Isolation and Northern Transfer of Tomato RNA for Screening Plants against Heat Tolerance

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ABSTRACT

Heat distribution is the major factor that limits the area of tomato cultivation in Sri Lanka.Development of heat tolerant varieties are required to overcome the problem of heat sensitivity in tomato cultivation. It is reported that the gene responsible for heat tolerance in tomato is HsfA-1 (652 bp). The overall objective of the present study is to develop a method of marker based selection of tomato varieties for heat tolerance. Immediate objective is to establish a protocol for isolation of RNA from tomato. Three tomato varieties Ravi, T245, Hersutum, were selected for the study. Leaves from one month old plants grown in green house condition were used for RNA extraction. Grinding in liquid nitrogen disrupted tissues and RNA was finally precipitated from the nucleic acid solution using LiCl.

Concentration of RNA obtain from each variety was estimated using a Spectrophotometer. Concentration of RNA yields obtain from Variety Ravi, Hirsutum and T245 was 34.58µg/ml, 30.6µg/ml and 29.45µg/ml respectively. Using gel that contains agerose, 10x running buffer and formaldehyde, and agerose gel performed electrophoresis. Northern transfer of RNA was done. RNA could not be visualized under UV illuminator in the loading buffer, which contains formamid, but RNA was presented on the 1.2% agerose gel with less concentration of formamaid in loading buffer. Formamide change the secondary structure of RNA into linear structure, which lowers the binding of Ethidium bromide with RNA. DNA content in isolated RNA sample was treated with RNAse. RNA contamination with DNA was low in established protocol. To identify heat tolerant varieties, expression levels of the HsfA-1 can be monitored using Northern hybridization. That supports to reduce field experiment of tomato to identify heat tolerant nature of cultivated tomato varieties in Sri Lanka. Development of heat tolerant tomato varieties will expand the extent of tomato cultivation in Sri Lanka.

KEY WORDS: Tomato, RNA, CTAB, Isolation, Heat Tolerance, Gene Hsfa-1, Northern- Transfer.

INTRODUCTION

Tomato (*Lycopersicon esculentum miller*) is one of the most popular vegetables in Sri Lanka. It is cultivated in an area of 5,788 ha with an average yield of 7.6 t/ha. (Peiris, 2002) During the recent past, the farming community of Sri Lanka due to its multifarious benefits in income has focused much attention on this crop; export potential, human nutrition, health and employment avenues.

Expansion of the cultivation of tomato is restricted by heat distribution in Sri Lanka .As most tomato varieties have been bred originally for cooler climate (ag. Arizona.edu). Tomato is better growing in Sri Lanka high altitude regions such as Matale and Kandy. They do not grow well in hot areas. Therefore the development of heat tolerant varieties is required to over come the problem of temperature stress in tomato cultivation in Sri Lanka. Identification of heat tolerant tomato varieties is essential to shorten the conventional tomato breeding cycle. In the development of heat tolerant varieties, it will be necessary to study the varieties with expression of the heat stress induced genes. The major focus of this study is to establish a reliable RNA extraction method from tomato to enable such gene expression studies.

Ribo nucleic acid (RNA) is a single strand polynucliotide chain. It is mainly consisted of purine (adenine, guanine) and pyrimidin (cytosine and urasil). (Bu'lock and Kristianseen, 1987). A pool of RNA contains three main group of RNA viz; mRNA, tRNA and rRNA. Only the mRNA will be translated in to proteins that will be useful in gene expression studies. As well it helps to detect the expression of relevant stress induced genes in plants. In the present we are keen on heat-induced gene HsfA-1, in the complex family of heat stress transcription factor HsfA-1 has a unique role as master regulator of thermo tolerance in tomato. .By detecting level of expression of HsfA-1 we would be able to screen the tomato varieties according to their heat tolerant ability and heat susceptibility action.

The expression level of gene can be determined by the Northern hybridization technique. The RNA extracted will be separated by gel electrophoresis, immobilized and probe with the labeled HsfA-1 gene. (Sambrook et al.1987).

The whole experiment helps to check heat tolerant ability of tomato varieties that are grown in Sri Lanka. Successfulness of RNA isolation from tomato varieties will accelerate the other experimental steps. Therefore establishment of RNA isolation protocol is essential for marker-based selection of heat tolerant in cultivated tomato varieties in Sri Lanka.

Successful analyses of gene expression depend s on The RNA isolation and hybridization procedures. Therefore experiment were made with the key Objectives such as Establishment of RNA isolation protocol for tomato, Quantitative and qualitative analysis of total cellular RNA.Northern transfer and detection of heat tolerant tomato varieties through non radio active (Dig-labeling) method.

MATERIALS AND METHODS

The experiments were carried out at the Molecular Biology laboratory of the Plant Genetic Resource Center, Department of Agriculture, Gannoruwa, Peradeniya. (From 2nd of December 2004 to 30th July 2005).

Plant Variety establishment and maintenance

Two tomato varieties, T245, Ravi and one wild tomato species L.hursutem were selected for the study. Seed of those varieties were sown on separate nursery. Bedding mixture was prepared according to the recommendation of Department of Agriculture in Sri Lanka (DOA). After three weeks tomato varieties were transplanted in separate pots. Those pots were filled with topsoil and compost mixture according to the recommendation of DOA. Two seedlings per pot were planted. Transplanted tomato varieties were placed on ion racks around 2 feet above the ground. Each and every plant was marked according to their variety and date of transplanting. Plants were maintained through out the experiment. Fertilization, water management and disease control were done according to the recommendation of DOA.

Preparation of Solutions

The stock solutions of the chemicals were prepared using DEPC treated distilled water (1%). Extraction buffer; 1 M Trios-HCL, 50 mM EDTA, 1% (w/v) SDS, pH 9,Phenol: Chloroform:Isomylalcohol (PCI); 24 parts Phenol,24 parts Chloroform,1 parts Isomylalcohol,Chloroform:Isomylalcohol(CI);24 parts Chloroform , 1 parts Isomylalcohol,4M Lithium solution,10x running buffer ; 0.2 M MOPS , 10mM EDTA, 0.1 M Sodium acetate pH 7,10x dye mixture; 0.2 %Xylen xyanol ,0.2 % Bromophenol blue, 10mM E50% (w/v) Glycerol, 20x SSC solution; 3M NaCL, 300mM Sodium acetate pH 7,TBE running buffer (1 L); 54 g Tris,0. 5M EDTA, 27.5 g Boric acid, pH 8.

Amplification of Gene HsfA-1

a. DNA extraction

DNA was extracted from the varieties of Ravi, Thilina, Maglob and *L.hirsutum* using Cetyltriumethylamonium bromide (CTAB) extraction protocol (Doyle and Doyle.1990). Two grams of leaves were measured from each variety and leaves were placed in a mortar. The leaves were ground well using pestle until it becomes a powder with liquid nitrogen. The powder was transferred in to falcon tube (15 ml), followed by the addition of 20ml of 1.5x CTAB extraction buffer. Tubes were placed in shaking incubator at 200 rpm for 20 minutes at 56 ^{0}C. 20ml of Chloroform: isomylalcohol (24:1) solution was added into falcon tubes and mixed well. Tubes were centrifuged at 3000rpm for 25 minutes at 20 ^{0}C .Supernatant was transferred into new falcon tube and mixed with 2ml of pre warmed (65 ^{0}C) 10% CTAB solution. After addition of 20ml of CI solution, it was shaked for 20 minutes at room temperature. Solution was then centrifuged at 3000rpm at 20^{0}C for 25 minutes. The supernatant was transferred into new falcon tube and 20 ml of isopropanol was added. The pellet formed after centrifugation was washed with 70% ethanol. The mixture was centrifuged and pellet was dried in air for 30 minutes. Finally pellet was dissolved in 2 ml of TE buffer.

b. PCR ampliplication

For PCR reaction master mixture was prepared according to following combinations, for five samples.

Ingredients	volume per one sample (µl)
Taq polymease (5 u/µl)	0.25
Buffer (10 x)	1.25
DNTP2.5mM)	1.0
Primer 1(10 pmol)	0.65
Primer 2(10 pmol)	0.65
Water	7.70

The two primer of the HsfA-1 Specific primers were5'GCACCTGCTTAAAAGTATAAGAAGTCGG 3'(primer1)and5'CCTGAAGAGTGACTGACTCCTG AACAC3' (Primer 2).After making the master solution, 11.50 µl of master mixture was transferred into 5 different eppendorf tubes. Then, 1µl of each extracted DNA was added into each eppendorf tube and mixed well. Amplification was done at 94 ^{0}C for 30 sec, 56 ^{0}C for 1 min and 72 ^{0}C for 1 min 30 sec for 30 cycles using Perkins, Elmer PCR machine.

PCR products were analyzed on 1.4% agarose gel, stained with ethydium bromide. Finally amplified bands were visualized under UV light through BIORAD gel documentation system, using quantity one software package.

Isolation of total RNA

All equipments were treated with 0.1 % DEPC over night washed with 0.1 NaOH and autoclaved and dried (Potrykus and Spangenberg.1995). 2g of leaves were harvested from each variety just before flowering. The clean middle age leaves were ground in liquid nitrogen with a motor and pestle until it become into a fine power. Frozen powder was transferred into a medium size corex tube using metallic spatula and 3 ml of extraction buffer was added, followed by the addition of 3 ml PCI solution (24:24:1). Tubes were then placed on ice for one hour, with intermittent shaking.

The mixtures were centrifuged at 10000 rpm for 5 minutes, at 16 ^{0}C in an An 2 rotor. Then upper aqueous phase was transferred into new medium size corex tubes. The organic phase was re-extracted with 1 ml of extraction buffer. Centrifugation was repeated as above and two aqueous phases were pooled. 4ml of PCI solution was added into the pooled aqueous phase. After centrifugation, upper phase was transferred into a new medium size corex tube and 4ml of CI (24:1) solution was added. After centrifugation upper phase was transferred into a medium size corex tube for the precipitation of RNA.

Two hundred micro liters of LiCL was added to the upper aqueous phase followed by the addition of 4ml absolute ethanol. Then mixture was shaken well and kept for 30 minutes at –20 ^{0}C. The mixture was centrifuged.

The supernatant was discarded and pellet was dissolved in 2ml of DEPC treated distilled water and 2ml of LiCl.After that mixture was kept at –4^{0}C for overnight. The mixture was centrifuged as above and supernatant was discarded. Then pellet was washed with 70% ethanol and centrifugation was repeated. The

supernatant was discarded and pellet was air dried in a fume hood. The dried pellet was dissolved in 200 µl of DEPC treated distilled water, and transferred into 1.5 ml eppendorf tubes for long-term storage. Then tube was stored at –20 °C. Finally single stranded RNA concentration was measured at 260 nm

Quantification of extracted RNA

Spectrophotometer was warmed up with UV light on for 15 minutes. 1 ml of DEPC treated distilled water was added in to four cuvettes and 5µl of water was removed from three cuvettes while adding of 5µl of extracted RNA from each variety (Ravi, T245, *and L.hirsutum*). Cuvettes were placed in the Spectrophotometer and absorption readings were taken (260/280nm).

Electrophoresis of RNA

RNA was separated according to their molecular size using denaturing agarose gel, which contained following constituents.

For standard gel of 50ml volume, 0.5g of agarose was dissolved in 36ml DEPC treated distilled water. Then both samples were mixed well by microwave oven. The mixture was cooled up to 55^{0}C in a fume hood and 9ml of formaldehyde and 5ml of 10x running buffer was added.

For preparation of agarose gel of 150ml volume, 1.8g of agaros was dissolved in 150ml of 0.5x TBE buffer solution .As well as 50ml volume agarose solution was prepared by dissolving 0.6g of agarose in 50ml of 0.5xTBE solution. Gels were casted as above.

Gel loading

Samples were loaded in the gel according to the following combination to select the best resolution of plant RNA (Potrykus and Spangenberg.1995).

Table 1:constitution of the sample loading buffer.

a. Combination –1

Sample	Constituents (µl)				
	RNA	DEPC	Formald-ehyde	Forma-mid	Dye
1	2	2	2	3	1
2	2	5	2	-	1
3	2	4	-	3	1
4	4	7	-	-	1

b. Combination 2

Sample	Constituents (µl)				
	RNA	DEPC	Formalde-hyde	RNA-se	Dye
1	2	2	2	-	1
2	2	4	-	-	1
3	2	2	2	-	1
4	2	4	-	-	1
5	2	2	-	2	1
6	2	2	-	2	1

C. Combination -3

Sample	Constituents (µl)			
	RNA	DEPC	Formalde-hyde	Dye
Ravi - 1	2	4	-	1
T 245 - 2	2	4	-	1
Ravi - 3	2	2	2	1
T 245 - 4	2	2	2	1

Gel runing

Combination 1 and 2 were run in 50 ml volume agarose gels at 150v and 50mA for one hour and combination 3 was run in 150 ml agarose gel at 150 V and 50mA for one and a half hours, until the dark blue bromophenol blue dye has migrated one-half to two-thirds the length of the gel.

After electrophoresis the gel was soaked in ethidium bromide solution for 15 minutes and de-stained in distilled water for 5 minutes. Then stained gel was allowed to remove excess water. RNA on the gel was visualized under UV light through BIORAD gel documentation system with quantity one software package.

Northern blotting

RNA was transferred into a Nylon membrane following Northern-blotting steps (Sambrook et *al*, 1987). The gel was rinsed with DEPC treated distilled water and the unnecessary parts of the gel were cut off leaving the area where RNA bands are present. Gel was then soaked in 20XSSC solution for 1 hour. Materials used in blotting unit were treated with DEPC treated distilled water. A tray was placed on a flat table and a plastic box was placed on the middle of the tray as a supporter for the gel.

3mm Whatman paper was placed on the supporter and the tray was filled with 20 X SSC solutions until the level of the liquid reaches almost to the top of the supporter. Air bubbles were removed with a glass rod. Positively charged hybond Nylon membrane was cut about 1 mm larger than the gel in both dimensions by a fresh scalpel. Then, Nylon membrane was floated in de-ionized water until it was soaked completely and the membrane was immersed in 20 X SSC solutions for 5 mins. The bottom left hand corner of the membrane was cut to recognize the gel orientation. The gel was placed on 3 mm Whitman paper in an inverted position and air bubbles were removed in between Whatman paper and the gel. Surround of the gel was covered with Para film. The Nylon membrane was placed on the top of the gel to align the cut corners. The membrane was then covered with five Sheets of 3 mm Whatman paper, cut to the size of the gel, soaked in 2 X SSC solutions. Followed by a stock of paper towels. A weight of 500g was placed on top of this arrangement and left 12 hrs to allow blotting.

The position of the gel was marked on the membrane. The membrane was soaked in 6XSSC solutions for 5 minutes and allowed to drain. Dried Nylon membrane was exposed to UV illuminator. The gel after transferring was soaked in ethidium bromide for 45 minutes and de-stained for 10 minutes. Finally it was observed under UV light using the BIORAD gel documentation system in order to confirm the transfer of RNA to the membrane.

RESULTS AND DISCUSSION

Plants were maintained under the plant house condition to minimize the environmental stress, and to maintain disease free condition.

Pure DNA was extracted from each variety by CTAB method (Doyle & Doyle.1990). Use of liquid nitrogen reduces the degradation of nucleic acid.

HsfA-1 gene specific primers Amplified a 652 bp fragment from all the varieties of tomato Ravi, Marglob and T 245, (figer 1) indicating the presence of Hsfa-1 related genes in their genomes. The absence of genetic polymorphism as detected by PCR hinders the use of there primers as a source of genetic markers for selection for heat tolerance.

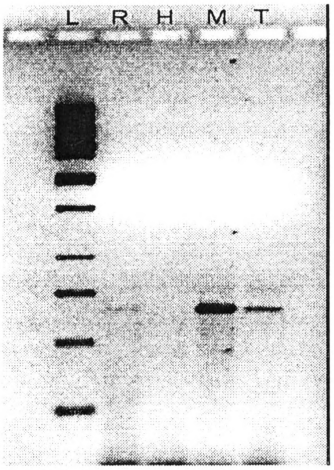

Fig. 01. Amplified gene HsfA– 01
L=Ladder R=Ravi, H=Hirsutum, M=Marglob, T=T245

Precautions were taken to minimize the RNA degradation by the RNAse during the RNA extraction procedure .The measures taken in this study, such as DEPC treatment were adequate in obtaining total RNA in good quality and quantity. As mentioned above, liquid N_2 increased the harvest of Nucleic acid while Phe/ch/ISo act as a de-proteination agent. LiCl helps to precipitate the cellular RNA. As well as it is accelerated by low temperature .So it is advised to keep the solution at -70°C. Finally, dried pellet of RNA is dissolved in DEPC treated distilled water that helped to reduce the RNAse reaction on extracted RNA. Low temperature reduces the degradation of RNA therefore RNA is usually stored at –70°C but this study the RNA was stored at -20°C for long-term storage. Concentration of RNA was measured at 260 nm wavelength using Bedmate 3 Spectrophotometer. The RNA concentrations were 30.6 µg/ml in L.hirsutum, 34.58µg/ml in Ravi and 29.45 µg /ml in T245, RNA purity is less in T 245 due to its high polyphenol content than in other varieties. Figure 2 shows the RNA electrophoresis on 1.2 % agarose gel using the different gel loading buffers, containing different concentration of Formaldehyde and formamid. RNA was best detected in lane 4 in combination 1.When both formaldehyde and formamid present RNA could not be detectable indicating .RNA was poorly visualized with loading buffer containing formamid but comparatively better visualization was obtained with the loading buffer containing formaldehyde. The results show the formamid on RNA structure, which affect the binding of stain to the RNA molecules.

Sample No. 4 showed better resolution than sample No 3. Therefore, formaldehyde also has ability to interfere on RNA structure. But it is less effective than formamid. Formamid form hydrogen bonds with nucleic acid and changed their structure into linear structure. Therefore it is not good for visualization of RNA but it is a good chemical in hybridization. Because linearity of RNA molecule facilitates its binding ability with dig labeled probe. (See Figure 02)

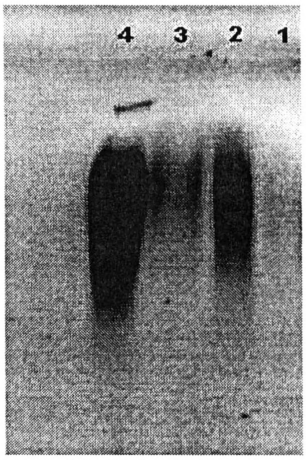

Fig. 02. 1.2% Agarose gel, Samples were loaded according to the combination 01

Contamination of RNA extraction with DNA is a common problem in RNA extraction protocols. The RNA samples extracted were tested for DNA contamination by treating the samples with the RNAse (figer 3). Loss of Ethidium bromide presence in RNAse treated samples (lane 5 & 6) suggests that there was no DNA contamination in the RNA extracted using the current protocol.

To optimize the loading buffer combinations for better resolution of RNA on electrophoresis different loading buffers were used (Table 1 combination 3). The best resolution was shown by sample No. 2, T 245. However, other samples were good enough for Northern transfer. (See Fig. 04)

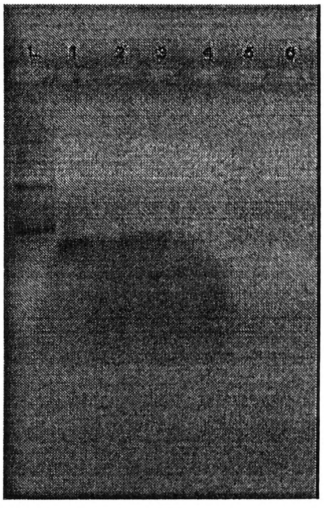

Fig. 03. 1.2% Agarose gel, Samples were loaded according to the combination 02 Samples 05 & 06 has treated with RNAs.

In Northern blotting, capillary reaction helps to transfer RNA molecules from the gel to Nylon membrane. After transferring process, the gel was soaked in ethidium bromide for 45 minutes and checked under UV light to confirm the RNA transfer.

The absence of RNA on the gel proved the better transfer of RNA in to Nylon membrane (see Fig. 5,6,7).

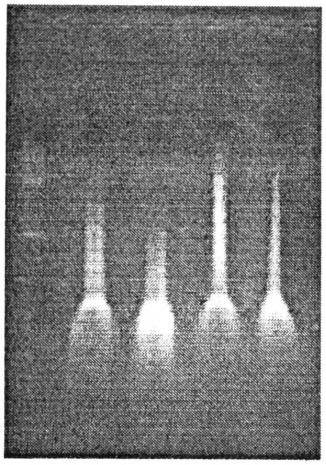

Fig. 04. 150 Volume 1.25% Agarose gel were loaded according to the combination 03(150V, 50mA, $1^{1/2}$ hrs)

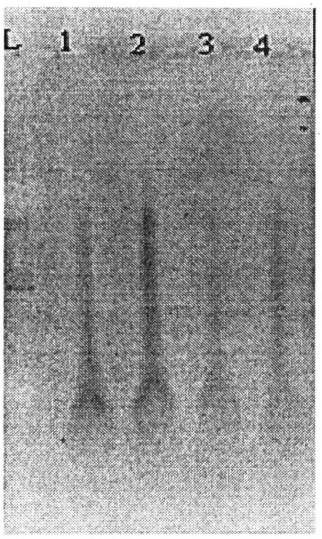

Fig. 05. Gel Before Northern transfer

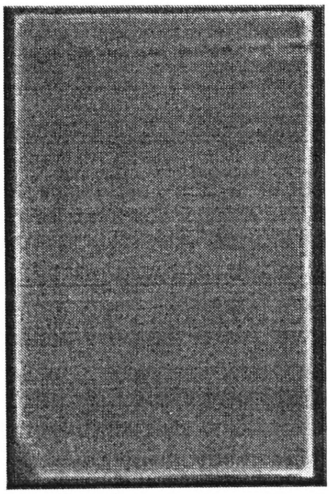

Fig. 06. Gel after Northern transfer.

CONCLUSION AND FUTURE DIRECTION

Better yield of RNA with the least contamination with DNA was resulted through the established protocol Therefore this protocol is suitable for the extraction of RNA from tomato. Formamid is not suitable to mix with loading mixture for visualizing the RNA but it's a good source to linearize

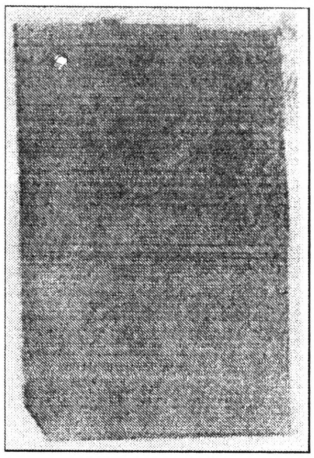

Fig. 07. Transferred Nylon membrane

the secondary structure. Quick handling and better sterilization with DEPC resulted in good RNA harvest. Nylon membrane has better binding affinity with RNA. Complete transfer of RNA into Nylon membrane was confirmed by the absence of visualized RNA on the resoaked gel in Ethidium bromide.

It is planning to label the amplified gene HsfA-1 by non-radioactive method, and hybridize with immobile RNA molecules on Nylon membrane. That will detect the heat expression level of the gene HsfA-1 in different heat tolerant tomato varieties. This will increase the efficiency of the conventional breeding of tomato to select heat tolerant varieties through field experiment. This helps to extend the land area of tomato cultivation due to development of heat tolerant tomato varieties.

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