

Construction and analysis of cDNA library for tospovirus resistance genes

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Abstract

In order to isolate a cDNA clone of tospovirus resistant R gene, a cDNA library was constructed in Uni-ZAP XL vector with poly(A) RNA purified from leaves of *Lycopersicon peruvianum* EC 52071. The library showed around 40 fold more white plaques than blue plaques. To get the probe for screening the library, PCR of cDNA was conducted using the gene specific primers designed for a portion of the tospovirus resistance genes based on the already available sequence from the *L. peruvianum* Sw 5 gene. The eluted 943 bp cDNA product obtained from RT-PCR using the above gene-specific primers were cloned in pTZ57R/T vector using TA cloning strategy and sequenced. The cDNA library was screened with the amplified fragment as a probe. Positive signals corresponding to around 150 plaques were seen in all 20 plates. All the 150 positive plaques were subjected to one more round of screening by replating and rehybridizing with the same probe. The presence of cDNA of interest in plaques selected at random were confirmed by PCR amplification. The cDNA library constructed using the ZAP Express® cDNA synthesis kit is a source for many genes in tomato. The library can also be utilized for isolation of other resistant genes and also of genes of agronomic importance.

Keywords: Tospovirus, cDNA library, *L. peruvianum*, resistance, RT-PCR.

Introduction

Tomato spotted wilt disease is one of the important virus diseases affecting tomato (*Lycopersicon esculentum* Mill). The causal agent, Tomato spotted wilt virus (TSWV) causes serious losses in the yield of groundnut and many other crops in Australia, India, Nepal, China, Thailand and USA (Reddy, 1985). Griep *et al.*, (2000) estimated an annual crop loss due to TSWV at over one billion US dollars, which ranks TSWV among the top ten of the most damaging plant viruses in the world. Singh and Tripathi (1991) recorded a loss of 1.26 t in fruit yield per hectare due to infection by TSWV in tomato. The disease was first reported in Southern Australia and is now widespread in temperate and subtropical regions throughout the world (Brittlebank, 1919). Tospoviruses belong to the family Bunyaviridae (Van Regenmortel *et al.*, 2000; Moyer, 1999). It is vectored by four species of thrips in a persistent manner (Francki and Halta, 1981).

Attempts made to control TSWV by vector control have met with only little success (Carter, 1973). Since new infection often depends on thrips migration in tomato fields (Paterson, 1987), it was recognized that host resistance offered the best long term solution through development of resistant cultivars. Resistance to TSWV was reported to be available in some wild species of tomato. Many attempts have been made to breed commercial tomato varieties resistant to TSWV utilizing the wild species but met with limited success. The most effective way to manage tospoviruses is through genetic engineering. This is especially useful in crops where natural sources of resistance are not available or if available, are not amenable to transfer into agronomically superior cultivars by conventional breeding methods (Pappu, 1997).

The wild species *L. peruvianum* has been reported to have broad resistance to different isolates of TSWV (Smith, 1944). Gilbert and Tanaka (1971) released 'Anahu' as a TSWV resistant tomato cultivar that had *L. peruvianum* in its background.

The first genetic studies of TSWV resistance in tomato reported five resistance genes from *Lycopersicon* spp. *L. pimpinellifolium* (Samuel *et al.*, 1930) and *L. esculentum* (Holmes, 1948). Two dominant genes were designated Sw-1a and Sw-1b, and three recessive genes were designated sw-2, sw-3 and sw-4. In all cases the resistance was overcome quickly, and further work with these genes has not been pursued. The Sw-5 gene, first identified in *L. peruvianum* has proven more stable than all other reported resistance genes for TSWV.

Brommonschenkel *et al.*, (2000) reported that Sw-5 is a single gene capable of recognizing several tospovirus isolates and species. Analysis of the predicted protein suggested that it is a cytoplasmic protein, with a potential nucleotide binding site (NBS) domain and a C terminal end consisting of leucine-rich repeats (LRRs) (Spasova *et al.*, 2001). With the availability of more durable resistance (R) genes from wild sources, there is a scope to transform elite cultivars to develop disease resistant plants. So it becomes necessary to isolate new resistance genes from wild species and to improve the quality of elite cultivars by transforming these genes into cultivars.

The objectives of the present study were to identify the presence of resistance (R) genes effective against tospoviruses from a wild species of tomato *L. peruvianum* EC52071 by construction and screening of cDNA library.

Materials and methods

Plant material

The wild species of tomato *Lycopersicon peruvianum* EC 52071 used in the study was obtained from National Bureau for Plant Genetic Resources (NBPGR), New Delhi.

RNA isolation

Total RNA was extracted from leaf using TRIzol reagent (Chomczynski and Sacchi, 1987). Poly(A)+ RNA was isolated separately from total RNA using GenElute™ mRNA Miniprep Kit (Sigma Aldrich Chemicals Pvt. Ltd., USA). Poly(A)+ RNA was resuspended in water, quantified by UV absorbance, and immediately frozen at 72°C until use.

Construction of cDNA library

The cDNAs were prepared from 5 µg poly(A)+ with the ZAP Express® cDNA synthesis kit and ZAP Express cDNA Gigapack III Gold Cloning Kit (Stratagene Inc., La Jolla, California, USA). The cDNAs were then size fractionated on a 1% w/v agarose gel. The cDNAs ranging from 0.8 to 3 kb were recovered from the gel using a DEAEcellulose membrane (Sambrook *et al.* 1989). The cDNAs were ligated in an approximately stoichiometric amount of *EcoRI*/*XhoI* digested vector and in vitro packaged with packaging extract according to the instructions of the manufacturer. *Escherichia coli* super competent cells (Stratagene) were transformed with the ligation products. After *E. coli* transformation, 2 × 10⁵ primary clones were obtained. All clones were then pooled and aliquots were frozen at -72°C until amplification.

Generation of probe for screening cDNA library

For probe preparation, reverse transcription of the isolated RNA was performed in a 20 µl reaction mixture containing: 50 ng of mRNA, RNase inhibitor 1.0 µl, 0.1 M DTT 1.0 µl, RT buffer (5X) 4.0 µl, 30 mM dNTP mix 2.0 µl, AMV reverse transcriptase 0.5 µl (Bangalore Genei). The samples were incubated at 42 °C for 1 hr and thereafter the reverse transcriptase was inactivated by heating at 97 °C for 5 min.

Gene specific primers were designed for a portion of the tospovirus resistance genes based on the already available sequence from the *L. peruvianum* Sw 5 gene. The primer sequences were as follows:

TNAU T F: 5' GCCTTCCATCAACACTT 3'

TNAU T R: 5' TTTCTTCCTTCATTCTCACA 3'

T - Tospovirus, F - Forward, R - Reverse.

PCR reactions were performed in a total volume of 50ml containing: First strand cDNA 3.0 µl, 10X PCR buffer 5.0 µl, 30 mM dNTP mix (7.5 mM each) 1.0 µl, Forward gene specific primer (10 pmol/µl) 1.5 µl, Reverse gene specific primer (10 pmol/µl) 1.5 µl, Taq DNA polymerase (3 U/µl) (Genei) 1.0 µl, Sterile water 37.0 µl in 0.2 µl thin walled tubes, using a thermal cycler (Eppendorf). The PCR cycling profile was denaturation at 92°C for 1 min, annealing at 60°C for 1 min and an extension at 72°C for 1.5 min for 30 cycles. The amplification products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and photographed using Alpha Imager TM 1220 Documentation and Analysis Systems. The amplified cDNA products

obtained in PCR reactions were loaded separately on 1.5% low melting agarose gel, electrophoresed and eluted using GenElute™ Gel Extraction Kit (Sigma Aldrich Chemicals Pvt. Ltd., USA).

The eluted 943 bp cDNA product obtained from RT-PCR using the above gene-specific primers were cloned in pTZ57R/T vector (InsT/A clone™ PCR Product Cloning Kit, MBI Fermentas Inc., USA) using TA cloning strategy. Plasmid DNA was isolated from selected recombinant clones using alkaline mini-prep procedure (Birnboim and Doly, 1979). The recombinant plasmid DNA was used for PCR amplification using gene specific primers to confirm the presence of gene. Sequencing of the clone was done using the M13 primer sequences present in the pTZ57R/T vector in an automated sequencer model 3100 version 3.0 (ABI PRISM) at the DBT- supported DNA sequencing facility at University of Delhi South Campus, New Delhi. PCR amplified cDNA fragments were used as probe for screening cDNA library. The probe DNA was radio labeled by the random priming method (Random Primer Labeling Kit, Bangalore Genei Pvt. Ltd., Bangalore, India).

Screening of cDNA library

Blotting

All the twenty plates (150 x 150 mm) each having ~ 5 × 10⁴ pfu were selected for screening. These were kept at 4 °C for 2 hr before use. Twenty nylon membranes were cut according to size of plates and placed on top of agarose carefully. After marking the plates and membranes, the membranes were lifted after 2 min and air dried for 2 min. They were kept for 2 min each in denaturing solution (0.5 M NaOH/1.5 M NaCl) and in neutralization solution (0.5 M Tris-Cl (pH 8.0)/1.5 M NaCl). The membranes were placed finally in 2 x SSC for 2 min and air dried for 30 min. The membranes were blotted briefly on a whatman 3 MM paper and the DNA were cross linked to the membranes at 120,000 µJ of UV energy for 30 seconds. The membranes were finally wrapped in aluminium foil and kept at room temperature until prehybridization and hybridization steps. Prehybridization and hybridization of the membranes were followed as described by Sambrook *et al.*, 1989.

In vivo excision of the pBK-CMV phagemid vector from the ZAP express vector

pBK-CMV phagemid vector was excised from the ZAP express vector as per the procedure described in ZAP Express® cDNA synthesis kit (Stratagene Inc., La Jolla, California, USA).

Results

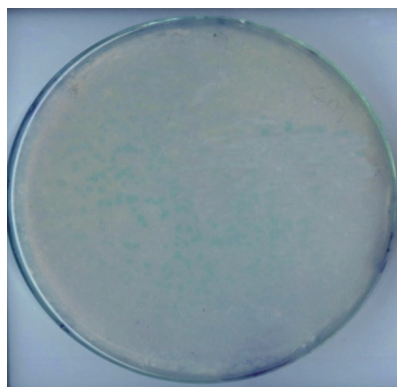
cDNA library construction in *L. peruvianum*

cDNA library of *L. peruvianum* EC 52071 was constructed as per the protocol supplied with Stratagene ZAP Express cDNA synthesis kit (Stratagene Inc., USA). The library showed around 40 fold more white plaques than blue plaques (Fig. 1).

Screening of cDNA library

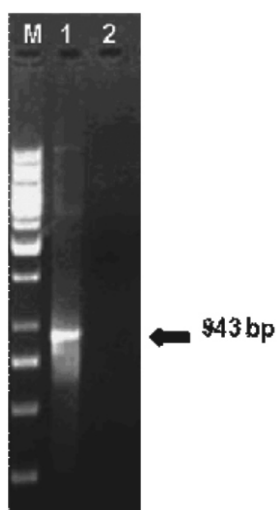
RT-PCR analysis (with primer TNAU T F/R) with cDNA extracted from leaf of wild species *L. peruvianum* EC52071 gave rise to a 943bp

Fig 1: cDNA library of *L. peruvianum* EC 52071



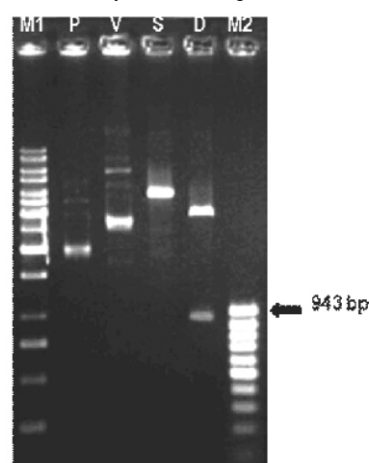
amplicon. The 943 bp amplicon corresponding to the tospovirus resistance gene from *L. peruvianum* was cloned into the pTZ57R/T vector and transformed into *E. coli* cells. The recombinant cDNA clones were confirmed for the presence of insert using restriction digestion and PCR analysis (Fig. 2a, Fig. 2b). The confirmed recombinant

Fig. 2a. Confirmation of recombinant clones by PCR



M 1 Kb DNA ladder; 3 PCR amplified products from recombinant plasmid

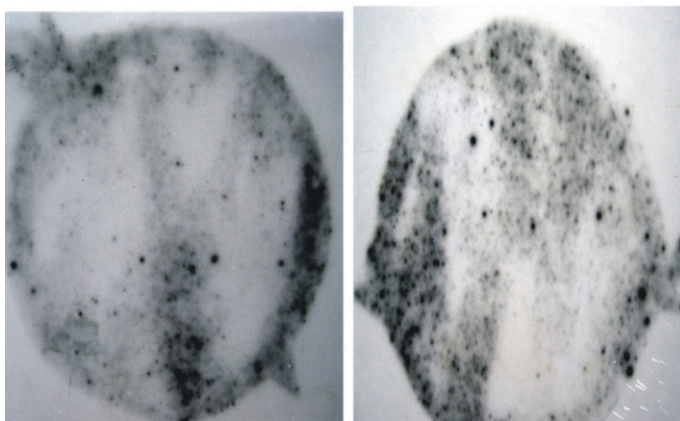
Fig. 2b. Confirmation of recombinant clones by restriction digestion



M1 1 Kb DNA ladder; M2 100 bp DNA ladder; P Undigested plasmid with insert; V pTZ 57R/T vector; S Restricted plasmid digested with Xba I D Restricted plasmid digested with Xba I and BamH I Arrows indicate released cDNA fragments after digestion

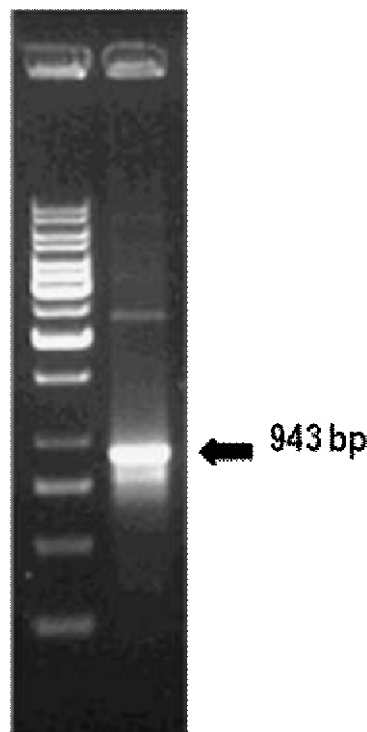
clone was sequenced and named as TNAU T. The sequence was submitted in the National Center for Biotechnology information

Fig 3: Autoradiogram showing positive signals of cDNA library



(NCBI) database. The genebank accession number for the sequence corresponding to the resistance gene, TNAU T from *L. peruvianum* is DQ 914638. In order to screen the cDNA library for tospovirus R gene, gel eluted PCR amplified partial gene product was used as the probe.

Fig. 4 Confirmation of recombinant plaques by PCR after secondary cDNA library screening



M 1 Kb DNA ladder; 3 PCR amplified products from recombinant plaques

The positive plaques were selected by aligning the plates with autoradiogram (primary screening) (Fig. 3). Positive signals corresponding to around 150 plaques were seen in all 20 plates. All the 150 positive plaques were subjected to one more round of screening by replating and rehybridizing with the same probe (secondary screening). The presence of cDNA of interest in plaques selected at random were confirmed by PCR amplification of recombinant plasmids (Fig. 4).

Discussion

Complementary DNA (cDNA) libraries represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason,

the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then is inserted into a self-replicating lambda vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease. In the present study cDNA library from leaf mRNA of *L. peruvianum* EC 52071 was constructed to pull out full length clone corresponding to tospovirus resistant R gene.

Lambda ZAP vector was used to construct the library. The ZAP Express vector allows both eukaryotic and prokaryotic expression, with increased cloning capacity. It also has a number of unique lambda cloning sites. Kwon *et al.* (2000) isolated a cDNA clone of RIP from *Amaranthus viridis* cDNA library, constructed in Uni-ZAP XL vector with poly(A) RNA purified from the leaves of *A. viridis*. The number of recombinant (white) plaques was around 40 times more than the non recombinant (blue) plaques. This suggests that the cDNA library constructed was a good representation of *L. peruvianum* cDNA transcripts. Based on reported values in the literature, the expected size of tospovirus R gene in *L. peruvianum* used in the study is around 3.7 kb. Hence lambda vector is more suitable.

Around 150 positive plaques were selected by primary screening using radiolabelled partial cDNA probe. All the plaques were subjected to secondary screening by replating and rehybridising with the same probe. In secondary screening around 70 plaques showed complete hybridization. Inserts cloned into the ZAP Express vector can be excised out of the phage in the form of phagemid vector. Hence phagemid vector was excised and multiplied. Further confirmation for the presence of right inserts in the hybridized clone were done by PCR amplification by picking plaques at random and amplifying using the same primer pairs that were used for partial gene amplification. Amplified products of the same size (943 bp) were obtained confirming the presence of the right gene inserts. Sequencing of these clones will confirm the presence of full length gene. cDNA probes were used for screening the genomic library of PAP and a genomic clone encoding PAP was picked up from the genomic library (Kataoka *et al.*, 1992a Kataoka *et al.*, 1992b).

The present study was helpful to isolate tospovirus resistant R genes from wild species of tomato *L. peruvianum* EC 52071. The R genes from wild species can be transformed into varieties/cultivars susceptible to tospovirus infection by various transformation techniques to develop tospovirus resistant plants. The cDNA library constructed using the ZAP Express cDNA synthesis kit is a source for many genes in tomato. The library can be utilized for isolation of other resistant genes and also of genes of agronomic importance.

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