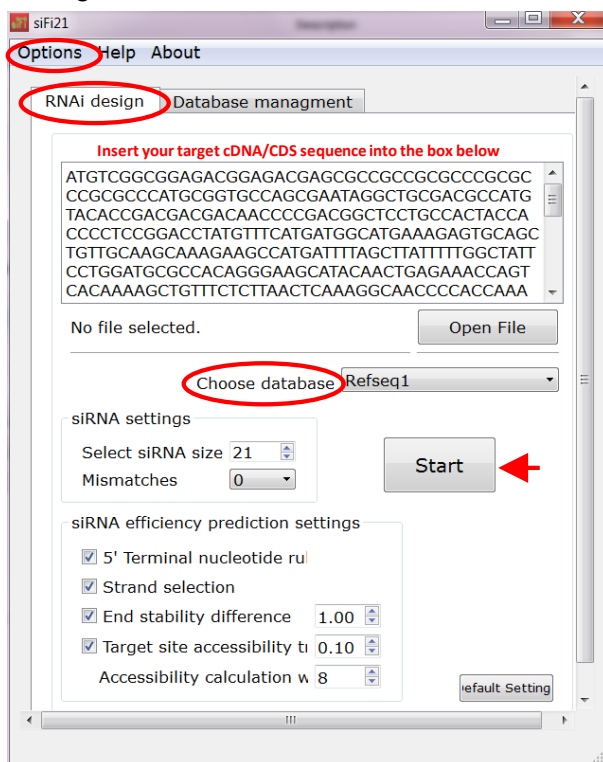

Figure 2: RNAi design using si-Fi

5. When a new window pops up listing all cDNAs targeted by siRNAs potentially generated from your input cDNA/CDS, select all true targets and click "OK". In the example below all selected transcripts (including alternative transcripts) are from the three homoeologous copies of the wheat *TaRar1* gene.
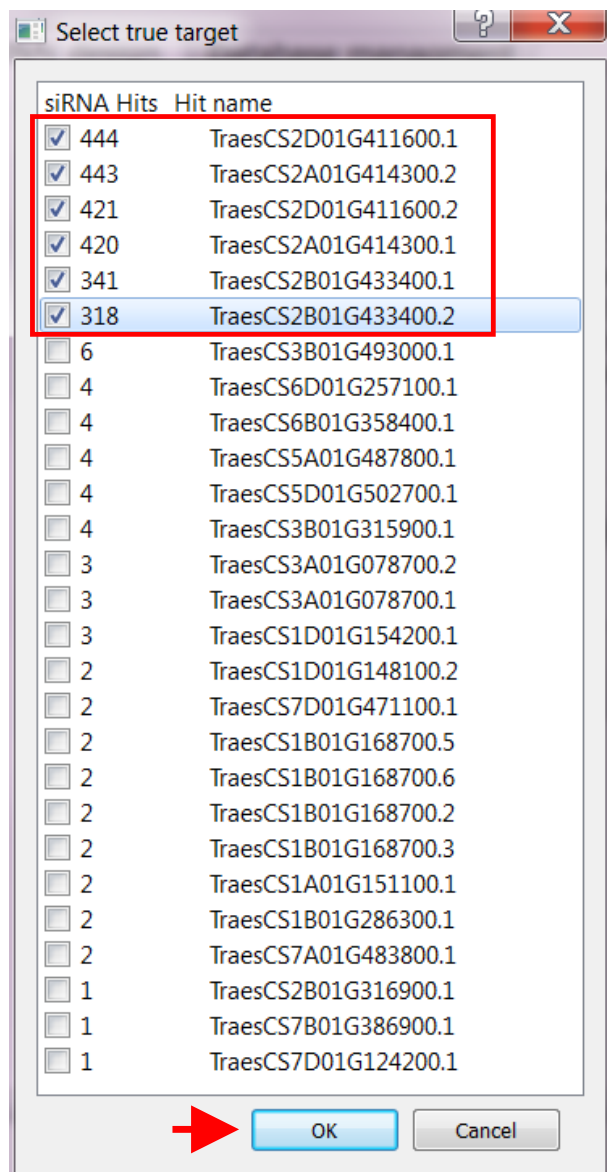

Figure 3: Identification of wheat mRNAs potentially targeted for silencing by siRNAs derived from a single RNAi construct

6. A new window presenting an RNAi design plot will pop up. Use this to select regions for use in VIGS. Whenever possible avoid selecting the regions producing siRNAs that can target genes other than your intended target. The regions to avoid are shown in pink. Also, try to select 150-350 nt regions predicted to produce silencing efficient siRNAs (shown in red). In the example shown below one of the regions that fits the above criteria lies between the nucleotides 220-530.
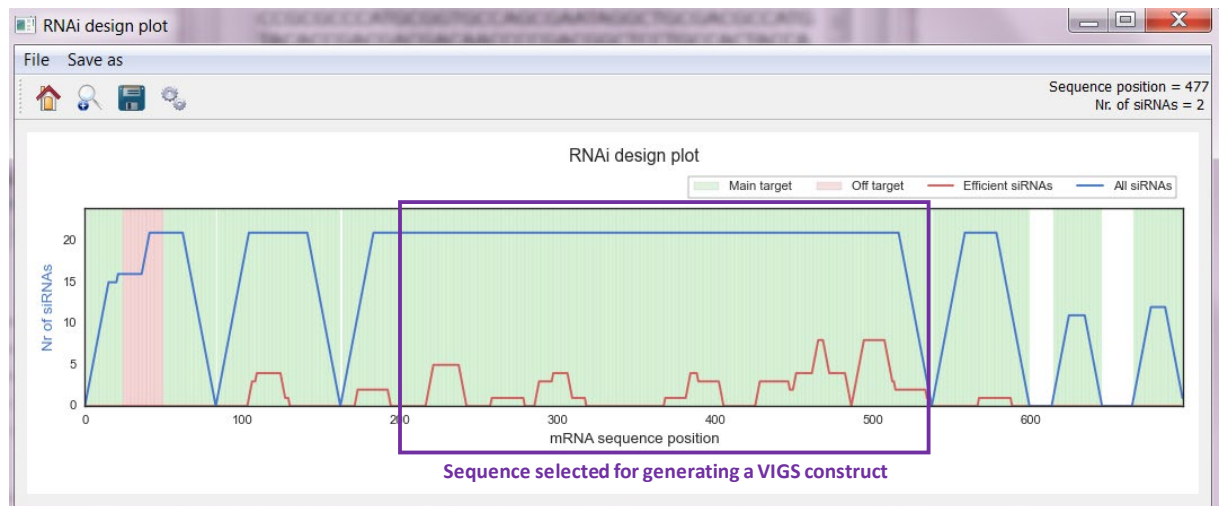
Figure 4: Selecting best mRNA sequence regions for developing VIGS constructs

7. In "Options" select an "Off-target prediction", and paste the selected candidate VIGS fragment (in our example, it is a 311-nt region spanning nucleotides 220-530 on *TaRar1* CDS) into the sequence box, then click the "Start" button.
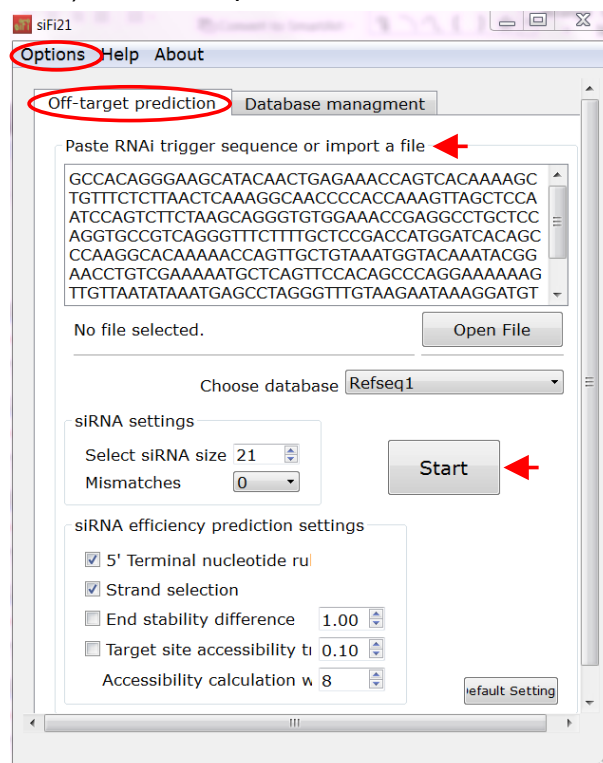
Figure 5: Off-target prediction using si-Fi

8. A new pop-up window will appear reporting numbers of predicted siRNA hits (total and efficient) to each corresponding target and off-target (if any) in the tabular and graphic formats.
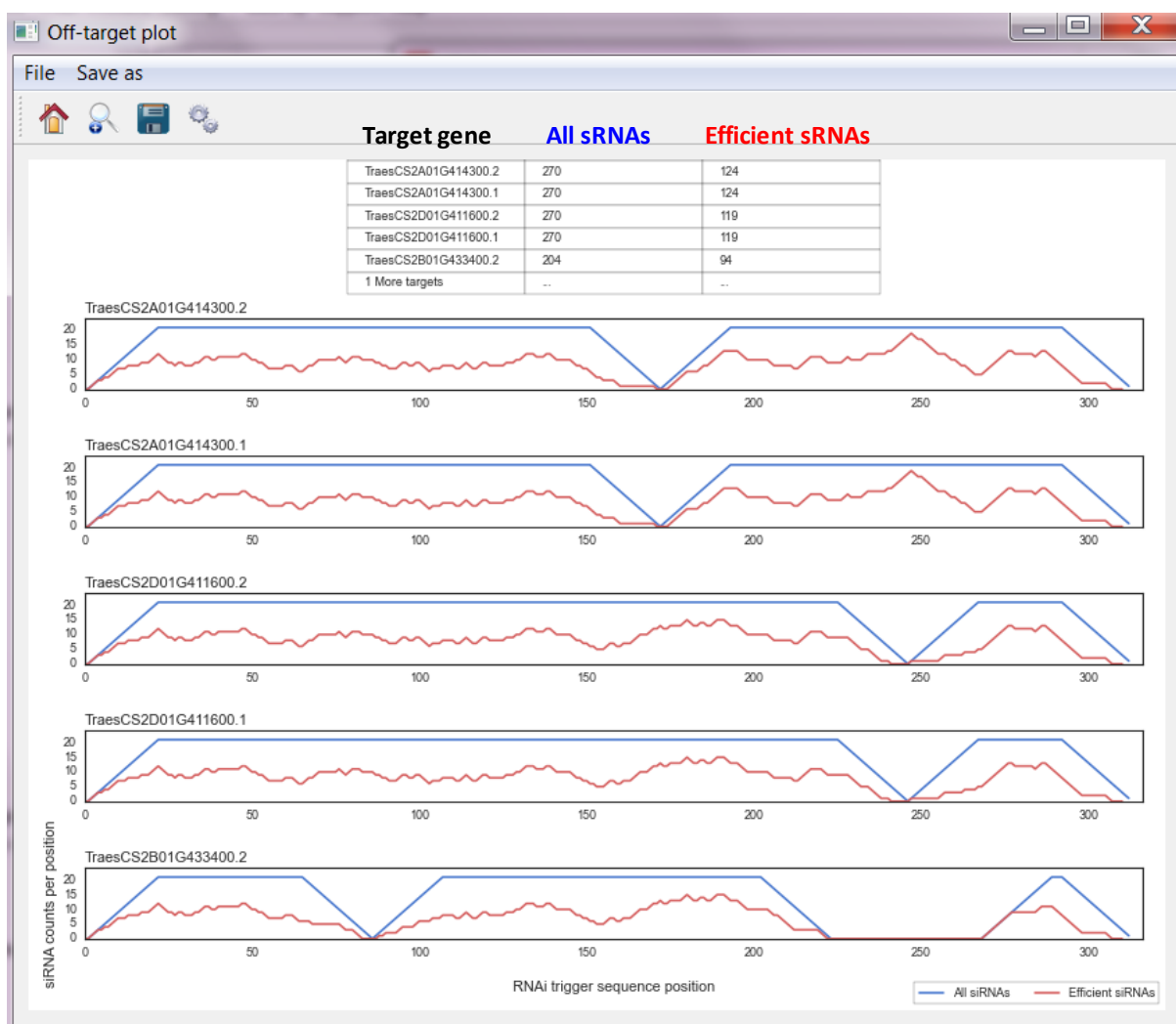
Figure 6: Off-target plot

Generation of BSMV VIGS Constructs

There are several versions of BSMV vectors available for VIGS with some requiring in vitro production of viral RNA genomes and others allowing production of viral RNA directly in planta. We prefer the latter, utilising the BSMV VIGS system developed by Yuan et al., (2011) and comprising the three binary vectors pCaBS-α, pCaBS-β and pCa-γbLIC, that contain full-length cDNAs corresponding to the wild-type RNAα, RNAβ, and modified RNAγ cDNA, respectively, flanked at the 5'-end by the double CaMV 35S promoter and at the 3'-end by a ribozyme sequence derived from another plant virus.

A gene fragment selected using the si-Fi software can be cloned in either antisense or sense orientation into a Ligation Independent Cloning (LIC) site of the BSMV RNAγ vector pCa-γbLIC using a LIC techniques as specified below. Note, there is some anecdotal evidence that antisense constructs are somewhat more efficient in inducing gene silencing.

1. PCR amplify the selected gene fragment either from the total tissue/organ/stage specific wheat cDNA or from the cloned/gene synthesised target cDNA (if available) using sequence specific primers carrying 5' extensions permitting LIC into the RNAγ vector. Add the following sequence extensions to the 5'-ends of the PCR primers: 5'-AAGGAAGTTTAA-3' (F-primer) and 5'-AACCACCACCACCGT-3' (R-primer).

2. Purify the resulting PCR product following an agarose gel-electrophoresis using QIAquick Gel Extraction Kit from Qiagen or similar products from other manufacturers.

3. Linearize pCa-γbLIC with ApaI at 25°C for >2 h. Inactivate the enzyme by incubating at 65°C for 20 min.

4. Treat both the gel-purified PCR product and linearized pCa-γbLIC with T4 DNA polymerase to generate the complimentary sticky ends as follows:

   a. *Incubate 200-250 ng of the PCR product at 22°C for 30 min in a total volume of 10 µl with 0.6 U of T4 DNA polymerase in 1x reaction buffer supplemented with 100 ng/µl bovine serum albumin and 5 mM dATP.*

   b. *Incubate 500 ng of linearized pCa-ybLIC at 22°C for 30 min in a total volume of 50 µl with 3 U of T4 DNA polymerase in 1x reaction buffer supplemented with 100 ng/µl BSA and 5 mM dTTP.*

   c. *Heat-inactivate the T4 DNA polymerase at 75°C for 15 min.*

5. Mix together 10 µl and 2 µl of T4 DNA polymerase treated PCR product and pCa-ybLIC, respectively, and heat to 65°C for 2 min. Allow the complementary sticky ends of the VIGS vector and the PCR product to anneal by incubating the mixture for 10 min at room temperature. Store any linearized and T4 DNA polymerase pre-treated vector at -20°C for future use.

6. Use an 1.5-2 µl aliquot for transformation of 20 µl chemically competent *E. coli* JM109. Plate the entire transformation mixture onto one or two LB agar plates supplemented with kanamycin (50 µg/ml), and incubate at 37°C overnight.

7. Pick 6-8 colonies and identify those carrying pCa-γbLIC containing the desired target plant sequence using a colony PCR with primers 2235.F (5'-GATCAACTGCCAATCGTGAGTA-3') and 2615.R (5'-CCAATTCAGGCATCGTTTTC-3') flanking the LIC site in pCa-γbLIC. This primer pair spans a region of 381-bp in the original pCa-γbLIC vector, whereas PCR products originating from colonies carrying recombinant vectors should be between 150-350 bp longer (depending on the size of the inserted plant sequence).

8. Miniprep plasmid DNA from 2 independent PCR-verified transformants and confirm the vector integrity by Sanger sequencing with the primers 2235.F and 2615.R.

# Production of recombinant BSMV

The binary BSMV vectors can be delivered to plants using *Agrobacterium tumefaciens* mediated inoculation. As wheat is a poor host for *Agrobacterium*, the VIGS procedure involves an additional step of a recombinant BSMV build-up in *Nicotiana benthamiana* (Figure 7). This model dicot is a good host for both *A. tumefaciens* and BSMV.
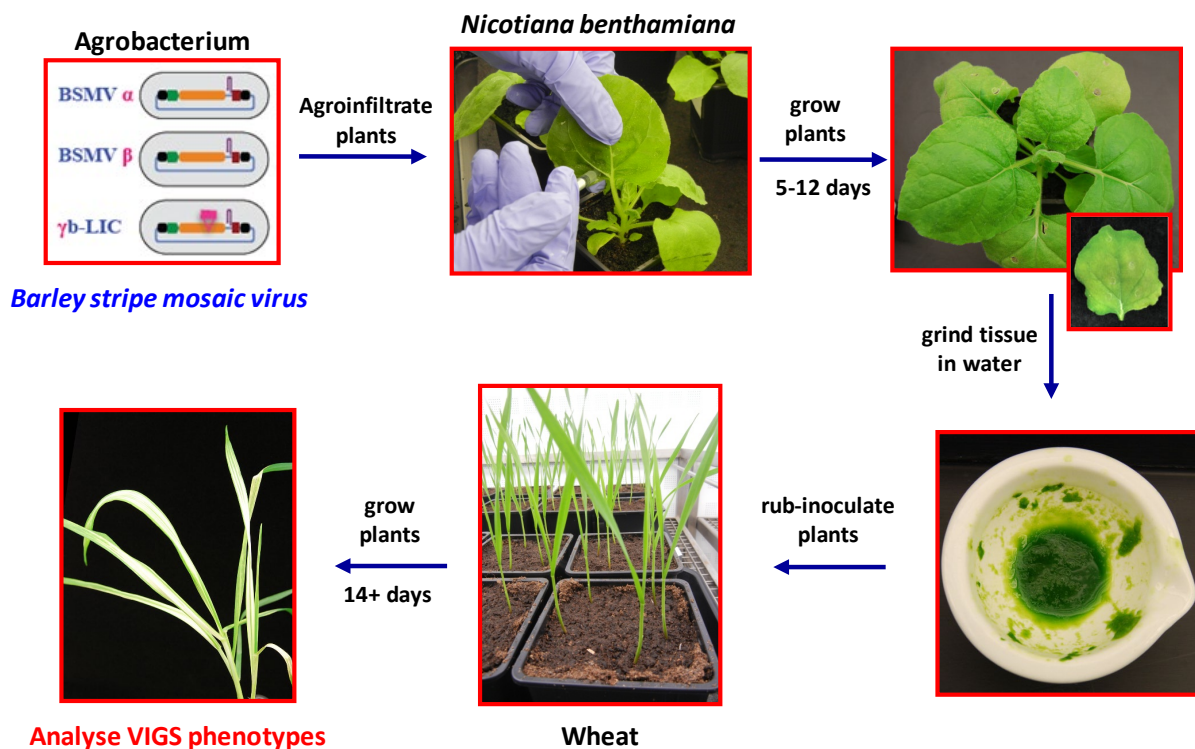
Figure 7: Schematic diagram of the VIGS procedure

1. Transform the sequence verified pCa-γbLIC VIGS construct into *A. tumefaciens* strain GV3101 using electroporation. Home-made electro-competent cells can be prepared for example using a procedure described in Lin (1995). We use the MicroPulser from Bio-Rad and 0.1 cm gap cuvettes for electroporation of 25-30 µl aliquot of competent cells using one 2.2 kV pulse.

2. Plate an aliquot of the transformation mixture on LB agar supplemented with 25 µg/ml gentamycin and 50 µg/ml kanamycin and incubate the plates for 2-4 days at 28°C until *A. tumefaciens* colonies appear.

3. Use the same procedure to produce *A. tumefaciens* GV3101 strains containing pCaBS-γ and pCaBS-β.

4. Consider also developing new or using pre-existing pCa-γbLIC constructs that can be used as negative or positive controls for VIGS. Negative control constructs should carry 150-350-nt sequences showing no homology to any of the wheat mRNA, such as for example fragments of GFP or GUS genes from *Aequorea victoria* or *E. coli*, respectively. pCa-γbLIC constructs targeting wheat *TaPDS* or *TaChlH* genes, available from our lab or elsewhere, may be useful as positive controls as these induce striking photobleaching or yellow-orange coloration of the silenced tissue (Figure 8) due to depletion of enzymes involved in biosynthesis of carotenoid pigments or chlorophyll, respectively (Lee *et al.*, 2012).
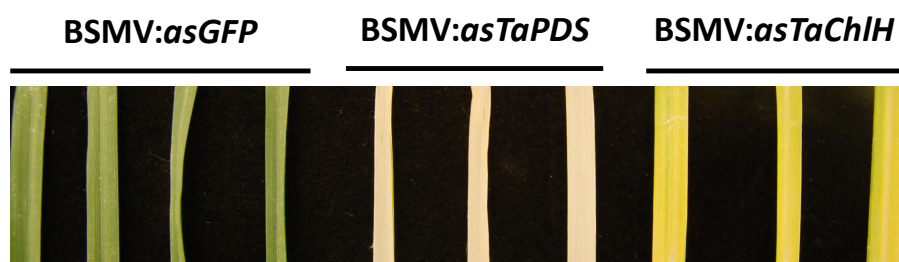
Figure 8: Phenotypes induced by negative and positive control VIGS constructs in wheat leaves.

BSMV:*asGFP* has no targets in wheat and only induces mild viral mosaic. On the other hand, silencing of genes involved in chlorophyll and carotenoid biosynthesis (*TaPDS* - phytoene desaturase) or in chlorophyll biosynthesis (*TaChlH* - magnesium chelatase subunit H) induces strong photobleaching or yellowing, respectively.

5. Grow from single colonies 5-ml liquid cultures (LB supplemented with 25 µg/ml gentamycin and 50 µg/ml kanamycin) of individual *A. tumefaciens* strains carrying pCa-γLIC VIGS constructs, pCaBS-γ and pCaBS-β overnight at 28°C with constant shaking at 220 rpm.

6. Pellet the *A. tumefaciens* cells at 2500 rcf for 20 min in a Centrifuge 5810 R (Eppendorf) or similar, and re-suspend the pellets in the infiltration buffer [10 mM MgCl2, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.6] supplemented with 150 µM acetosyringone to a final optical density of 1.5 at 600 nm. Incubate the cultures at room temperature without shaking for ≥3 h.

7. Combine equal volumes of *A. tumefaciens* strains carrying pCa-γLIC VIGS construct, pCaBS-γ and pCaBS-β and pressure infiltrate the bacteria into the abaxial side of fully expanded leaves of 25-30 days old *N. benthamiana* seedlings using a 1-mL needleless syringe (Figure 7). Approximately 0.5-1 ml of *Agrobacterium* suspension is required for infiltration of the whole area of one leaf.

8. In case of successful agroinfiltration, BSMV induced mosaic symptoms should become clearly visible in the upper uninoculated leaves by 7-9 days post agroinfiltration. Harvest directly infiltrated or the first systemically virus-infected *N. benthamiana* leaves at 5-12 or 7-12 days post agroinfiltration, respectively. These tissues can be used fresh for preparing the virus inoculum or stored at -80°C following snap freezing in liquid nitrogen for wheat inoculation at later dates.

Inoculation of wheat with BSMV and induction of gene silencing

When designing a VIGS experiment in wheat it is important to take into consideration that it takes approximately 2-3 weeks for gene silencing to be initiated after plants inoculation with BSMV VIGS constructs. Moreover, BSMV-mediated VIGS is relatively transient and the gene silencing is particularly efficient in the first 2-4 systemically infected leaves developed above the inoculated leaves and in the intermediate stem tissues when young plants are inoculated. The gene silencing in newer leaves often becomes patchy and incomplete. However, as the BSMV infection of wheat plants of different stage of development (up to and including the ear emergence Growth Stage GS50-59) is robust in most of the casers it should be possible to time the virus inoculation appropriately as to allow VIGS and hence analysis of the induced phenotypes in either young, older, or maturing plants. For instance, by inoculating flag or penultimate leaves of wheat plants in the mid to late

booting stage (GS45-49) it is possible to trigger gene silencing in wheat ear / flowering tissues and likely also in the seed (Lee et al. 2012). Note that there may be some variation in the efficiency and/or persistence of VIGS amongst the different wheat genotypes, and for example in a rapidly developing wheat USU Apogee BSMV-induced gene silencing initiated in the young 14-day old seedlings often persist throughout the developing plant and into the flag leaf and spike. Also, the level and persistence of silencing may also be dependent on the VIGS construct itself and for that reason it is advisable to test at least two independent VIGS constructs targeting different (preferably non-overlapping) regions of the same target mRNA/gene CDS.

1. Pre-germinate wheat seeds on pre-wetted filter paper or sterile sand. Select germinated seeds with root radicles at a similar point of emergence to equilibrate maturity of plants to be used in virus inoculations.

2. Plant the germinated seeds in pots filled with plant growth media/compost suitable for cereals. As there may be some variation in the efficiency of VIGS from plant to plant and from experiment to experiment, aim to test at least 10-12 plants with each VIGS construct and replicate each experiment at least twice and preferably trice.

3. Grow wheat until the desired growth stage.

4. Grind virus-infected *N. benthamiana* leaf tissue using a prechilled mortar and pestle in water (or 10 mM potassium phosphate buffer pH 7) containing 1-2% w/v Celite 545 AW (Sigma-Aldrich) abrasive until a fully homogenous slurry/sap is produced (Figure 7). Use 2.5-3 ml of buffer per each 1 g of *N. benthamiana* leaf tissue.

5. Inoculate either one or the two consecutive youngest fully expanded leaves with the sap as follows. Hold the lower stem of the plant firmly with one hand, dip forefinger of the other hand in the sap inoculum and give each leaf 3-6 gentle firm passes between forefinger and thumb. A slight squeaking sound should be audible as the wax is being scraped-off from the leaves. No sound may indicate you are rubbing far too gently, and this often leads to poor or no viral infection. But also, important not to squeeze the leaves too hard as this may terminally damage the leaves and consequently also result in no viral infection.

6. Let the plants absorb the virus for 5-10 min before gently spraying the leaves using a fine mist water spray bottle.

7. Cover plants with plastic bags, propagator lids or keep plants in suitably sized plastic boxes in low light or dark overnight to allow recovery from the rub-inoculation stress. No or little damage to the leaves should be visible the following day.

8. Grow plants under conditions favouring BSMV-mediated VIGS, which are: day/night temperature of 23-24/20°C, 16 h photoperiod, and light intensity of 120-300 µmol·m–2·s-1. Under these conditions, the first symptoms of virus infection, typically light green or slightly yellowish short streaks dotted along the leaf blade, should appear on the upper uninoculated leaves by 7-11 days post inoculation whereas effects of gene silencing should become apparent on from 14-21 days post inoculation. Note that temperature is one of the critical environmental factors influencing VIGS.

Assessment of virus-induced gene silencing

Assess the efficiency of silencing of the wheat gene targets by phenotyping and / or using a quantitative reverse-transcription PCR (qRT-PCR).

The primers used for qRT-PCR should bind outside the mRNA region targeted for silencing to prevent amplification from the abundant recombinant viral RNA templates. Adhere to the standard guidelines for relative gene expression analyses (Bustin et al., 2009) when designing qRT-PCR assays. Carefully consider the wheat reference genes for target gene expression normalization. Select genes showing stable expression under a range of conditions in your experiments. We typically use *TaCDC48* (*TraesCS4A02G035700*) and TaeIF4E (*TraesCS3D02G527800*), as these genes show no or negligible change in expression in response to BSMV and many other biotic stresses.

Analyse several individual plants using qRT-PCR as the efficiency of VIGS can vary from plant to plant.