Chapter 22

A Cytological Analysis of Wheat Meiosis Targeted by Virus-Induced Gene Silencing (VIGS)

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Abstract

Virus-induced gene silencing (VIGS) is a rapid and cost-effective reverse genetic technology that can be used to assess gene function in wheat. This chapter contains a detailed description of how to target wheat meiotic genes by VIGS. The timing of this technique is critical and has been optimized to silence meiotic genes at peak expression, evidenced by silencing of *Triticum aestivum disrupted meiotic cDNA1* (*TaDMC1*). We also describe cytological techniques that have been adapted for the preparation and analysis of meiocytes in wheat, including fluorescent in situ hybridization (FISH) with directly labeled, synthetic oligonucleotide probes, and immunolocalization on spread material.

Key words VIGS, Meiosis, Cytology, Wheat, FISH, Chromosomes, Immunolocalization

1 Introduction

The model diploid plant *Arabidopsis thaliana* has enabled the identification and characterization of over eighty genes required to ensure accurate homologous recombination, synaptonemal complex formation, and correct chromosome segregation during meiosis [1, 2]. As sequencing technologies have improved over the past 5 years, a considerable number of genomes are now available on open databases, such as Ensembl (http://plants.ensembl.org/index.html) and Phytozome (http://phytozome.jgi.doe.gov), so that orthologous genes in wild species and crops may be identified. In particular, completion of the draft allohexaploid bread wheat (*Triticum aestivum*) genome ($2n = 6x = 42$, AABBDD) has provided opportunities for a bioinformatic analysis to be performed [3], and consequently key meiotic genes that are essential for mediating crossover formation can be identified. Approaches and technologies to investigate gene function by mutagenesis have concurrently been developed in bread wheat including the extensive panel of TILLING lines [4] and gene targeting approaches including CRISPR/Cas (Subheading 3 in [5]). These techniques

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are extremely effective at creating null mutants, and the TILLING lines may also produce hypomorphic phenotypes. The major limiting factor is the time and cost required to produce such knockouts, which limits the number of genes that can be screened. An alternative approach for investigating meiotic orthologous gene function in wheat is to utilize virus-induced gene silencing (VIGS) [6–9]. This provides a rapid, efficient approach for determining gene function in wheat, so further analyses using TILLING or CRISPR/Cas may be employed on promising targets. Therefore, in this chapter we describe the methodology of how to generate VIGS knockdowns of meiotic genes in wheat along with the approaches used to analyze the phenotype cytologically.

2 Materials

2.1 Plant Material and Cultivation

1. Hexaploid bread wheat (*Triticum aestivum*) cultivar Bobwhite is routinely used for VIGS experiments due to its susceptibility to barley stripe mosaic virus (BSMV) infection, but many other genotypes can also be utilised.
2. Tobacco (*Nicotiana benthamiana*) is used as an intermediate host.
3. The plants are grown in a Level 3 biological containment facility under controlled environmental growth room conditions: photoperiod 16 h, temperature 20 °C (night) and 23 °C (day), light intensity (at the soil level) ~180 μmol m^{-2} s^{-1}, and relative humidity ~60%.

2.2 Virus Induced Gene Silencing (VIGS)

2.2.1 BSMV VIGS Binary Vectors Are Described in [10]

1. pCaBS-α: plasmid for BSMV RNA alpha; contains kanamycin resistance gene for selection in bacteria.
2. pCaBS-β: plasmid for BSMV RNA beta; contains kanamycin resistance gene for selection in bacteria.
3. pCa-γbLIC: plasmid for BSMV RNA gamma; contains a ligation independent cloning (LIC) site for insertion of wheat gene fragments for VIGS, and kanamycin resistance gene for selection in bacteria.

2.2.2 VIGS Construct Design

1. Software si-Fi version siFi21 (downloadable from http://labtools.ipk-gatersleben.de/).
2. An entire wheat cDNA annotation TGACv1 or IWGSC RefSeq v1.0 in FASTA format (downloadable from Ensembl Plants http://plants.ensembl.org/Triticum_aestivum/Info/Index, or from the Wheat Portal at URGI https://wheat-urgi.versailles.inra.fr/Seq-Repository/Annotations, respectively).

2.2.3 PCR and Purification of PCR Products

1. PCR of target sequence for VIGS: ~100 ng of template DNA, 1 U of high-fidelity DNA polymerase (e.g., Phusion DNA Polymerase), 1× reaction buffer, 0.4 mM dNTPs, and 0.4 μM primers containing the following 5′ sequence extensions: 5′-AAGGAAGTTTAA-3′ (F-primer) and 5′-AACCACC ACCACCGT-3′ (R-primer).
2. DNA gel extraction kit (e.g., QIAquick Gel Extraction Kit (Qiagen) or similar).

2.2.4 Ligation-Independent Cloning

1. T4 DNA polymerase mixture for vector: 200 ng of *Apa*I-digested pCa-γbLIC DNA, 5 mM dTTP, 1× BSA, 1× NEBuffer 2.1, 1.2 U of T4 DNA polymerase. For 20 μL: add 5 μL of ApaI-digested pCa-γbLIC, 1 μL of 100 mM dTTP, 0.2 μL of 100× BSA, 2 μL of 10× NEBuffer 2.1, 0.4 μL of 3000 U/mL T4 DNA polymerase, and make up to 20 μL with ddH_20.
2. T4 DNA polymerase mixture for insert: 200–250 ng of PCR product, 5 mM dATP, 1× BSA, 1× NEBuffer 2.1, 0.6U of T4 DNA polymerase. For 10 μL: add *x* μL of PCR product, 0.5 μL of 100 mM dATP, 0.1 μL of 100× BSA, 0.2 μL of T4 DNA polymerase, and make up to 10 μL with ddH_2O.

2.2.5 Generation of Recombinant BSMV RNA Gamma Constructs

1. Chemically competent *E. coli* strain JM109 or DH5α.
2. Super Optimal broth with Catabolite repression (S.O.C.) medium: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. Add glucose after autoclaving broth with the remaining ingredients and sterilize the final solution by passing it through a 0.2 μm filter.
3. Lysogeny Broth (LB) agar plates supplemented with kanamycin: agar (15 g/L), NaCl (10 g/L), tryptone (10 g/L), yeast extract (5 g/L), kanamycin (50 μg/mL), pH to 7.0 with 5 N NaOH.
4. Liquid LB medium supplemented with kanamycin: NaCl (10 g/L), tryptone (10 g/L), yeast extract (5 g/L), kanamycin (50 μg/mL), pH to 7.0 with 5 N NaOH.
5. Colony-PCR: 1 U of *Taq* DNA polymerase, 1× reaction buffer, 0.4 mM dNTPs, and 0.4 μM primers (2235.F and 2615.R).
6. Colony-PCR/sequencing primers: 2235.F (5′-GATCAACTG CCAATCGTGAGTA-3′) and 2615.R (5′-CCAATTCAGG CATCGTTTTC-3′).
7. Miniprep plasmid DNA Kit.

2.2.6 Transformation of BSMV Vectors into Agrobacterium tumefaciens

1. Electrocompetent *A. tumefaciens* strain GV3101 (or EHA105).
2. Electroporator and cuvettes.
3. LB agar plates supplemented with kanamycin (50 μg/mL) and gentamycin (25 μg/mL).

2.2.7 *Agroinfiltration of* Nicotiana benthamiana *Seedlings*

1. Liquid LB medium supplemented with kanamycin (50 μg/mL) and gentamycin (25 μg/mL).
2. Infiltration buffer: 10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES; pH 5.6), 150 μM acetosyringone.
3. Young 25–30-day-old *N. benthamiana* seedlings.
4. 1-mL needleless plastic syringes.

2.2.8 *Inoculation of Wheat Plants with BSMV*

1. 26–30 days old (4–4.5 leaf stage) wheat cultivar Bobwhite plants (Fig. 1).
2. Prechilled mortars and pestles.
3. Distilled or reverse osmosis water, or 10 mM potassium phosphate buffer pH 7.

Fig. 1 Bread wheat cultivar *Bobwhite* at the 4–4.5 leaf stage (~28 days post-sowing) ready for inoculation with BSMV. First symptoms of viral infection (yellow spots or stripes) will appear on upper leaves 5–11 days postinoculation, and target genes are silenced at ~14 days postinoculation. Photograph courtesy of Wing Sham Lee

4. Celite 545 AW (Sigma-Aldrich) abrasive.
5. Suitably sized plastic bags or incubation boxes large enough to accommodate individual or groups of inoculated plants.

2.3 Preparing Meiotic Spreads

1. Fixative solution: three parts of absolute ethanol to one part of acetic acid fixative (v/v). Prepare fresh.
2. Citrate buffer (0.01 M): 445 µL of 0.1 M sodium citrate, 555 µL of 0.1 M citric acid, made up to 10 mL with ddH_2O, pH 4.5.
3. Enzyme solution: 1% cellulose, 1% pectolyase in 0.01 M citrate buffer, pH 4.5. Store in 333 µL aliquots at −20 °C. Working concentration: mix 333 µL of enzyme solution with 667 µL of 0.01 M citrate buffer, pH 4.5.
4. Acetic acid (70%).
5. VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

2.4 FISH

1. Denaturation mixture: 50% formamide, 10% dextran sulfate, 2× SSC, made up to 40 µL per slide. For 40 µL: add 20 µL of 100% formamide, 8 µL of 50% dextran sulfate, 4 µL of 20× SSC, and make up to 40 µL with ddH_2O.
2. Saline–sodium citrate (SSC; 20×): 3 M sodium chloride, 0.3 M trisodium citrate, pH 7. For 1 L: dissolve 175.3 g of NaCl and 88.2 g of $Na_3C_6H_5O_7$ in 1 L of ddH_2O, adjust pH with a few drops of 14 N HCl. Sterilize by autoclaving. Dilute in ddH_2O to prepare 2× SSC.
3. Oligonucleotide probes directly labeled with fluorophores:
   Oligo-pSc119.2-2 [11]:
   5′-[A488]TTCCACGATTGACGATTCCGGGGGTGCGTTTACGTGTCCGTCGTC-3′.
   Oligo-pTa794–1:
   5′-[T×Rd]YRGAGTTCTGATGGGATCCGGTGCTTTAGTGYTGSTATGATCGCA-3′.
   Oligo-pTa71-2 [11]:
   5′-[A647]GGGCAAAACCACGTACGTGGCACACGCCGCGTA-3′.
4. Probe mixture: 10% dextran sulfate, 2× SSC, 50 ng of each probe. For 40 µL: add 8 µL of 50% dextran sulfate, 4 µL of 20× SSC, 2 µL of 25 ng/µL probe(s), and make up to 40 µL with ddH_2O (*see* **Note 1**).
5. 2T: 2× SSC, 0.2% Tween 20.
6. VECTASHIELD® Mounting Medium with DAPI.

2.5 Immunolocalization on Spread Material

1. Citrate buffer: 10 mM trisodium citrate, pH 7. For 500 mL: dissolve 1.47 g of trisodium citrate in 500 mL of ddH_2O, adjust pH to 7 using citric acid granules.
2. Phosphate-buffered saline (PBS; 1×): 1 preprepared tablet of PBS (100×) per 100 mL of ddH_2O. Prepare 500 mL.
3. PBST: 1× PBS, 0.1% Triton X-100. For 500 mL: add 0.5 mL of Triton X-100 to 499.5 mL of 1× PBS.
4. Blocking solution: 1× PBS, 3% Bovine Serum Albumen (BSA). For 10 mL: dissolve 0.3 g of BSA in 10 mL of 1× PBS.
5. Primary antibodies: make up to the preferred dilution (e.g., 1:200, 1:500) in 1× PBS containing 3% BSA. We typically use 1:500 for linear axis (e.g., ASY1, ASY3) and synaptonemal complex proteins (e.g., ZYP1), but 1:200 for focal recombination machinery proteins (e.g., MLH3, HEI10).
6. Secondary antibodies: make up to the preferred dilution (e.g., 1:200) in 1× PBST containing 3% BSA (e.g., goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 594, goat anti-guinea pig Alexa Fluor 647 (Invitrogen)).
7. VECTASHIELD® Mounting Medium with DAPI.

3 Methods

3.1 VIGS

1. Conduct an in silico analysis of the whole coding sequence (CDS) of the target meiotic gene using si-Fi21 and select 250–400 bp regions with good predicted silencing efficiency (*see* **Notes 2** and **3**).
2. Design primers to the selected region and amplify the target gene sequence by RT-PCR from wheat spike RNA or, if contained within a single exon, by PCR from total genomic DNA. Ensure ligation independent cloning (LIC) adaptor sequences are appended to the 5′ ends of the primers.
3. Resolve the amplicon by gel electrophoresis, excise the band and purify using a commercially available extraction kit.
4. Meanwhile, linearize 1 μg of pCa-γbLIC plasmid with *Apa*I at 25 °C for 2 h in a 25 μL reaction. Stop the reaction by heat inactivating the restriction enzyme at 65 °C for 20 min.
5. In separate tubes, treat ~200 ng of the *Apa*I-linearized pCa-γb-LIC vector and ~200–250 ng of the gel-purified PCR product with T4 DNA polymerase in 20 μL and 10 μL reactions, respectively, and incubate at 23 °C for 30 min (*see* **Note 4**). Stop the reaction by heat-inactivating the T4 DNA polymerase at 75 °C for 15 min.
6. To clone the insert into the vector, add 2 μL of treated vector (~20 ng) to 10 μL treated insert (200–250 ng), incubate at

65 °C for 2 min, and then leave at RT for 15 min to allow the annealing of complementary ends.

7. Transform the recombined plasmid into chemically competent *E. coli* cells, plate out and grow at 37 °C under kanamycin selection (50 μg/mL).
8. Confirm inserts in transformants by colony PCR with a pair of flanking primers, 2235.F and 2615.R, and verify by sequencing from isolated plasmid DNA.
9. Once verified, transform the pCa-γbLIC VIGS construct into electrocompetent *A. tumefaciens* cells with a 2.2 kV pulse on an electroporator, plate out and grow at 28 °C under gentamycin (25 μg/mL) and kanamycin selection (50 μg/mL).
10. In addition to BSMV RNAγ, α, and β genomic segments are also required for successful infection. It is therefore necessary to generate additional *A. tumefaciens* transformants with pCaBS-α and pCaBS-β (as in **step 9**).
11. Grow 5 mL cultures of *A. tumefaciens* overnight at 28 °C with constant shaking (220 rpm) under gentamycin (25 μg/mL) and kanamycin (50 μg/mL) selection. Three different transformants are grown, one containing the pCa-γbLIC VIGS construct, one containing pCaBS-α and one containing pCasBS-β.
12. Spin the overnight cultures down for 20 min at 2500 × *g*, discard supernatant, and resuspend the pellets in the infiltration buffer to a final OD_{600} of 1.5. Incubate suspension at RT, without shaking, for a minimum of 3 h.
13. Mix the α, β, and γ strains together at a 1:1:1 ratio, and then pressure infiltrate 0.5–1 mL of the suspension directly into the underside of *N. benthamiana* leaves using a needleless 1 mL syringe (*see* **Note 5**).
14. 3–5 days later collect directly infiltrated leaves from infected *N. benthamiana* plants, and grind them using a cold pestle and mortar with 3 mL of potassium phosphate buffer (or water) per g of leaf tissue.
15. To inoculate wheat plants dip forefinger into the sap inoculum, and gently rub each leaf between forefinger and thumb, 3–6 times. Let the plants absorb the virus for 5–10 min, mist with water, cover with plastic bags or place inside incubation boxes and keep under low light overnight to allow the plants to recover from the inoculation stress. Next day, return plants to standard growth conditions.
16. To knockdown meiotic genes wheat plants are inoculated 14 days prior to harvesting the anthers for cytological analysis, when the effects of VIGS are typically at its strongest. When using wheat cultivar Bobwhite inoculate the plants at the

~4–4.5 leaf stage (~28 days post-sowing; Fig. 1). Early anthers are ready for sampling by fixation from ~14 days post inoculation.

3.2 Preparing Meiotic Spreads

1. At the booting stage (~42 days post-sowing), harvest immature wheat spikes, and place them on a moist 9 cm Ø filter paper in a 9 cm Ø petri dish.
2. Dissect early anthers (0.5–1.5 mm length) from the spike with watchmaker's forceps and a fine mounted needle, and place them in 1 mL of fixative solution.
3. Replace the fixative solution after 1 h and again at the end of the day. Leave at RT for at least 24 h, and store at 4 °C for up to 3 months.
4. Transfer the anthers to a watch glass, remove the fixative solution, and wash with 2 mL of 0.01 M citrate buffer (3 × 5 min).
5. Remove the final wash, add 1 mL of enzyme solution, place a lid on the watch glass, and incubate at 37 °C for 60 min.
6. After an hour has passed stop the reaction by removing the enzyme solution and replacing it with 2 mL of ice-cold sterile distilled water. Keep the anthers on ice until ready to use.
7. Using a calibrated eyepiece graticule sort the anthers by size, typically into 0.1 mm piles.
8. Transfer ~6 anthers of the same size onto a slide, with a minimum amount of water, and quickly macerate them with a mounted needle.
9. Add 10 μL of 70% acetic acid to the material, mix into a suspension, place on a 45 °C hot plate, spread horizontally with a mounted needle, add a further 10 μL of 70% acetic acid, spread again, allow suspension to collect into a single droplet, and leave for 45 s.
10. Take the slide off the hot block, and add 200 μL of fixative solution in a ring around the material. Then pick the slide up, hold it at a 45° angle over a waste container, and add a further 200 μL of fixative solution directly over the entirety of the spread area.
11. Dry the preparation by blowing the back of the slide with a commercial hair dryer.
12. Mount the preparation in 10 μL of DAPI in Vectashield and apply a cover glass (24 × 40 mm; No. 1) to visualize immediately (Fig. 2a), or leave unmounted for downstream applications (*see* Subheadings 3.3 and 3.4). Unmounted slides can be kept for several months at −20 °C.

3.3 FISH

The meiotic spreads prepared in Subheading 3.2 can be used for FISH with oligonucleotide probes directly labeled with fluorophores.

1. Dehydrate the preparations by washing the slides in 100% ethanol (2 × 5 min) in a Coplin jar, remove the slides and allow to air-dry.
2. Add 40 μL of denaturation mixture onto the spread area, cover with a plastic coverslip (24 × 30 mm), and incubate on a 62 °C hot plate for 4 min to denature the chromosomal DNA.
3. Immediately snap chill the slides in a Coplin jar containing ice-cold 2× SSC, to prevent the DNA from reannealing, transfer to a 4 °C cold room and float off the coverslips.
4. Without delay remove slides from the ice-cold 2× SSC solution, drain off any excess, pipette 40 μL of probe mixture onto the spread area, cover with a plastic coverslip (24 × 30 mm), and incubate them overnight in a humid chamber at 37 °C.
5. The next day take the slides out of the 37 °C incubator, and float off the plastic coverslips in a Coplin jar containing 2× SSC.
6. Wash the slides in 2× SSC in a Coplin jar at 40 °C (3 × 5 min), and then in 2T in a Coplin jar at RT (2 × 2 min).
7. After completing the posthybridization washes remove the slides from the Coplin jar, drain off any excess 2T, mount in 10 μL of DAPI in Vectashield and apply a glass coverslip (24 × 40 mm; No. 1).
8. View the FISH preparations on a fluorescence microscope with filters for DAPI, FITC, Texas Red, and Cy5, and one that is equipped with an image capture and analysis system (Fig. 2b).

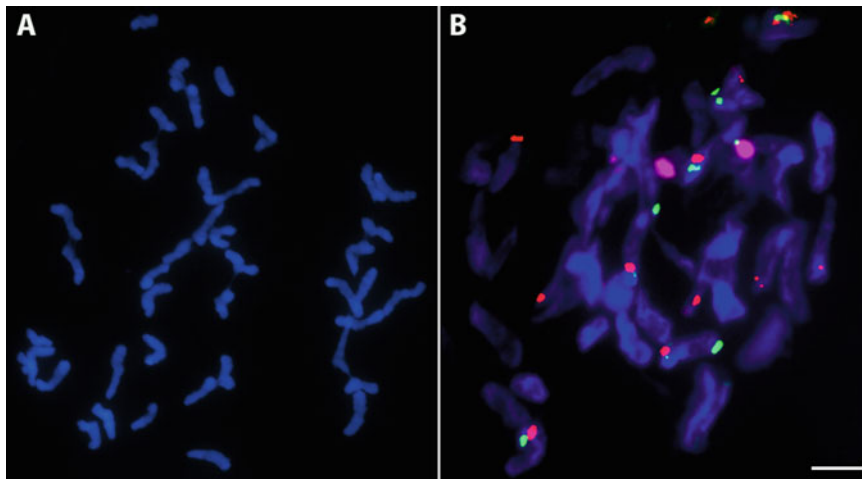

Fig. 2 Meiotic metaphase I chromosome spreads in cultivar *Bobwhite* BSMV:*asTaDMC1* VIGS knockdown lines. (**a**) DAPI only. (**b**) Probed by FISH with directly labeled synthetic oligonucleotide probes: pSc119.2-2 (green), pTa794-1 (red), and pTa71-2 (purple). The meiotic phenotype is variable between cells, but in the most severe knockdowns the majority of homologous chromosomes remain unpaired, visible as univalents. Scale bar = 10 μm

3.4 Immuno-localization on Spread Material

The meiotic spreads prepared in Subheading 3.2 are used for immunolocalization of meiotic proteins, adapted from [12].

1. Place the slides in a staining rack and, in a suitable container, wash in 1× PBS at RT (3 × 5 min).
2. While on the final wash, microwave citrate buffer on maximum power until boiling (typically ~5 min) and, while still bubbling, submerge the slides in it and leave for 45 s.
3. After 45 s has elapsed remove the slides, and wash immediately in 1× PBST at RT for 5 min.
4. Drain off any excess 1× PBST, add 50 μL of blocking solution to the spread area, cover with Parafilm, and incubate at RT for 30 min in a humid chamber.
5. Carefully remove Parafilm with tweezers, drain off any excess blocking solution, add 50 μL of primary antibodies at the desired concentration, cover with Parafilm, and incubate at 37 °C for 30 min (or overnight at 4 °C) in a humid chamber.
6. Carefully remove Parafilm and wash slides in a Coplin jar in 1× PBST at RT (2 × 5 min).
7. Drain off any excess 1× PBST, add 50 μL of secondary antibodies at the desired concentration, and incubate at 37 °C for 30 min in a humid chamber.
8. Carefully remove Parafilm and wash slides in a Coplin jar in 1× PBST at RT (2 × 5 min).
9. Drain off any excess 1× PBST, mount in 10 μL of DAPI in Vectashield, and apply a glass coverslip (24 × 40 mm; No. 1).
10. View the immunocytological preparations on a fluorescence microscope with filters for DAPI, FITC, Texas Red, and Cy5, and one that is equipped with an image capture and analysis system (Fig. 3).

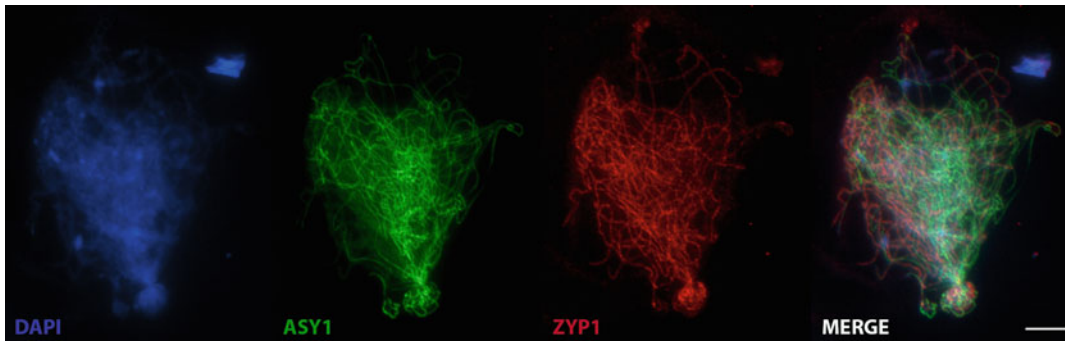

Fig. 3 Dual immunolocalization of ASY1 (green) and ZYP1 (red) to a late prophase I nucleus in a cultivar *Bobwhite* BSMV:*asTaDMC1* VIGS knockdown line, showing lengths of unaligned chromosomes that lack ZYP1. Scale bar = 10 μm

4 Notes

1. Formamide is deliberately omitted from the probe mixture as it can cause instability of probe–target hybrid molecules involving short oligonucleotides. It is present in the denaturation mixture as it is necessary to reduce the melting temperature of the chromosomal DNA, but must be washed away in ice-cold 2× SSC prior to the addition of the probes.
2. It is optimal to select and make VIGS constructs from at least two nonoverlapping fragments for each gene target. These provide independent tests of each other, and similar results with both constructs increases the confidence that the observed phenotype is due to specific silencing of the intended target gene rather than any off-target or aberrant effects.
3. In each experiment at least one negative control VIGS construct containing a 250–400 nt gene fragment of non–plant origin gene, such as the *Aequorea victoria Green Fluorescent Protein* gene (*GFP*), should also be included.
4. The 3′ → 5′ exonuclease activity of the T4 DNA polymerase will generate complementary overhanging ends between the vector and the insert.
5. Unlike wheat, *N. benthamiana* is fully susceptible to *A. tumefaciens* infection and is used to accumulate high levels of BSMV. Sap extracted from its leaves is then used to infect wheat plants.

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