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Full Length cDNA Library Construction Towards Identification Of Fusarium Wilt Resistance Genes In Chickpea (*Cicer arietinum*)

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ABSTRACT

Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is one of the major yield limiting factors in chickpea. The disease causes10–90% yield loss annually in chickpea. Eight physiological races of the pathogen (0, 1A, 1B/C, 2, 3, 4, 5 and 6) are reported so far whereas additional races are suspected from India. Mapping of wilt resistance genes in chickpea is difficult because of minimal polymorphism; however, it has been facilitated to great extent by the development of sequence tagged microsatellite site (STMS) markers and EST's that have revealed significant interspecific and intraspecific polymorphism. Markers linked to six genes governing resistance to six races (0, 1A, 2, 3, 4 and 5) of the pathogen have been identified and their position on chickpea linkage maps were elucidated. These genes lie in two separate clusters on two different chickpea linkage groups. While the gene for resistance to race 0 was situated on LG 5 and those governing resistance to races 1A, 2, 3, 4 and 5 spanned a region of 8.2 cM on LG 2. Cloning of wilt resistance genes is desirable to study their evolution, mechanisms of resistance and their exploitation in wilt resistance breeding and wilt management. So for the present study full length cDNA library was constructed from wilt resistance cultivar of Chickpea i.e., WR315 against most virulent race of Fusarium i.e., Race1, and subsequently paved way to clone few of the wilt resistance genes such as PR proteins, MAP kinases and Transcription factors.

Keywords: *cDNA library, Chickpea, and Fusarium*

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the most important biotic stresses of chickpea (*Cicer arietinum* L.) and has the potential to cause 100% yield losses. An annual loss in chickpea grain yield of about 10 to 15% has been reported for this disease (Jimenez-Diaz *et al*., 1989 and Singh and Reddy 1991). The disease is prevalent in the Indian subcontinent, Ethiopia, Mexico, Spain, Tunisia, Turkey, and the United States ((Kraft *et al*., 1994). Chickpea is most widely grown in South Asia and the Mediterranean region (Saxena,1990). It ranks third in the world among pulse crops after peas and beans with an area of 10,374 thousand ha with total production of 7,123 thousand MT and an average yield of 687 kg/ha. Despite economic importance and strong national and international breeding programmes, average yields of chickpea have not improved considerably over the years. One of the major constraints in realization of full yield potential of chickpea is wilt caused by a Deuteromycetes fungal pathogen *Fusarium oxysporum* Schlechtend.: *Fr*. f. sp. *ciceris* (Padwick) Matuo & K. Sato. The pathogen penetrates the vascular bundles of roots of chickpea plants and stops or reduces water uptake to the foliage. The infected plants ultimately wilt and die (Beckman, 1987) . The disease is highly destructive worldwide in occurrence (Kraft *et al*., 1994). It has been reported from almost all chickpea growing areas of the world including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey and US (Westerlund *et al*., 1974 and Nene *et al*. 1989 and Halila and Strange, 1996). The disease is capable of causing 100% yield loss. *F.oxysporum* f. sp. *ciceris* is a vascular pathogen that perpetuates in seed and soil and hence is difficult to manage by the use of chemicals. Pathogenic and genetic variability in the pathogen was characterized using differential lines and DNA markers for certain races. Efforts were also made to develop race-specific DNA-based markers for certain races . The pathogen has eight races. Races 1A, 2, 3, and 4 have been reported from India, and races 0, 1A, 1B/C, 5, and 6, from the United States

and Spain. Cultivar specialization (race) of *F. oxysporum* f. sp. *ciceris* was first reported in India. Subsequently, resistance to wilt in chickpea has been shown to be race specific and governed by major resistance genes. But efforts have not been made to clone genes governing Fusarium resistance in Chickpea plants.

MATERIAL AND METHODS

Plant growth conditions

Seeds of *Cicer arietinum* cultivars WR315, were surface sterilized with 0.1% $HgCl_2$ for 3 min, followed by three washes with70% alcohol for 30 sec each. After each wash with ethanol, seeds were rinsed with sterile water to remove any remnants of ethanol. Following the final wash seeds were soaked in sterile water and kept in dark. Overnight soaked seeds were then germinated in test tubes containing MS basal medium pH 5.8 supplemented with 3% sucrose and 0.6% agar. Plants were allowed to grow in a growth room maintaining 28º C day temperature 25º C night temperature, 16hr/8hr light and dark cycles and 60% relative humidity for approximately 3 weeks.

Inplanta infection and tissue collection

Fusarium oxysporum ciceri race 1, the most virulent race of wilt pathogen on chickpea plants was routinely sub cultured on PDA slants and the virulence is checked from time to time by infecting the susceptible cultivar JG62. For inplanta infection single spore derived mycelium was inoculated into PDB and grown for a week at 28ºC and 180 RPM and grownup culture was filtered through two layers of cheese cloth and spores were diluted to a final concentration of 10^6 spores/ml. Three weeks old well grown uniform seedlings were taken out from test tubes rinsed in sterile water to remove any adhering medium and kept in another set of test tubes containing approximately 10^6spores/ ml of spore suspension. For the control treatment plants were kept in test tubes containing sterile water diluted with PDB in the same propotion. Following infection plants were put back into the growth room and root tissue was harvested at different time point viz. 0 h, 6h, 12 h, 18 h, 24h, 48h and 5 days after post infection.

RNA isolation for full length cDNA library construction

Precautions were taken to keep RNases out of working area. All glassware used, were baked at 300ºC overnight. The 50ml tubes were treated with 3% H_2O_2 for few hours, rinsed with RNase-free water thrice, autoclaved for 30 min and dried before use. All plastic wares and gel running tank, gel tray, comb were treated with 3% H_2O_2 and rinsed thrice with autoclaved water before use. Water used for RNA work was treated with 0.1% DEPC for 10-12hrs at room temperature and then autoclaved for 30min. All solutions were prepared in DEPC treated water and autoclaved for 30min.

Total RNA isolation using GITC method

1. Approximately 3 gm of plant tissue was ground with liquid nitrogen in pre-chilled mortar and pestle.
2. The powdered tissue was transferred to a 50ml oak ridge tube and immediately10ml of Guanidium isothiocyanate (GTC) buffer was added to it.
3. This was vortexed vigorously and then centrifuged for 10 min at 10,000rpm.
4. The supernatant was decanted into a fresh tube and 1ml of NaOAc pH 4.0 was added to it. The contents were mixed by inverting the tube few times.
5. Equal volume of water saturated phenol and chloroform in 1:1 ratio was added to the same tube and was vortexed.
6. Centrifugation was done at 10,000rpm for 10min at 4 °C.
7. The upper aqueous phase was transferred to a new RNase free tube leaving the inter-phase. To it equal volume of Chloroform was added followed by vortexing and centrifugation at 10,000 rpm at 4 ° C for 10 min.
8. The aqueous phase was transferred to a new tube and equal volume of isopropanol was added to it.
9. The tube was inverted few times to mix the contents properly and kept in –20°C overnight.
10. Next day centrifugation was carried out at 12000 rpm for 15min at 4°C.
11. The supernatant was discarded. To the pellet 3ml of guanidium buffer was added and the pellet was dissolved in it.
12. 3ml of isopropanol was added to the tube and after mixing the contents by inverting the tube few times then it was kept in –20°C for 4 hrs to allow RNA precipitation.
13. Centrifugation was carried out at 12,000 rpm for 15min at 4°C. The supernatant was discarded and pellet was washed with 75% ethanol
14. The pellet was air dried and dissolved in 50-75 ìl of DEPC treated water by incubating at 55-60°C for 10- 15 min.

15. The dissolved RNA was stored in -80°C. The yield of RNA obtained was 100-150ìg and concentration 2-3 ìg/ìl.

Poly A+ isolation from total RNA preparation using Dynabeads.

For isolating Poly A+ representing the mRNA fraction in total RNA, Dynabeads were used and mRNA was isolated following the steps as outlined below.

Conditioning of Dynabeads oligo (dT)25

1. Dynabeads oligo (dT) 25 were resuspended by gentle vortexing. A 250 ìl aliquot was withdrawn from the stock tube suspension to an RNase-free 1.5mleppendorf tube.
2. The eppendorf tube was placed on the magnetic stand and as the beads moved towards the wall the suspension was left clear. The clear buffer was pippetted off and replaced by 100ìl of binding buffer.
3. The tube was removed from the magnetic stand and Dynabeads oligo (dT)25 were suspended in 100 ìl of binding buffer (20mM Tris-HCl pH 7.5, 1.0M LiCl, 2mMEDTA) by pipetting up and down several times.
4. To collect the beads, magnetic stand was used. The supernatant was removed and Dynabeads oligo (dT)25 were resuspended in 100ìl of fresh binding buffer.

Preparation of total RNA

1. The volume of total RNA (100-250 ìl) was adjusted to 100 ìl using DEPC treated water.
2. RNA was denatured by heating at 65°C for 2 min and quick chilled on ice.

mRNA isolation from total RNA

1. Total RNA (250 ìg) from the above step was added to the Dyna beads oligo (dT)25 which were already suspended in the binding buffer.
2. Thorough mixing was done by pipetting up and down. Incubation was carried out at room temperature for 5 min to allow binding of mRNA to beads.
3. The eppendorf was placed in magnetic stand for 2 min and supernatant removed.
4. The beads were washed twice with 200ìl of washing buffer (10mM Tris-HCl pH7.5, 0.15 M LiCl, 1mM EDTA) using the magnet. Care was taken to remove the traces of wash buffer completely.
5. The elution of bound mRNA was carried out by suspending the Dynabeads oligo(dT)25 in 15ìl of elution buffer (10mM Tris-HCl pH7.5) and heating to 65-70°Cfor 2min. Followed by quickly placing the tube in the magnetic stand. As the beads moved towards the magnetic side the eluate with mRNA was left at the bottom of eppendorfs. The eluate was transferred to fresh RNase free eppendorf tube.
6. The concentration of the eluted mRNA was estimated by measuring the absorbance A260 of the final preparation. From 250 ìg of total RNA at least 1.5-2ìg of mRNA was isolated.

Regeneration of dynabeads

1. Dynabeads oligo (dT)25 original volume 250ìl were resuspended in 200ìl of reconditioning solution (0.1M NaOH) and suspension was transferred to new RNase free tube.
2. The suspension was incubated at 65°C for 2 min to remove any mRNA bound to the beads. It was then placed on the magnetic stand and the supernatant was discarded.
3. The same steps for washing in reconditioning solution were followed twice. In the last wash the beads were suspended in 200ìl of storage buffer.4. The beads were washed in storage buffer thrice or until the pH of supernatant was below 8.0.
5. The beads were stored in 150ìl of storage buffer (250mM Tris-HCl, pH 7.0, 20mM EDTA, 0.1%Tween-20, 0.02% Sodium azide).

Construction of Clontech SMART cDNA Library

Full length cDNA library was constructed in Lambda TriplEx2 vector following protocol.

In brief the protocol consists of first strand synthesis coupled with dc tailing by power script RT followed by second strand synthesis using either Long distance PCR or Primer extension. cDNA was run on gel and it was digested with Sfi1 enzyme followed by size fractionation using chromaspin columns and ligated to vector arms and finally packaged. The titer value of the library is checked for per cent recombinant clones.

Converting TriplEx to pTriplEx Library Conversion Protocol

The day before conversion of the entire phage cDNA library, check the titer of the lTriplEx cDNA library, and proceeded as per the steps given below.

1. Grew BM25.8 cells overnight from a single colony in 10 ml of LB medium at **31°C** with shaking at 190 rpm.
2. Removed 1 ml of overnight culture and use it to inoculate 10 ml of LBmedium (1:10 dilution). Continue growing at **31°C** with shaking until the OD600 reaches 1.2 (2–2.5 hrs). Transfect cells on the same day.
3. Added 100 ml of 1 M MgCl2 to the 10 ml freshly grown BM25.8 culture (10mM final concentration).
4. Mixed 200 ml of BM25.8 cells and lTriplEx cDNA containing 2 x 10^6pfu in a sterile 5-ml tube. Mixd gently by pipetting.
5. Incubated this mixture of cells and phage for 1 hour at **31°C** without shaking.
6. After incubation is completed, added 500 ml of LB medium and incubated for 1 hr at **31°C** with shaking at 190 rpm. At this point, conversion of the entire library to plasmid form was completed.
7. Using a sterile spreader, spread 10 and 100 ml of converted cDNA library (diluted 1:100 in LB medium) on 150-mm LB agar plates containing 50 mg/ml carbenicillin.
8. Grown the LB agar plates overnight at **37°C**.
9. Counted colonies and determine the total number of converted recombinant clones per ml. counted the total number of recombinant clones present in the whole volume of the converted plasmid library generated at Step 6.
10. Prepared 150–200 150-mm LB agar plates with 50 mg/ml carbenicillin.
11. Plated 20,000–30,000 converted clones on each 150-mm LB-carb plate (3–5 x 106 clones) and grown overnight at **37°C** (not longer than 14 hr).

D. Plasmid DNA isolation from converted pTriplEx cDNA library

1. Added 10 ml of LB medium to each 150-mm plate to wash the grown cells from the surface of agar plate.
2. Gently removed the cells from the surfaces of the agar plates with a sterile spreader and transfered the cells in the medium from all the plates to sterile 500-ml centrifuge bottles.
3. Centrifuged at 10,000 rpm for 20 min at 4oC.
4. At this point, followed a protocol for CsCl preparation of plasmid DNA, or used a NucleoBond Plasmid Kit.

RESULTS AND DISCUSSION

Isolation of total RNA: Total RNA was obtained from plant tissue and its quality was checked by running onto Formaldehyde gel (Figure 1.1)

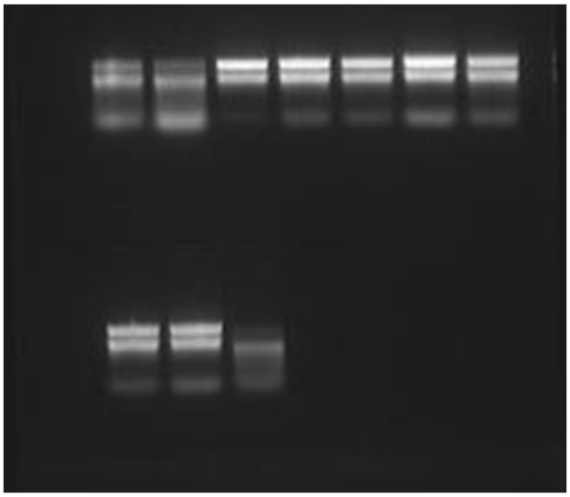

Figure1.1 Total RNA isolated from plant tissue and running onto gel

Isolation of mRNA: PolyA$^+$ RNA was isolated from total RNA using Dynal mRNA isolation kit, following manufacturers guidelines.

Synthesis of cDNA: First and second strand was synthesized using Clontech SMART cDNA synthesis kit following manufacturers protocol. In brief, First cDNA was synthesized by adding SMART IV oligo CDS III/3 primer and first strand cocktail containing dNTP's, buffer,DTT, Power script in a total volume of 10 ml and incubated at 42 ^{0} c for 1hr. After incubation dscDNA was synthesized using primer extension and or Long distance PCR(Figure1.2)

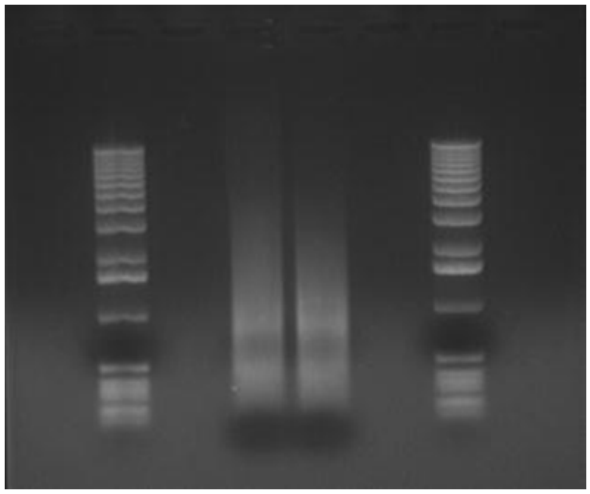

Figure1.2 dscDNA run on0.9% agarose gel

Following Proteinase K digestion and SfiI digestion cDNA was size fractionated using Chromaspin columns and fractionated cDNA was run on 1.1% agarose gel (Figure 1.3).

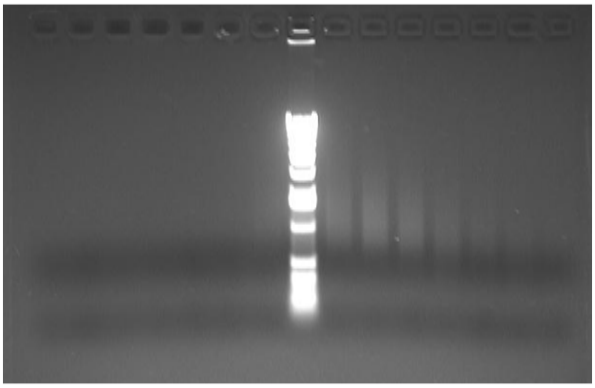

Figure 1.3: Size fractionated ds cDNA run on 1.1% agarose gel

TrippleX2 vector arms and each ligation was packaged and titre value of library was checked.

Determining the Percentage of Recombinant Clones: PCR insert screening of library was carriedout and found to be 80% recombinent clones(Figure1.4)

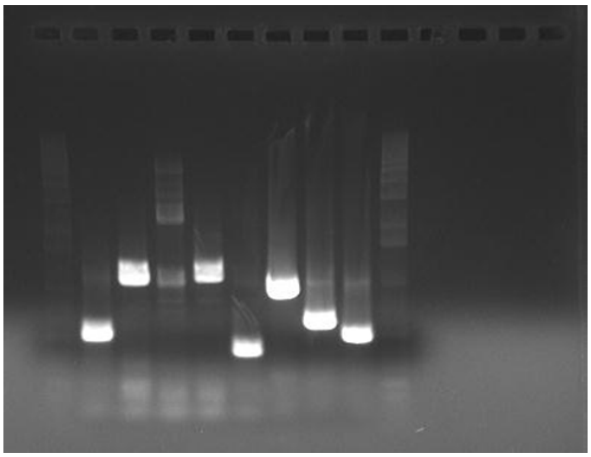

Figure1.4: Agarose gel showing Plaque PCR amplified clones

Converting λ TriplEx to pTriplEx: l TriplEx2 library was converted to pTriplEx library and plasmid DNA was isolated and sequenced for some of the clones and were fall in the range of PR proteins, MAP kinases, Transcription factors etc. and finally this facilitated in cloning Fusarium wilt responsive genes in chickpea cultivar WR315.

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