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Protocol for t-DNA insertional mutagenesis in the chickpea Fusarium wilt pathogen, Fusarium oxysporum f. sp. ciceris for identification of virulence genes

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ABSTRACT

Chickpea is the third most consumed grain legume in the world. It serves as a valuable source of protein and micronutrients, especially for the large vegetarian population in the Indian subcontinent. One of the major constraints in chickpea production is the Fusarium wilt disease caused by the soil-born necrotrophic pathogen Fusarium oxysporum f. sp. ciceris (Foc). The pathogen has been shown to interact with chickpea in a race-specific manner and evolved into multiple races. So far eight races of it have been reported including 0, 1A, 1B/C, 2, 3, 4, 5, and 6. When a pathogenic race and host genotype interact in a race-specific manner, the interaction follows the typical gene-for-gene interaction (R-AVR) hypothesis, where major genes control avirulence/virulence in the pathogen and resistance/susceptibility in the host. However, neither race-specific molecules (AVR) from the Fusarium oxysporum f. sp. ciceris nor the resistance gene (R) from the chickpea have been reported.
I used the Agrobacterium tumefaciens- mediated transformation (ATMT) for random t-DNA insertional mutagenesis of the chickpea Fusarium wilt pathogen to produce mutants.
Here, we describe a protocol for identifying pathogenicity and virulence genes through random insertional mutagenesis of the chickpea Fusarium wilt pathogen, Fusarium oxysporum f. sp. ciceris (Foc) as an example of the protocol.

Agrobacterium Minimal medium:
Dissolve K 2 HPO 4 × 3H 2 O (3.44 g), KH 2 PO 4
(1.45 g), NaCl (0.15 g), MgSO 4 × 7H 2 O (0.5 g), (NH 4)2SO4 (0.5 g) in 800 mL of water. Adjust the pH to 5.5, and the volume
to 900 mL with water and autoclave. In parallel, dissolve CaCl 2 × 2H 2 O (0.067 g), FeSO 4 × 7H 2 O (0.0025 g), glucose (2 g)
in 100 mL water and filter through a 0.2 m m Whatman filter. Once the autoclaved medium reaches ~50°C add filter sterilized
medium and mix well
Induction medium:
Dissolve K 2 HPO 4 × 3H 2 O (3.44 g), KH2PO4 (1.45 g), NaCl (0.15 g), MgSO 4 × 7H 2 O (0.5 g), (NH 4)2SO4 (0.5 g) in 800 mL of water. Adjust the pH to 5.5, and the volume to 900 mL with water and autoclave. In parallel, dissolve CaCl 2 × 2H 2 O (0.067 g), FeSO 4 × 7H 2 O (0.0025 g), glucose (1.8 g) MES (7.8 g) and glycerol (5 mL) in 100 mL water and filter through a 0.2 m m Whatman filter. Once the autoclaved medium reaches ~50°C add filter sterilized medium and mix well. Add 1.5% of technical agar for plates.

Selection medium: Autoclave PDA (39 g/L) and add hygromycin (50 m g/mL) and cefotaxime (200 m g/mL) immediately before pouring the plates.

Materials required

- Cefotaxim (200 mg/mL): dissolve the required amount in demineralized water and filter sterilize through a 0.2 m m Whatman filter (see Notes 3 and 4).
- Hygromycin (50 mg/mL): dissolve the required amount in demineralized water and filter sterilize through a 0.2 m m Whatman filter (see Notes 5 and 6).
- Kanamycin (50 mg/mL): dissolve the required amount in demineralized water and filter sterilize through a 0.2 m m Whatman filter (see Note 7).
- Acetosyringone (200 mM): dissolve the required amount in DMSO (see Note 8
- Buffer: TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS).
- Reagents: 5M NaCl, 10% CTAB, 5M NH 4 Ac, Chloroform: isoamyl alcohol (24:1), Isopropanol and 70% ethanol.
- Enzymes: Nco I (six cutters) and Msp I (four cutters), T4 DNA ligase and GO Taq DNA polymerase.
- Fungal species: Fusarium oxysporum f. sp. ciceris was used as the recipient species for transformation.
- Bacterial strain: *A. tumefaciens* strain EHA-105 with plasmid pCAMBIA1300 was used as a T-DNA donor strain.
- Host plant: Cicer arietinum, JG62 as a susceptible genotype and WR315 as a resistance
- Nitrocellulose membrane: Hybond-N + (GE Health Care).
- Heat sterilized microcentrifuge tube (1.5 mL).
- Sterilized glassware: 300 mL conical fl ask.
- Spreader.

1 Preparing Fusarium spore suspension

1.1 A wilting isolate (on the genotype JG62) of the *Foc* was cultured on PDA plates containing cefotaxime (50mg/L) at 25 °C for 7-10 days

1.2 Spores were collected from the plant by adding 4 mL of double-distilled autoclave water to the plate and gently brushing the mycelium with a clean brush and filtered through eight layers of muslin cloth

1.3 The 4 mL spores suspension was added to 2 mL tubs and pelleted at 2000 g at 4 C for 8-10 min. the pellet was washed twice with autoclaved distilled water.

1.4 The pellet was resuspended in 1 ml of sterile water, counted and diluted to the concentration of 1×10^6 spores/mL by using a hemocytometer

2 Fungal Transformation

2.1
- Primary culture: the primary culture of *A. tumefaciens* EHA-105 strain was prepared by inoculating a freshly isolated single colony of *A. tumefaciens* EHA-105 strain containing the pCAMBIA1300 in minimal medium with Kanamycin (50 mg/mL) antibiotic. Keep the culture for 36h at 28 °C with agitation at 250 rpm

2.2
- Take 1mL of overnight grown culture and inoculate in 10mL of minimal medium without Kanamycin and agitate at 250 rpm to the OD of 0.4–0.5

2.3 The *A. tumefaciens* cells were centrifuged at 2,000 g for 10 min and media was removed. The pellet was resuspended induction medium containing acetosyringone (200 µM) and grown until an OD of 0.15 (grow the cells in IM for about 6 h).

2.4 Place sterile Whatman filter paper on induction media plates. Take 100 µL of the *A. tumefaciens* culture growing in induction media (previous step) and mix it with 100 µL (1 × 10^6 spores/mL) of Foc spore suspension in a microfuge tube

2.5 Pour the Agrobacterium cells and Foc spores on the Whatman filter paper placed on induction medium plates containing AS (200 µM) for 2 days (48 h) to co-cultivate at 28

2.6 After 48 h of incubation, transfer the filter paper to the PDA plates containing 100 mg/L hygromycin and 300 mg/L cefotaxime and incubate the PDA plates under light conditions (16h) at 25 °C. observations were taken regularly over 5-7 days of incubation for the appearance of colonies.

2.7 Colonies appaired were serially transferred to PDA plates containing hygromycin to check for the stability of mutation.

2.8 Stable mutants were further checked for t-DNA insertion by hygromycin specific primers. Mutants which gave amplification were treated a putative transformants.The putative transformers were maintained as single conical culture in 25% glycerol and stored at -80