Chapter 4: TRN1 gene information
4.1 INTRODUCTION

In the 3rd chapter I have discussed about the differential expression of leaf developmental genes during Tomato leaf curl New Delhi virus infection. Among the 19 different developmental genes it was confirmed that the expression level of TRN1 gene was highly upregulated. Both the semi-quantitative PCR and Real time PCR data confirmed the identical result. It is also known that alteration in TRN1 expression results in leaf defects that resemble the tomato leaf curl virus symptoms (Cnops et al. 2006). Thus investigations were carried out to understand the mechanism of regulation of tomato TRN1 and the significance of this regulation during infection. Prior to the in depth analysis, information regarding gene map was required.

From the literature and database analysis it was known TRN1 gene codes for TORNADO1 protein of 1380 amino acids Arabidopsis thaliana. The N-terminal region of TRN1 gene contains a putative LRR ribonuclease inhibitor–like (LRR-RI) subfamily domain which is most similar to the animal cytosolic nucleotide binding oligomerization domain(NOD-LRR) proteins (Inohara et al. 2005). However, not much information regarding the tomato TRN1 gene is available in literature.

In order to know the details about the gene information cloning and sequencing of the full length gene, followed by analysing the presence of different motifs bioinformatically is required. In addition analysis of expression pattern of the gene in different plant parts at the course of development and evolutionary relatedness of the gene needed to be carried out to confirm the identity of the cloned product.
4.2 MATERIALS

4.2.1. Plant materials: Same as mentioned earlier in chapter 2

4.2.2. Common reagents of RNA isolation, cDNA preparation: Same as mentioned earlier in chapter 3

4.2.3. pGEM-T- easy vector

![Diagram of pGEM-T Easy Vector](image)

Fig 1: Diagrammatic representation of pGEM –T Easy vector (Promega)

4.2.4. Bacterial strain

*E. coli* are gram-negative bacillus bacteria. They reproduce by successive binary fission with a generation time of approximately 30 minutes with optimum growth occurring at 37°C. This strain of *E. Coli* (DH5α) is not a pathogen, and was developed for laboratory cloning use. This strain was developed by D. Hanahan as a cloning strain with multiple mutations that enable high-efficiency transformations.
4.2.5. Primers used for full length cloning of TRN1 gene

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4.3. METHODS

4.3.1 Isolation of total RNA and preparation of cDNA

For isolation of total RNA from leaf tissue same protocol was followed as described in chapter 3. For isolation of RNA from hypocotyls and root following steps were done.

Hypocotyls: The tomato seeds were allowed to germinate in soilrite. After 7 days of germination the hypocotyls were harvested, and RNA was isolated from 200 mg tissue.

Roots: similar growth condition was followed. The roots of 10 day old plant was harvested and washed in Millipore water for the removal of debris.

4.3.2 Preparation of E.Coli (DH5α) competent cells by CaCl₂ solution

- Preparation of calcium chloride solution: 60 mM CaCl₂ + 15% Glycerol was prepared autoclaved and cooled before use.
- Bacterial culture: From the overnight grown 5 ml culture, 1 ml bacterial culture is added in fresh 100 ml liquid LB media and allowed to grow at 37°C, in a shaker at 180 rpm. After 3 hrs of bacterial growth when the O.D reached 0.4 the cells were harvested.
Procedure:

1. 50ml culture tube is taken and pre-cooled in ice for 10 min. 50 ml of log phase culture was poured in the pre-cooled culture tube.
2. The culture was centrifuged at 3500rpm at 4°C for 10 min, soup was discarded.
3. The remaining culture was poured in the culture tube followed by centrifugation at the same parameter.
4. Within the bacterial pellet (~10ml) ice cold calcium chloride solution was added. The bacterial pellet was resuspended in it.
5. Centrifugation was done at 4°C for 10 min soup was removed.
6. The pellet was again resuspended in 2ml ice cold calcium chloride.
7. 100μl of competent cells were aliquoted in 1.5ml eppendorf tube.
8. The competent cells were snap chilled in liquid N₂ and stored in -80°C freezer.

4.3.3. Transformation of E. Coli (DH5α) competent cells

1. The competent cells were thawed in ice for 5 min.
2. 2μl of plasmid DNA was added in 100μl of competent cells and kept in ice for 15min.
3. The competent cells were kept in a 42°C water bath for 60 sec. The cells were immediately transferred in ice after heat shock.
4. The cells were kept in ice for another 15 min.
5. 1ml of LB media is added within the competent cells and allowed to grow at 37°C for 1hr at 280rpm shaking.
6. After 1hr the cells were harvested and the transformed cells were screened with proper antibiotic.
4.3.4. Cloning of gene fragment in pGEM-T easy vector

The gene of interest for cloning in TA vector was PCR amplified by standard Taq Polymerase (it incorporates an extra "A") analysed in 1% agarose gel. The specific band was gel purified and ligation was done following the kit provided protocol.

- 2X Rapid Ligation Buffer.................................5μl
- pGEM – T Easy vector(50ng)...............................1μl
- PCR product................................................3μl
- T4 DNA ligase..............................................1μl

**Total volume...........................................10μl**

The reaction mixture was mixed by pipeting and the ligation was performed at 4°C over night. In the next day 5μl of ligation mix was transformed in DH5α competent cells for screening of the transformed cells.

4.3.5. Construction of phylogenetic tree

I have collected known and putative TRN1 (or TRN1 like) protein sequences of different plants including representatives of monocotyledonous, dicotyledonous, tree, various herbs and a Bryophyte. Phylogenetic analysis was done using MEGA 6 algorithm with 1000 bootstraps.
4.4. RESULTS

4.4.1. Full length amplification and cloning of TRN1 gene

Full length coding sequence of TRN1 was amplified from cDNA pool synthesized from tomato young leaf (1 month old plant) tissue RNA using OligoD primer and a type of M-MuLV reverse transcriptase. The amplified fragment was cloned into a suitable vector and subsequently sequenced.

Fig 2: Full length amplification and cloning of TRN1 gene

A. Full length amplification of TRN1 CDS (4371bp) from cDNA using (TRN1 FL R and TRN1 FL F) primers. B. Schematic representation of position of exons and introns in the TRN1 gene; the next panel shows the gel picture showing the TRN1 fragment (980bp, 1080bp, 2311bp) after digestion with EcoRI enzyme. Arrows indicating the respective cloned fragment.
transcriptase. I could amplify the whole CDS (4371 bp estimated size) using a Taq-based DNA polymerase having proofreading activity (Fig. 2A) and attempted to clone the 4371 bp fragment in pGEM-T easy vector however, the clones were not stable. For easy cloning and sequencing, I have designed primers to amplify 980bp fragment, 1080bp fragment and 2311bp fragment from cDNA followed by cloning of the fragments in pGEM-T easy vector. The positive clones were confirmed by digested with EcoRI, followed by sequencing. The assembled full-length sequence has been deposited in GenBank (KJ398402.1).

**Solanum lycopersicum putative tornado 1 protein (TRN1) mRNA, complete cds**

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Chapter 4  TRN1 gene information

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4.4.2. TRN1 gene Structure

SolGenomics database was searched using Arabidopsis TRN1 protein sequence yielded a homologous sequence with Locus ID Solyc03g112750; however, it was not annotated as TRN1 in SOL genomics network website. The TRN1 gene having 5 exons (138bp, 93bp, 1513bp, 1241bp and 1386bp ) and 4 introns (86bp, 496bp, 91bp and 134bp) respectively (Fig. 2B). The TRN1 gene having 1456 aa, the amino acid sequence were analysed in (EXPASY tool) for the identification of different protein domains present in the TRN1 gene. In analysis of the protein sequence using Expasy tool 6 LRR domains (208-235, 236-263, 368-395, 396-423, 432-461 and 521-549 aa) and one GTPase domain (572-717) have been identified (Fig. 3B).

Fig 3: TRN1 gene structure  A. Position and distribution of 5 exons of TRN1 gene, B. Position of the 6 LRR and 1 GTPase domains in TRN1 gene as predicted by Expasy tool.
4.4.3. Phylogenetic Tree of TRN1 gene

I have collected known and putative TRN1 (or TRN1 like) protein sequences of different plants including representatives of monocotyledonous, dicotyledonous, tree, various herbs and a Bryophyte based on lowest e-value of the local alignment. Phylogenetic analysis was done using MEGA 6 algorithm with 1000 bootstraps (Fig. 4). Emergence of TRN1 sequences followed the

**GenBank accession numbers of the genes used in this analysis noted beside the species abbreviations.**


**Fig4. Phylogenetic analysis of TRN1 gene of different plant species.**
evolutionary path, TRN1 gene of *Physcomitrella patens* seemed to be the ancestor of all others analyzed in this study.

I could not include any Gymnosperm TRN1 sequence due to nonavailability in NCBI. TRN1 of flowering plants seemed to have originated from *Amborella trichopoda*, the most basal flowering plant. While all monocotyledonous plants TRN1 are related and grouped together, a date palm sequence was branched out from the rest of the monocotyledonous group. The TRN1 gene of Dicotyledonous plants are grouped together. The TRN1 gene of tomato plants were grouped with other plants of solanaceae family.

### 4.4.4. Tissue specific and developmental stage specific expression of TRN1 gene

For the determination of relative expression of TRN1 gene in different tissues, RNA was isolated by TRIZOL reagent (Invitrogen). I have chosen leaf, root and hypocotyls tissues for the isolation of total mRNA. Equal amount of RNA (5μg) of RNA was used for the preparation of cDNA using oligo d(T) primer. The cDNA was diluted 5 times with nuclease free water. The semi-quantitative PCR was performed from 1μl of cDNA from each sample. The expression level of housekeeping gene Actin was used as a internal calibrator for the determination of the relative expression level of TRN1.

![Fig5. Tissue specific expression of TRN1 gene](image_url)

**A.** Figures showing the different tissues used in this study, **B.** The semi-quantitative PCR product analysed in 1.5% agarose gel, **C.** Graph shows the relative expression of TRN1 gene in leaf, root and hypocotyls. Data presented as percent of band intensity relative to actin in the same sample.
gene in different tissues. The PCR product was analysed in 1.5% Agarose gel (Fig5.B) followed by quantification gene in the respective tissue was normalised by the expression of Actin gene in that tissue (Fig5.C). From the graph in (Fig5.C) it is evident that the TRN1 gene is expressing almost at equal level in leaf, root and hypocotyl. For the developmental stage specific experiment similar procedure was followed. This is evident from the data that TRN1 gene is expressed at basal level in all tissue. The expression of TRN1 gene in radicle is relatively higher than cotyledonary leaf and first true leaf (Fig6B).

Fig6. Developmental stage specific expression of TRN1 gene, A. Figures showing the different developmental stages(Radicle, cotyledonary leaf and first true leaf) B. The Realtime PCR product analysed in 1.5% agarose gel. Graph shows the relative expression of TRN1 gene in Radicle, cotyledonary leaf and first true leaf.
4.5 DISCUSSION

Till date, experimental evidence of tomato TRN1 gene expression and transcript sequence information was not available. My search of the tomato SolGenomics database using Arabidopsis TRN1 protein sequence yielded a homologous sequence with locus ID Solyc03g112750, however, it was not annotated as TRN1. Moreover, the predicted sequence in tomato had additional 240 bp in the 5’-end of the coding sequence compared to the TRN1 CDS of Arabidopsis. TRN1 full-length gene is found in the chromosome 3 (tomato genome database version 2.5) between 63087645 to 63093187 nucleotide positions, in which the CDS spans from 63087801 to 63092171 nucleotide positions. Interestingly tomato TRN1 has additional 80 aa to its 5’-end compared to the gene in Arabidopsis. Analysis of the sequence in Expasy did not reveal the existence of any additional domain in this region. For this reason, it is difficult to predict the functional advantage due to the presence of this extended N-terminus. The sequenced clones had a G instead of A, and a C instead of T at positions 50 nt and 1371 nt downstream to A of ATG compared to the TRN1 gene in tomato genomic sequences. These nucleotide substitutions would cause only Lys17Arg alteration and introduce a silent mutation in the other position.

Emergence of TRN1 sequences followed the evolutionary path, Physcomitrella patens TRN1 seemed to be the ancestor of all others analyzed here. TRN1 of flowering plants seemed to have originated from Amborella trichopoda, the most basal flowering plant. While all monocotyledonous plants TRN1 are related and grouped together, a date palm sequence was branched out from the rest of the monocotyledonous group. Dicotyledonous plants TRN1 are genetically related and tomato TRN1 gene is closely related to other Solanaceous crops (Fig.4).

Relative expression level of TRN1 in different tissues was determined by semi-quantitative RT-PCR analysis. In this experiment, the expression level of β-actin in various tissues used as an internal standard. TRN1 is ubiquitously expressed and the level was similar in leaf, root and hypocotyl tissues that were tested (Fig. 5).
4.6 REFERENCE
