Agrobacterium-Mediated Virus-Induced Gene Silencing Assay In Cotton

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Short Abstract (50 words)

We present the detailed protocol for Agrobacterium-mediated virus-induced gene silencing (VIGS) assay in cotton. The tobacco rattle virus (TRV)-derived VIGS vectors were deployed to induce RNA silencing of cotton *GhCLA1*, *Cloroplastos alterados 1* gene. The photobleaching phenotype caused by silencing *GhCLA1* was observed at the seedling stage within 2 weeks after inoculation.

Long Abstract (400 words)

Cotton (*Gossypium hirsutum*) is one of the most important crops worldwide. Considerable efforts have been made on molecular breeding of new varieties. The large-scale gene functional analysis in cotton has been lagged behind most of the modern plant species, likely due to its large size of genome, gene duplication and polyploidy, long growth cycle and recalcitrance to genetic transformation[1]. To facilitate high throughput functional genetic/genomic study in cotton, we attempt to develop rapid and efficient transient assays to assess cotton gene functions.

Virus-Induced Gene Silencing (VIGS) is a powerful technique that was developed based on the host Post-Transcriptional Gene Silencing (PTGS) to repress viral proliferation[2, 3]. *Agrobacterium*-mediated VIGS has been successfully applied in a wide range of dicots species such as *Solanaceae*, *Arabidopsis* and legume species, and monocots species including barley, wheat and maize, for various functional genomic studies[3, 4]. As this rapid and efficient approach avoids plant transformation and overcomes functional redundancy, it is particularly attractive and suitable for functional genomic study in crop species like cotton not amenable for transformation.

In this study, we report the detailed protocol of *Agrobacterium*-mediated VIGS system in cotton. Among the several viral VIGS vectors, the tobacco rattle virus (TRV) invades a wide range of hosts and is able to spread vigorously throughout the entire plant yet produce mild symptoms on the hosts[5]. To monitor the silencing efficiency, *GhCla1*, a homolog gene of *Arabidopsis Cloroplastos alterados 1* gene (*AtCLA1*) in cotton, has been cloned and inserted into the VIGS binary vector pYL156. *Cla1* gene is involved in chloroplast development[6], and previous studies have shown that loss-of-function of *AtCla1* resulted in photobleaching phenotype on true leaves[7], providing an excellent

visual marker for silencing efficiency. At approximately two weeks post *Agrobacterial* infiltration, the photobleaching phenotypes started to appear on the true leaves, with 100% silencing efficiency in all replicated experiments. The silencing of endogenous gene expression was also confirmed by RT-PCR analysis. Significantly, silencing could potently occur in all the cultivars we tested, including various commercially grown varieties in Texas. This rapid and efficient *Agrobacterium*-mediated VIGS assay provides a very powerful tool for rapid large-scale analysis of gene functions at genome-wide level in cotton.

Protocol Text:

1.) Grow cotton seedlings

1.1) Sow the seeds of several upland cotton (*Gossypium hirsutum*) varieties Fibermax 832, Phytogen 425RF, Phytogen 480WR and Deltapine 90 in pots (7 cm in diameter) containing Metro Mix 700 (SunGR, Beavile, WA);

1.2) Keep the pots in a tray covered with a plastic dome at 23^{o}C, 120 μE m^{-2} S^{-1} light, with a 12 hour light/12 hour dark photoperiod in a growth room.

1.3) Remove the dome when two cotyledons have emerged.

1.3) Approximately two weeks later, use the plants with two fully expanded cotyledons for VIGS assay. At this stage, the true leaves haven't yet emerged (Figure 1).

2.) Construct VIGS vector carrying GhCla1 gene

2.1) Amplify the *Cloroplastos alterados 1* gene of cotton (*GhCLA1*) by PCR with primers *GhCLA1-F*, 5'-GCCCTTTGTGCATCTTC-3' and *GhCLA1-R*, 5'-CTCTAGGGGCATTGAAG-3' from a cDNA library of *G. raimondii* leaf tissues.

2.2) Digest the PCR products of *GhCLA1* with EcoRI and KpnI and insert into pYL156 (pTRV-RNA2) vector by ligation.

2.3) Screen the clones on LB agar plates containing 50 μg/mL of Kanamycin. Verify the constructs by restriction enzyme digestion and sequencing.

2.4) Transform the plasmid into *Agrobacterium tumefaciens* GV3101 by electroporation and recover in LB liquid medium at 28^{o}C. Select the transformants on plates with LB+Kanamycin (50 μg/mL) + Gentamycin (25 μg/mL). The bacteria can be stored in 25% glycerol at –80˚C for long term usage.

3**.) Perform VIGS inoculation**

3.1) Three days before inoculation, streak frozen glycerol stocks of *Agrobacterium tumefaciens* carrying pYL156 (TRV):RNA1, pYL156 (TRV):RNA2-pYL (vector alone) and pYL156(TRV):RNA2-*GhCla1* on LB agar plates containing 50 μg/mL of Kanamycin and 25 μg/mL of Gentamycin. Incubate the plates at 28^{o}C for 24 hours.

3.2) Two days prior to VIGS infiltration, pick a single colony for each construct from the above plates and inoculate it into 5 mL of LB medium supplemented with 50 μg/mL of

Kanamycin and 25 μg/mL of Gentamycin; Grow the bacterial culture at 28^{o}C for overnight in a roller drum at a speed of 50 rpm.

3.3) Transfer the above culture to a flask with 50 mL of LB medium supplemented with 50 μg/mL of Kanamycin and 25 μg/mL of Gentamycin, plus 10 mM MES (2-(4 morpholino)-ethane sulfonic acid) and 20 μM acetosyringone. Grow the culture at 28^{o}C for overnight in a shaker with a speed of 50 rpm.

3.4) On the following day, spin down the agro-bacterial cells at 4000 rpm for 5 minutes; re-suspend the culture in the infiltration buffer containing 10 mM $MgCl_2$, 10 mM MES and 200 μM acetosyringone. Adjust the OD 600 of the culture to 1.5.

3.5) Leave the culture on the bench at room temperature for 3 hours.

3.6) Prior to *Agrobacterial* infiltration, punch the underside of cotyledons of cotton plants with a 25 G needle without piercing through the cotyledons. One or two holes (depends on the softness of tissue) were punched on each section of cotyledon (Figure 2).

3.7) Mix *Agrobacterial* culture suspension of pYL156 (TRV):RNA1 and pYL156 (TRV):RNA2-pYL or pYL156(TRV):RNA2-*GhCla1* in a 1:1 ratio; hand infiltrate the mixture from the underside of cotyledons through the wounding sites using a 1 mL needleless syringe (Figure 3).

3.8) Cover the plants with a plastic dome and leave the infiltrated plants at room temperature under dim light condition for overnight.

3.9) Transfer plants to a growth room with the temperature of 23^{o}C, 120 μE m^{-2} S^{-1} light with a 12 hour light /12 hour dark cycle.

3.10) Examine the silencing phenotype at 7~8 days post infiltration. The true leaves of silenced plants by pYL156 (TRV):RNA2-*GhCla1* started to display the photobleaching phenotype (Figure 4). The plants infiltrated with *Agrobacteria* carrying pYL156 (TRV): RNA2-pYL serve as a control.

3.11) Verify the gene silencing efficiency by examining the expression level of endogenous genes using reverse transcription polymerase chain reaction (RT-PCR) with RNA isolated from control and silenced cotton plants.

Representative Results:

Approximately two weeks after hand-infiltration with *Agrobacterial* mixture, the photobleaching phenotype caused by *GhCLA1* silencing was clearly observed on the true leaves. The silenced plants displayed 100% silencing efficiency with a uniformly distributed and very strong photobleaching phenotype on the entire true leaves in all cultivars tested (Figure 4).

Figure Legends

Figure 1. A cotton seedling at about two-week old stage with two fully expended cotyledons used for *Agrobacterial* infiltration.

Figure 2. Punching tiny holes on the underside of cotyledons of cotton plants using a 25 G needle to facilitate *Agrobacterial* infiltration.

Figure 3. Hand infiltration of *Agrobacterial* mixture into the cotyledons of cotton through the wounding sites using a needleless syringe.

Figure 4. Photobleaching phenotype appeared on VIGS silenced cotton plants. Four cultivars were shown. A) Fibermax 832; B) Phytogen 480WR; C) Phytogen 425RF; D) Deltapine 90.

Discussion:

VIGS has been proven to be a powerful tool in functional genomics analysis by transiently knocking down the expression of endogenous genes. In this study, we developed an *Agrobacterium*-mediated VIGS by utilizing a TRV-based binary vector.

The cotton *Cla1 (GhCla1)* gene was developed as a visual marker to monitor the silencing efficiency. We have consistently obtained 100% of gene silencing efficiency, demonstrated by the photobleaching phenotype appeared on the true leaves in all varieties tested, starting from about two weeks post infiltration. The successful development of cotton VIGS provides an alternative to readily silence the genes of interest for loss-of-function assays and lays foundation for cotton functional genetics/genomics in the fast approaching post-genome era[8].

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Disclosures: We have nothing to disclose.

Table of specific reagents and equipment:

Name of the reagents and equipments	Company	Catalogue/serial number	Comments
Roller drum	Glas-Col, LLC.	099A TC108	Agro-bacterium culture
Incubator	Sheldon Manufacturing, Inc.	01046209	Agro-bacterium culture
UV/Vis spectrophotometer	Beckman Coulter	Model: DU530	Measuring OD
Gene Pulser	BioRad	Model: 1652076; Serial: 154BR3880	Electroporation
Pulser Controller	BioRad	Model: 1652098; Serial: 232BR4833	Electroporation
Micropulser Electroporation cuvette	BioRad	165-2081	Electroporation
1 ml Syringe	BD Biosciences	30962	Inoculation of agro-bacterium
Metro Mix 700	SUNGR	SKU# 553001	Growing seedling
Terra Cotta pot	T.O.Plastics	GPS 3001B2	Growing seedling
MES momohydrate	USB	18886	Infiltration buffer
Acetosyringone	Sigma	D134406	Infiltration buffer

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