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Functional Genomic Analysis of Cotton Genes with *Agrobacterium*-Mediated Virus-Induced Gene Silencing

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Abstract

Cotton (*Gossypium* spp.) is one of the most agronomically important crops worldwide for its unique textile fiber production and serving as food and feed stock. Molecular breeding and genetic engineering of useful genes into cotton have emerged as advanced approaches to improve cotton yield, fiber quality, and resistance to various stresses. However, the understanding of gene functions and regulations in cotton is largely hindered by the limited molecular and biochemical tools. Here, we describe the method of an *Agrobacterium* infiltration-based virus-induced gene silencing (VIGS) assay to transiently silence endogenous genes in cotton at 2-week-old seedling stage. The genes of interest could be readily silenced with a consistently high efficiency. To monitor gene silencing efficiency, we have cloned cotton *GrCla1* from *G. raimondii*, a homolog gene of *Arabidopsis Chloroplastos alterados 1* (*AtCla1*) involved in chloroplast development, and inserted into a tobacco rattle virus (TRV) binary vector pYL156. Silencing of *GrCla1* results in albino phenotype on the newly emerging leaves, serving as a visual marker for silencing efficiency. To further explore the possibility of using VIGS assay to reveal the essential genes mediating disease resistance to *Verticillium dahliae*, a fungal pathogen causing severe Verticillium wilt in cotton, we developed a seedling infection assay to inoculate cotton seedlings when the genes of interest are silenced by VIGS. The method we describe here could be further explored for functional genomic analysis of cotton genes involved in development and various biotic and abiotic stresses.

Keywords

Cotton; Virus-induced gene silencing; Functional genomic; *Agrobacterium*; *Verticillium dahliae*

1. Introduction

Cotton (*Gossypium* spp.) is widely planted around the world for its significant economic value of the textile fiber, feed, foodstuff, oil, and biofuel products (1). The upland cotton, *G. hirsutum*, dominates world cotton commerce with more than 90% of the annual fiber

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¹³By using VIGS approach, we have demonstrated that *GhNDR1* and *GhMKK2* are required for *Verticillium* resistance in cotton (20).

production (2, 3). As of 2009/2010, cotton cultivation was estimated to be about 30 million hectares worldwide, producing a value of approximately 39 billion US dollars, while its production in the United States alone was estimated at approximately five billion dollars (4).

The major concern for cotton production is the significant loss of yield caused by many devastating diseases and pests. Cotton has been considered as the world's *dirtiest* crop due to the heavy application of pesticides and fungicides. The soilborne pathogen *Verticillium dahliae* causes severe *Verticillium* wilt diseases on cotton (5). Because of extremely persistent resting structures, such as microsclerotia, this pathogen can survive in soil for many years. Most notably, this fungus is very difficult to be reached by fungicides because the fungi reside in the woody vascular tissues and can be transmitted systemically in cotton plants (5). Despite the significant efforts towards understanding the biology of this pathogen and identifying the genetic determinants responsible for cotton *Verticillium* resistance (6, 7), to date, the genetic and molecular mechanisms underlying cotton resistance to *Verticillium* infection remain poorly understood.

In recent years, significant advances in cotton genetics and genomics have been achieved towards the molecular breeding and genetic engineering of new cotton varieties to increase the sustainable yield and fiber quality as well as to improve the traits combating various pathogen infections (8, 9). Understanding cotton gene functions and regulations constitutes a critical step for manipulating cotton genes in agriculture. A persistent challenge in cotton functional genomic studies is the lack of molecular and genetic tools partly due to the large genome size, the long growth cycle, and the unstable transformation efficiency (9). Virus-induced gene silencing (VIGS) has been demonstrated as a rapid and efficient approach to study gene functions at whole-genome level in various plant species (10–12). VIGS, a type of RNA-mediated posttranscriptional gene silencing, functions as an antiviral defense mechanism in plants (10–12). Through *Agrobacterium* infiltration, the T-DNA containing the partial viral genome and gene of interest is delivered into host cells. The production of double-stranded RNAs between the endogenous gene and DNA fragment delivered from T-DNA vector results in a chain reaction to generate robust silencing signals (12). With the time, the silencing of endogenous genes occurs both locally and systemically throughout the plant tissues.

To date, different plant virus vectors have been deployed for VIGS assays in dicotyledonous plant species, including *tobacco mosaic virus* (TMV), *potato virus X*, *tomato golden mosaic virus*, *tobacco rattle virus* (TRV), and *cotton leaf crumple virus* (CLCrV) vectors (13–16). In monocotyledonous plants, *barley stripe mosaic virus* has been applied to silence genes in barley and wheat and *brome mosaic virus* (BMV) in rice (17–19). Among these viruses, TRV invades a wide range of hosts and spreads vigorously throughout the entire plants but usually triggers a mild symptom, which makes it a good candidate as a VIGS vector (13). TRV belongs to the Tobravirus containing a bipartite positive-sense single-stranded RNA: RNA1 and RNA2. RNA1 contains genes of the viral replicase, RNA-dependent RNA polymerase, and movement protein, which are required for replication and movement (13). RNA2 contains genes encoding the coat protein and other nonessential structural proteins, which can be engineered to insert a target gene fragment to be silenced. Both RNA1 and RNA2 cDNAs have been cloned into T-DNA cassette between duplicated 35S promoter and

the nopaline synthase (NOS) terminator (13). Here we describe a detailed method of *Agrobacterium* infiltration-based VIGS assay in cotton seedlings. We further provide an example of using VIGS assay to understand gene functions in cotton seedling resistance to *Verticillium dahliae* infection. The protocol established here could be potentially adapted to study a diverse array of biotic and abiotic stress responses in cotton and provides a powerful tool in cotton functional genomics.

2. Materials

2.1. Plants, Growth Conditions, and Pathogen Strain

1. Cotton seeds: upland cotton (*Gossypium hirsutum*) variety FM9160 seeds obtained from Bayer CropScience (Lubbock, TX, USA).
2. Soil: Metro Mix 700 (SunGR, Beaville, WA, USA).
3. Growth room conditions: 23–25°C and 120 $\mu\text{E m}^{-2} \text{s}^{-1}$ light, with a 12 h light/12 h dark photoperiod.
4. Pathogen strain: *Verticillium dahliae* (isolate King).

2.2. Plasmid Construction and Cloning

1. PCR amplification reagents: 10× reaction buffer, 10 mM dNTP, and Phusion high-fidelity DNA polymerase (New England BioLabs, MA, USA).
2. Restriction enzymes: EcoRI and KpnI (New England BioLabs, MA, USA).
3. DNA ligation kit: 10× T4 DNA ligase buffer and T4 DNA ligase (4 U/ μl) (New England BioLabs, MA, USA).
4. VIGS RNA2 vector: pYL156 (pTRV2:RNA2).
5. QIAquick Gel Extraction Kit (QIAGEN).
6. LB liquid medium.
7. LB plates containing antibiotics.
8. Kanamycin (50 mg/ml stock) and gentamicin (50 mg/ml stock).
9. *Agrobacterium tumefaciens* GV3101 electro-competent cells stored in 10% glycerol at -80°C .

2.3. *Agrobacterium* Infiltration for VIGS Assay and Confirmation of Gene Silencing

1. *Agrobacterium tumefaciens* GV3101 containing pTRV1 (pTRV-RNA1).
2. *Agrobacterium* induction culture solution: LB liquid medium containing 50 $\mu\text{g/ml}$ of kanamycin, 50 $\mu\text{g/ml}$ of gentamicin, 10 mM of MES (2-(4 morpholino)-ethane sulfonic acid), and 20 μM acetosyringone.
3. *Agrobacterium* infiltration solution: 10 mM MgCl_2 containing 10 mM of MES and 200 μM acetosyringone.
4. 1 ml needleless syringes.

5. Syringe needles (20 Gauge).
6. Spectrum™ Plant Total RNA Kit (Sigma).
7. cDNA synthesis kit (Invitrogen).
8. PCR machine.

3. Methods

3.1. Grow Cotton Plants

1. Fill the soil in square pots (7 cm in diameter) and put the pots in a tray.
2. Sow the seeds in the soil (one seed per pot).
3. Soak the potting soil by pouring water in the tray.
4. Cover the tray with a plastic dome; grow the seedlings in the growth room until two cotyledons have emerged and remove the dome. Growth room condition: 23–25°C and 120 $\mu\text{E m}^{-2} \text{s}^{-1}$ light with a 12 h light/12 h dark photoperiod (see Note 1).

3.2. Clone *Clal* and Other Genes of Interest into pYL156 Vector

1. Search your genes of interest through blast against the *Gossypium* unigenes database at <http://www.cottontdb.org/blast/blast.html>. Design a pair of primers that could amplify about 500 bp of target genes with EcoRI at 5' end and KpnI at 3' end. We used *Arabidopsis Chloroplastos alterados 1* (*AtClal*, AT4G15560) as a query for the blast search for cotton *Clal* gene (20) (see Notes 2 and 3).
2. A 500 bp fragment of *Clal* gene or other genes of interest could be amplified by PCR with Phusion high-fidelity DNA polymerase from the cDNA library synthesized with RNA isolated from cotton leaf tissues (see Notes 4 and 5).
3. PCR products are purified with ethanol precipitation and digested with EcoRI and KpnI, together with pYL156 vector. The digestion is done in 15 μl mixture containing 0.2 μl of each enzyme, 1.5 μl of NEBuffer 4, and 0.15 μl of 100 $\times$ BSA. The digestion is conducted at 37°C for 2–3 h.
4. Recover the digested vector and PCR fragments from DNA agarose gel using QIAquick Gel Recover Kit following the manufacturer's instruction.

¹Maintaining the temperature of growth room at approximately 23–25°C and light at 120 $\mu\text{E m}^{-2} \text{s}^{-1}$ is important to obtain a consistent and uniform silencing efficiency. The cotton seedlings are also easy to be infiltrated with *Agrobacterium* when grown under these conditions (14, 20).

²More than one gene of interest could be inserted into pYL156 vector to silence multiple genes at once in one vector. The DNA fragment for each gene is about 200–300 bp to be inserted into the vector. A three-way ligation could be used to insert two DNA fragments in the vector. According to our experience with *Arabidopsis*, efficiency of silencing two genes is higher with *Agrobacterium* containing two genes inserted into one vector than the mixture of *Agrobacterium* with individual genes from two vectors.

³The primer for cloning cotton *Clal* gene into pYL156 vector is *Clal*-F, 5'-GGAATTCACAACATCGATGATTAG-3', *Clal*-R, 5'-GGGGTACCATGATGAGTAGATTGCAC-3'. The PCR product should not contain internal EcoRI and KpnI sites.

⁴We cloned cotton *Clal* from *G. raimondii* cDNA library, and it worked very well in all upland *G. hirsutum* cottons we tested, suggesting the high sequence conservation among different cotton genomes.

⁵The DNA fragment used for silencing ranges from 250 to 500 bp in length. Further shortening the length of fragment will reduce the silencing efficiency.

5. Ligate the digested PCR products with vector: the ligation is done in a 15 μ l mixture containing 1 μ l of vector DNA (5–10 ng), 5 μ l of insert DNA (20–50 ng), 1.5 μ l of 10 $\times$ T4 DNA ligase buffer, and 0.2 μ l of T4 DNA ligase (4 U/ μ l). The reaction is incubated at room temperature for $\sim$ 2 h.
6. Add 50 μ l of *E. coli* competent cell into the ligation mixture, heat shock at 37°C for 2 min; chill on ice; add 200 μ l LB liquid; and recover at 37°C for 30 min with a roller drum.
7. Spread the *E. coli* culture on LB plate containing 50 μ g/ml kanamycin, and incubate the plate at 37°C overnight.
8. Screen the clones by miniprep DNA isolation and digestion of the DNA with EcoRI and KpnI. Finally, sequence the clones to confirm the insertion.
9. The confirmed clones are transformed into *Agrobacterium tumefaciens* GV3101 by electroporation method.
10. Spread the culture on LB plate containing kanamycin (50 μ g/ml) and gentamicin (50 μ g/ml); incubate at 28°C for 2 days. Culture the *Agrobacteria* with LB liquid, and store the culture with 25% glycerol at -80°C for further use.

3.3. VIGS Processes

1. Select a single colony from the fresh LB plates containing *Agrobacterium tumefaciens* carrying pTRV1, pYL156 (empty vector control), pYL156-GrCla1, and pYL156 carrying Your Favorite Gene (pYL156-YFG).
2. Inoculate the single colony with 5 ml of LB liquid containing kanamycin (50 μ g/ml) and gentamicin (50 μ g/ml); culture at 28°C overnight on a roller drum at 50 rpm.
3. Add 45 ml of *Agrobacterium* induction culture solution into the above culture; incubate at 28°C overnight in a shaker at 100 rpm.
4. Harvest the bacteria at $1,180 \times g$ for 5 min, resuspend the pellet in *Agrobacterium* infiltration solution, and adjust the OD 600 to 1.5.
5. Leave the bacterial cultures on the bench at room temperature for 3 h (see Note 6).
6. Gently punch a couple of holes on the backside of the cotton cotyledons using a fine-tip needle without piercing through the tissue (see Note 7).
7. Mix the *Agrobacterial* culture suspension of pTRV1 with pYL156, pYL156-GrCla1, or pYL156-YFG at a 1:1 ratio.
8. Hand infiltrate the mixture into the cotyledons through the wounding sites using a needleless syringe.

⁶Incubation for 3 h at room temperature will enhance the silencing efficiency. It can be extended up to 24 h.

⁷Direct infiltration of the *Agrobacterial* culture into the cotton cotyledons often results in severe wounding effect. Punching small holes on the backside of cotyledons greatly facilitates the infiltration and reduces the wounding damage.

9. Cover the infiltrated plants with a plastic dome and leave at room temperature overnight under the dimlight ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) (see Note 8).
10. Transfer the plants to the growth room and remove the dome.
11. About 7–10 days later, examine the silencing phenotype for *Clal* or *YFG*. The true leaves on the plants infiltrated with pYL156-GrClal will show albino phenotype (see Note 9).
12. Harvest the true leaves from silenced and control plants; isolate RNA with Spectrum™ Plant Total RNA Kit (Sigma).
13. Synthesize cDNA with cDNA synthesis kit (Invitrogen), and perform semiquantitative or real-time RT-PCR analysis to confirm the silencing of endogenous genes (see Note 10).

3.4. Verticillium Infection of VIGS Silenced Cotton Plants

1. Culture *Verticillium dahliae* (isolate King) on potato dextrose agar (PDA) plates at room temperature (23°C) for 3–4 days.
2. When pYL156- *GrClal*-silenced plants show albino symptoms, prepare the spore suspension of *V. dahliae*.
3. Collect the fungal spores by scratching the fungal mycelium on the plate surface using sterile dH₂O. The spores are filtered with autoclaved cheesecloth, counted under a microscope using a hemacytometer, and diluted to the concentration of 1×10^6 /ml in sterile H₂O containing 0.001% Tween 20.
4. Infiltrate 100 μl of spore suspension to the stems of silenced cotton plants at a position approximately 1 cm below cotyledons using a 22 Gauge needle syringe (see Note 11).
5. Score the Verticillium wilting phenotype by examining the wilting symptoms appeared on the true leaves of plants (see Notes 12–14).

⁸After infiltration, covering the plants with a dome to keep the high humidity under dim light and relatively low temperature (21–23°C) facilitates the plants to recover from the wounding effects and the *Agrobacterial* infiltration.

⁹The albino phenotype on the newly emerging leaves will be observed approximately 10 days post-infiltration. One month later, 100% of *GrClal*-*Agrobacteria*-infiltrated plants will exhibit a strong albino phenotype. This was repeatedly observed in many varieties of upland cotton (20).

¹⁰The primers for cotton *Clal* semiquantitative RT-PCR are *Clal*-F, 5'-GCCCTTTGTGCATCTTC-3', *Clal*-R, 5'-CTC TAGGGGCATTGAAG-3'. *GhActin9* could be used as an internal standard control (20).

¹¹There are several other approaches available for *Verticillium* infection, such as uprooted dip inoculation (21) and cotyledonary node drop inoculation (22). When performing the stem inoculation, it is important to pierce through halfway of the stem and infiltrate the spore suspension into the stem.

¹²It usually takes 10–14 days to observe the *Verticillium* wilting phenotype after inoculation. However, the symptom development depends on the varieties, plant growth conditions, and pathogenicity of *Verticillium*. The wilting phenotype can be examined by either counting the percentage of wilting plants or measuring the stunting appearance (20).

¹⁴The *Agrobacterium*-infiltrated plants should be kept under contained quarantine conditions, and plants should be properly autoclaved and disposed after the assays as TRV has a broad host range and is a notifiable pathogen.

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