Identification of key genes involved in root development of tomato using expressed sequence tag analysis

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Abstract Root system of plants are actually fascinating structures, not only critical for plant development, but also important for storage and conduction. Due to its agronomic importance, identification of genes involved in root development has been a subject of intense study. Tomato is the one of the most consumed vegetables in the world. Tomato has been used as model system for dicot plants because of its small genome, well-established transformation techniques and well-constructed physical map. The present study is targeted to identify of root specific genes expressed temporally and also gene(s) involved in lateral root and profuse root development. A total of 890 ESTs were identified from five EST libraries constructed using SSH approach which included temporal gene regulation (early and late) and genes involved in morphogenetic traits (lateral and profuse rooting). One hundred sixty-one unique ESTs identified from various libraries were categorized based on their putative functions and deposited in NCBI-dbEST database. In addition, 36 ESTs were selected for validation of their expression by RT-PCR. The present findings will help in shedding light to the unexplored developmental process of root growth in tomato and plant in general.

Keywords Root development · EST Library · Tomato · Stress related genes · Root elongation

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Introduction

Hidden underground, plant roots often receive less attention than they deserve (Maloof 2004). They are the critical part of plant structure and developmental process (Grierson and Schiefelbein 2002). Faster and more extensive root growth is important for good plant growth under the most adverse soil conditions. Despite their important role in plant development (Parker et al. 2000) relatively little work has been done to understand molecular basis of root development. Very limited number of root specific genes and genes involved in root development have been isolated and identified. Most of the root specific genes isolated are from *Arabidopsis* and Maize (Hochholdinger et al. 2004) while only few root specific genes have been identified from tomato. Evans et al. (1988) isolated root-specific cDNA clones from pea and concluded that root-specific mRNA species are present at a very low level. Fuller et al. (1983) have cloned and characterized a number of nodule-specific genes. Bogusz et al. (1988) isolated a haemoglobin gene expressed specifically in roots of non-nodulating plants. Keller and Lamb (1989) isolated a gene encoding a cell wall hydroxyproline rich glycoprotein expressed during lateral root initiation. Lerner and Raikhel (1989) reported the cloning and characterization of a barley root-specific lectin. Several tobacco cDNAs and the corresponding genomic clones of genes that are expressed in roots but not in leaves have been isolated (Conkling et al. 1990). Even though most of the root specific genes have been isolated from *Arabidopsis*, few root specific genes have also been isolated from Maize, Barley, *Gossypium hirsutum* and *Cucumis sativus* (Sakuta et al. 1998).

Recently, the focus of studying developmental processes has been shifted to tomato, *Solanum lycopersicum* belonging to Solanaceae family. Tomato is the second most consumed vegetable worldwide and represent a major agricultural

industry. Tomato has become a model system of dicot plants for genomic studies because of its small genome, well-established transformation techniques, well-constructed physical map and large number of cDNA sequences (Mueller et al. 2005). The International Tomato Genome Sequencing Project started in 2004 by an international consortium including participants from Korea, China, United Kingdom, India, Netherlands, France, Japan, Spain, Italy and the United States and completed in 2012 (Tomato Genome Consortium 2012). Since then many databases and online tools have been developed to annotate full tomato genome (D'Agostino et al. 2009; Grennan 2009; Fei et al. 2011; Suresh et al. 2014). Preliminary comparisons between the different Solanaceae members revealed synteny among various homologous genes (Wang et al. 2008). This makes tomato a good entry point for characterizing the genes of other Solanaceae members, and associating them with agronomic traits. In tomato, Liu et al. (1998) isolated two root specific phosphate transporters LePT1 (*Lycopersicon esculentum* Phosphate Transporter 1) and LePT2. Both genes were highly expressed in roots, although there was some expression of LePT1 in leaves. The encoded peptides of the LePT1 and LePT2 genes belong to a family of 12 membrane-spanning domain proteins and show a high degree of sequence identity to known high-affinity Pi transporters. In situ hybridization studies demonstrate cell-specific expression of LePT1 in tomato root. The LePT1 mRNA was detectable in peripheral cell layers such as rhizodermal and root cap cells (Daram et al. 1998). Berezky et al. (2003) isolated Lenramp1 (*Lycopersicon esculentum* natural resistance-associated macrophage protein 1) and Lenramp3 genes from tomato and demonstrated that these genes encode functional NRAMP metal transporters and localized mainly to intracellular vesicles. Lenramp1 and Leirt1 (iron-regulated transporter 1) revealed both root-specific expression and up-regulation by iron deficiency. Weeks et al. (2003) isolated a phosphate transporter StPT2 (*Solanum tuberosum* Phosphate Transporter 2) from potato, mainly expressing in the elongation zone at the root tip. A strict root-specific iron-regulated gene of tomato encoding a Lysyl-tRNA synthetase-like protein was isolated using subtractive-hybridisation approach by screening for cDNA clones specific for genes with altered expression in wild-type versus mutant root tissue (Giritch et al. 1997). Other reports include the isolation of metallothionein-like proteins from tomato (LEMT1, LEMT2, LEMT3 and LEMT4) (Giritch et al. 1998), root specific nitrate transporters and ammonia transporter (LeNr1-1, LeNr1-2 and LeAmt1) (Lauter et al. 1996), 2-oxoglutarate and Fe(II)-dependent dioxygenase superfamily (Jones et al. 2008) and FER (Fps/Fes related kinase) genes by Ling et al. (2002). Although, the above studies have identified few genes expressed only in roots, an extensive search for identification and analysis of root specific genes in tomato has not been done so far. Therefore, in the present study, we have

performed high through put screening of root development specific genes using differential expression methods SSH (suppressive subtractive hybridization). We could isolate stage specific ESTs expressed in root, early stage root, late stage root, lateral roots and profuse roots. In addition, stage specific expression of ESTs was validated using RT-PCR.

Materials and methods

Generation of root cDNA libraries

Root specific library

Root specific library was constructed with the aim to identify the genes specifically expressed in roots and not in shoots. Tomato seeds were grown. In 8d old seedling the root was found to be growing actively with proper primary root and root tip. cDNA was prepared from the root and shoot of 8 day old seedling. Using 8d old root cDNA as the tester and 8d old shoot cDNA as the driver, a library was constructed by Suppressive Subtractive Hybridization (SSH) approach. This has been referred as root specific library hereafter. SSH was performed according to the manufacturer's instructions (Clontech). After two rounds of subtraction, the subtracted secondary PCR product was cloned into T-tailed cloning vectors, pGEM-T Easy (Promega) and pXcmKn12 (National Institute of Genetics, Japan) and transformed to *E. coli*. One thousand clones were obtained from root specific library.

Temporal library

Cellular differentiation and development of various organs in multi-cellular organisms is highly coordinated by differential gene expression in time and space. Hence temporal cDNA library was constructed with the aim to understand the nature of the genes specifically involved in early and late stages of root development. The root phenotype of the seedlings grown for varying duration was observed. In 8 day (8d) old seedling, only one primary root with actively growing root tip was observed. In 40 day (40d) old seedling, the root growth was extensive with the formation of lateral roots. Two SSH libraries were made using RNA from 8d and 40d old roots of tomato. RNA from 8d old roots and 40d old roots were used as tester and driver respectively for constructing early library. Whereas RNA from 40d old roots was used as tester and 8d old roots as driver for constructing temporal late library. Both libraries have been referred as temporal library here after. The secondary PCR product was cloned in T-tailed vector pGEM-T Easy and transformed into *E. coli* (DH5 α strain). Two hundred and 250 clones were obtained from temporal early and late expression libraries, respectively.

Library for root morphogenetic traits (lateral and profuse library)

In order to understand the genes specifically involved in root morphogenetic traits like profuse and lateral rooting, tomato species with different root phenotypes were used. Seeds of *S. lycopersicum*, *S. peruvianum*, *S. cheesmani* and *S. hirsutum* were germinated and the root phenotypes were studied at different time intervals. At 40 days, the root morphology of all the tomato species showed pronounced differences. *S. lycopersicum* showed moderate root development with extensive lateral rooting. Rooting was found to be less in *S. hirsutum* and *S. cheesmani*. The root growth was extensive and without any lateral roots in the case of *S. peruvianum*. Therefore, RNA from 40d old roots of *S. peruvianum* and *S. lycopersicum* were used as tester and driver respectively to construct profuse root library. On the other hand RNA from 40d old roots of *S. lycopersicum* was used as tester and from *S. peruvianum* as driver for lateral root library. The secondary PCR product was cloned in T-tailed vector pGEM-T Easy and transformed into *E. coli* (DH5 α strain). Approximately 200 and 400 clones were obtained from lateral and profuse root libraries, respectively.

Sequencing

Plasmid DNA for sequencing was isolated using standard alkaline lysis method. All the sequencing experiments were carried out with automated DNA sequencer (ABI Prism 377, PE Applied Biosystems) using Big Dye terminator kit (Perkin Elmer, USA) at the sequencing facility at Delhi South campus, Delhi University, New Delhi and School of Biotechnology, Madurai Kamaraj University, Madurai.

In silico analysis of ESTs

The sequences longer than 150 bp sequence length were taken for in silico analysis using various bio-informatic tools like BlastN and BlastP from both NCBI and SGN databases. Redundancy and contig analysis of ESTs were done both manually and by means of clustering, using CAP3 EST assembler. Sequences that exhibited more than 80 % identity over total large sequence were considered identical or closely related and were assigned to a specific group. Sequence alignment of those highly similar sequences was confirmed by sequence alignment programs 'Clustal W' (ClustalW and ClustalX version 2007). Potential function of the sequenced ESTs was predicted using BlastP programme (default) at NCBI database. ESTs were grouped into different functional categories according to their predicted gene products based on sequence comparison with the current SWISSPROT/TrEMBL database. Annotation was also performed based on Gene

Ontology (GO) terms and functional categories were defined using Blast 2GO program.

Expression analysis of selected ESTs

S. lycopersicum/*S. peruvianum* seeds were surface sterilized with 0.01 % HgCl₂ and soaked in distilled water for overnight. The seeds were sown on the autoclaved vermiculate, in 200 ml cups or pods. The seedlings were grown for 10 days in growth chamber at 27 °C with a day/night cycle of 12 h/12 h at a light intensity of 30 $\mu\text{E m}^{-2}\text{s}^{-1}$ and humidity of 75–80 %. One hundred milligrams tissue from 8 and 40 day old seedlings was used to extract total RNA with TRIZOL reagent. DNase treated RNA was used for cDNA synthesis using M-MuLV reverse transcriptase (Gibco-BRL). The volume of the cDNA to be taken for further PCR amplification was normalized by performing control PCR reaction with the primers specific to tomato house keeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) using different volumes of cDNA (0.1, 0.2, 0.5 and 1 μl). Equal volume of PCR product from all samples was fractionated on a 1.5 % agarose gel and intensity of the bands compared. The normalized volume of different cDNA stocks, which gave equal intensity with GAPDH primers, were taken for further validation with EST specific primers (Table 1) which were designed using Web Primer software (<http://www.yeastgenome.org/cgi-bin/web-primer>).

Results

Features of EST libraries

Root specific cDNA libraries of many plant species have been made earlier in order to understand molecular basis of root development processes (Asamizu et al. 2000). In this study, a total of 575 ESTs studied in root specific (379), temporal (97), lateral rooting (72) and profuse root (27) libraries which had sequence longer than 150 base pair. BlastN analysis of these EST sequences in NCBI and Sol Genome network indicated 247 ESTs coding for rRNA and organelle genome whereas 328 ESTs represented cDNA sequence. Upon examining 328 EST sequences in CAP3 EST assembler and BlastN program, it was found that 81 (root specific), 42 (temporal), 32 (lateral rooting) and 6 (profuse root) ESTs represented non-redundant sequences. Most of the unique EST sequences were already present in the databases except 4 ESTs of root library and 3ESTs of profuse library who did not have any significant similarity to known sequences in both NCBI and SGN databases. All the non-redundant ESTs of root library (EX149573 – EX149653), temporal library (EX149660 – EX149701), lateral library (EX149540 – EX149571) and profuse library (EX149654 – EX149659) were deposited in dbEST database in NCBI.

Table 1 List of primers used for validation of selected ESTs

S.No.	EST Accession Number	Forward Primer	Reverse Primer	Expected Size of amplicon
1.	EX149632.1	AGA CAC AAC AGG ACC AGA ACC(21)	TGC GGC AGC AAT AGA ACC (18)	270
2.	EX149576.1	CTT GCT GCC TCA GAT TTC C(19)	CAT CAA CTT CCT TGG TGC TC (20)	273
3.	EX149577.1	AGG ACG AAG TGA ACA GAG TG(20)	GAA AGA GAA GCC CAT TAG C(19)	335
4.	EX149578.1	CGA ATA GCA GCA GCA GAT AG(20)	GCC TTC AGG ATT ACG CC(17)	256
5.	EX149581.1	TAG TCC TCC TTG TTC TCA GC(20)	GGG CTC CTT TTG ACC TC (17)	266
6.	EX149586.1	GGT GAA CAA CGA TGG CTA TG(20)	CTG GGA TTA GCA TTG GAACTG (21)	327
7.	EX149593.1	CAC TTC GGT CAA TGC GG(17)	TGT TTT GGA CTG TCT ATG CC (20)	275
8.	EX149614.1	AGA AGG ATA AGA TGT CGC C (19)	CAG GTG ACG CAA CTA AGC(18)	216
9.	EX149590.1	TCG CAA ACT CTG TGG CA(17)	CGC AGT GGC ATT GGA AG(17)	164
10.	EX149619.1	GCG TTC AGT TGT TGC G(16)	GCG AGT CTA TGG AAT GCT (18)	206
11.	EX149626.1	TCT CAC TTG CTC GCT CAC(18)	CAC TTG TAG CCG TGG ATA AC(20)	213
12.	EX149642.1	CAG CCA AAC AGG TGA AGG(18)	TCA GAG ATG GAT GGT CGG(18)	261
13.	EX149616.1	ATA GAA CTC CCA TTT GCC T (19)	CTT CGT TAT CGT TGA CAT TG(20)	194
14.	EX149594.1	GGC ACC AAA CAA GAA CTC G(19)	CCG TCA CAC TGG AAC AAG(18)	156
15.	EX149663.1	GCC GAG GTA GAT TAT GAA GGT G (22)	GAC ATC AGC AGG CAG AGA AC (20)	221
16.	EX149665.1	GAC GCA CCT CAT CTT CAG (18)	GGC ACC ATC CTC TTC ACT (18)	190
17.	EX149674.1	CGT AAT CGT CAC CAT CAC C (19)	TCT GCT CCT TCA GTA TCT CC (20)	392
18.	EX149677.1	GCA ACC CAA CTC TCA ACT C (19)	GCC TAA GGT ATC CGA CAA TG (20)	320
19.	EX149688.1	GGG CTG GAT ACA TTT CTC (18)	TCA ACA CTG ATA GGA CAC G (19)	172
20.	EX149695.1	AGA GGA AAA CTT ACA AAC AGA CAG(24)	CGA GAA ACC CAG ACT TAC CA (20)	204
21.	EX149696.1	TGT CTC CCC AAC TGA ATA CTC (21)	GTG CTG GTG CTA ACA TCC TC (20)	193
22.	EX149699.1	GGT TTC TCG TCG GCT AAG (18)	AGG AAT GGA TTC TGG CA (17)	252
23.	EX149661.1	GTC CCT TAC CCA TTC TGC C (19)	GGA TGA AGT TGT TCC TGA GC (20)	301
24.	EX149669.1	AGT GCT GCT CTG AAT GCT GG (20)	GTC TGC TGC TGC CTC TGT C (19)	303
25.	EX149675.1	CGC CTG AGA ATG GAT ACT TG(20)	AGG AAC TTG AGA AGG AGC C(19)	384
26.	EX149678.1	GCG GAA GAG ACG GAT TG(17)	GGA CCA TGT TGC CAG TAG(18)	274
27.	EX149541	CCT TCT CTG CTG TTT GCT G(19)	GTG CTG GTG TGA TGG GA (17)	237
28.	EX149540.1	AAA TCA GGG TGA ATG GC(17)	CCA GTG TCT ATG GCT CTC C(19)	298
29.	EX149544	TGC CAC TGG AAA TCG G (16)	CGG TTA CGC ACA AGC C(16)	248
30.	EX149546	TGG GCT GAA CCG TGT G (16)	AGG AAG GCA TCA ACC G(16)	254
31.	EX149567	TTT GAC CCC TCT TGC TG (17)	GGA CTC TTG ACC ATC TGC C(19)	259
32.	EX149569	CAT CTT CTC CAT CTG ACT CTC(21)	CAA AGC AGC AGC AAA CAC(18)	206
33.	EX149570	GCT TGG GTG TCA GGT TCT(18)	TGG GTT GTT CAA GGT GC (17)	202
34.	EX149553	CAC CTG GGA AAC TGT CAA G(19)	GAC TCG GAA TAG AAA CGC T(19)	217
35.	EX149559	GTG CTC GGC AAA GAT AAC(18)	AGA TGG CGA AGT GGT CC(17)	288
36.	EX149657	CCG TTC GTG ATT ACC TCG (18)	GGC ATC TCC CTT CTT CTA (18)	270
37.	GI:1931618	TGC CAT CAA GGA GGA ATC (18)	TCT CGG AAA GTG AAC AGG (18)	408

Functional characterization of libraries

Blast 2GO analysis of the non-redundant EST sequences from root library, temporal library, lateral library and pro-fuse library revealed that annotated ESTs were falling into stress related, metabolism, development, transport, signal transduction, protein synthesis and structural proteins categories (Fig. 1a, b, c and d; Tables 2, 3, 4 and 5). However, a total of 36 ESTs from different libraries fall in to

category of unknown and uncharacterized genes (Fig. 1a, b and d).

Validation of ESTs

An attempt has been made to validate ESTs generated in different libraries. For this, a total of 36 ESTs were selected from different libraries and were subjected to RT-PCR using EST specific primers. Out of 14 ESTs analyzed from root library, 7

Fig. 1 Functional distribution of Unigenes from (a) Root specific library, (b) Temporal library, (c) Lateral root library and (d) Profuse root library

ESTs (EX149632.1, EX149576.1, EX149578.1, EX149593.1, EX149619.1, EX149670.1 and EX149594.1) showed over expression in roots (Fig. 2a, b, d, g, j, l and n). Interestingly, two ESTs EX149581.1 and EX149586.1 were expressed only in roots (Fig. 2e and f). In temporal library, 4 out of 12 ESTs (EX149663.1, EX149665.1, EX149695.1, and EX149675.1) showed over expression in 40-day old roots (Fig. 3a, b, f and k), whereas one EST EX149678.1 was over expressing in 8d old roots (Fig. 3l). One EST (EX149669.1) expressed only in 40d old roots whereas no expression could be seen in 8d old roots (Fig. 3j). Similarly, two ESTs (EX149567 and EX149553) out of 9 selected ESTs of lateral library over expressed in 40 day root of *S. lycopersicum* (Fig. 4e and h) and 1 EST (EX149541) over expressed in 40 day root of *S. peruvianum* (Fig. 4a). In case of profuse library, out of six ESTs, only two ESTs (EX149654.1 and EX149657.1) were above 300 bp, and primer could be designed only for EX149657.1. Amplicons of expected size was observed in both *S. lycopersicum* and *S. peruvianum* cDNAs (Fig. 5).

Discussion

Development of *Arabidopsis* root has been well characterized at morphological, biochemical and genetic level as compared to other plants. Over the years, a number of genes related to root development have been identified

that are important for pattern formation, cell cycle, cell growth. Moreover several genes involved in hormonal basis of root development have also been characterized, especially auxin and ethylene (Kushwah et al. 2011). Several root mutants have been identified from different plants like *Arabidopsis* (Busov et al. 2008), rice (Kim et al. 2007), maize (Hochholdinger et al. 2004) wheat (Wang et al. 2006) and tomato (Negi et al. 2010). A great progress in understanding the molecular processes underlying root development has been achieved only through root mutants (Zhang et al. 2007; Busov et al. 2008). These genes are beginning to provide an understanding of the developmental processes. But still there is a long gap in understanding molecular basis of root development like temporal regulation and genes responsible for specific morphogenetic traits, like profuse rooting and later root formation.

Recent progress in plant genome sequencing and the parallel development of global gene expression analysis platforms provide the opportunity to understand developmental and physiological events at the genome level. Functional genomic approaches may provide powerful tools for identifying expressed genes. To isolate gene(s) with differential expression patterns, a variety of methods such as mRNA differential display polymerase chain reaction (DD PCR; Liang and Pardee 1992) representational difference analysis (RDA; Nikolai et al. 1993; Hubank and Schatz 1994), microarray (Chee et al. 1996; Chu

Table 2 List of ESTs from root specific library, putative gene product and their possible function

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
Development				
1	EX149588.1	387	leucine-rich repeat family protein / extensin family protein	BT013424.1
2	EX149576.1	490	Tubulin beta-8 chain	AK321713.1
3	EX149608.1	248	RCD1 but without the WWE domain	AK325207.1
4	EX149624.1	409	pectin methylesterase inhibitor family protein	AK323328.1
5	EX149651.1	341	pectin methylesterase inhibitor	AK323416.1
6	EX149642.1	418	tetratricopeptide repeat (TPR)- containing protein	AC232775.1
Stress				
1	EX149577.1	400	ubiquitin-conjugating enzyme	AK247532.1
2	EX149578.1	300	arginine decarboxylase	AK319876.1
3	EX149580.1	409	HSP70	X54030.1
4	EX149581.1	419	Heat shock protein 81-2	AK321904.1
5	EX149585.1	270	glycolate oxidase	AK325991.1
6	EX149587.1	271	ubiquitin-conjugating enzyme 2	AK325502.1
7	EX149590.1	493	gamma-amino-N-butyrate transaminase	AK328051.1
8	EX149582.1	331	serine decarboxylase	BT012721.1
9	EX149591.1	250	PR5-like protein	GU184125.1
10	EX149600.1	417	lectin-like protein kinase	AK324460.1
11	EX149612.1	339	phenylalanine ammonia-lyase 1	AK320002.1
12	EX149623.1	417	DNAJ heat shock N-terminal domain-containing proteins	AK323027.1
13	EX149637.1	336	Heat shock protein 81-2	AC210371.2
14	EX149638.1	275	ubiquitin-protein ligase 1 (UPL1)	No hit
15	EX149652.1	350	DnaJ proteins from PGR	AK321731.1
Translation				
1	EX149573.1	250	SUI1	AK321390.1
2	EX149596.1	206	60S ribosomal protein L10	AK323239.1
3	EX149602.1	366	60S ribosomal protein L34	AK321389.1
4	EX149633.1	454	NAP1Ps	AY496136.1like
5	EX149635.1	360	SUI1	AK246267.1
Transcription				
1	EX149575.1	300	WRKY	AK322575.1
2	EX149616.1	357	myb family	AK321449.1
3	EX149632.1	424	transcription factor jumonji (jnj) family protein	No hit
4	EX149643.1	419	myb-related transcription activator	AK321449.1
Metabolism				
1	EX149579.1	332	phosphoenolpyruvate carboxykinase	AY007226.2
2	EX149584.1	324	fatty acid multi functional protein like	BT014107.1
3	EX149589.1	261	beta-galactosidase precursor	AC238909.6
4	EX149603.1	362	UDP-lucuronosyl/UDP-glucosyl transferase	AK322767.1
5	EX149609.1	388	2-dehydro-3-de- oxy phosphoheptonate aldolase	Z21793.1
6	EX149610.1	365	acetoacetyl CoA thiolase	AK327019.1
7	EX149611.1	444	Tubulin alpha-6 chain	BT012711.1
8	EX149614.1	438	Methylcrotonyl-CoA carboxylase alpha chain	U07745.1
9	EX149615.1	396	AtNADP-ME2	AK325158.1
10	EX149619.1	436	xyloglucan endotransglycosylase	AK319959.1
11	EX149621.1	437	ATP synthase beta chain 2	AK321619.1
12	EX149625.1	423	acetyltransferase (GNAT) family protein	AP010940.1
13	EX149626.1	401	Ketol-acid reductoisomerase	EU616546.1
14	EX149627.1	217	ATP synthase delta' chain, mitochondrial precursor	AC212275.3

Table 2 (continued)

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
15	EX149628.1	277	S-adenosylmethionine synthetase	AK320008.1
16	EX149634.1	270	formate dehydrogenase	AK321203.1
17	EX149639.1	417	RSH-like protein	AK319321.1
Structural proteins				
1	EX149640.1	330	Similar to histone H3.2 protein	AK246205.1
Signal Transduction				
1	EX149601.1	451	CDPK isoform 7	AY904339.1
2	EX149604.1	397	Calmodulin	AK246240.1
3	EX149617.1	240	calcium-binding protein	AK321186.1
4	EX149647.1	393	CDPK	XM_002322580.1
5	EX149648.1	409	Probable gibberellin receptor	No hit
Transport				
1	EX149586.1	408	amino acid carrier	AF013280.1
2	EX149592.1	245	phosphate-responsive protein	AK326164.1
3	EX149593.1	489	nitrate transporter	X92853.1
4	EX149594.1	270	mitochondrial substrate carrier family	AK324731.1
5	EX149620.1	419	amino acid carrier	AF013280.1
6	EX149629.1	306	ABC transporter family	AB075550.1
7	EX149631.1	434	sodium/calcium exchanger family protein	AK321303.1
8	EX149650.1	393	lysine and histidine specific transporter	AK320685.1
9	EX149653.1	218	sodium/calcium exchanger family protein	AK321303.1
Uncharacterized				
1	EX149583.1	254	Not annotated	AK322503.1
2	EX149595.1		Not annotated	AK320916.1
3	EX149597.1	396	Not annotated	AK319301.1
4	EX149598.1	326	GYF domain-containing protein	CU459062.7
5	EX149599.1	416	glycine-rich protein	AK322835.1
6	EX149605.1	173	3-oxoacyl-[acyl-carrier-protein]	AK325714.1
7	EX149607.1	428	Not annotated	AK329740.1
8	EX149613.1	414	Not annotated	AK319191.1
9	EX149618.1	403	Not annotated	No hit
10	EX149641.1	347	zinc finger (CCCH-type) family protein	AK327303.1
11	EX149630.1	421	Not annotated	AK329740.1
12	EX149636.1	388	Not annotated	AC215492.2
13	EX149644.1	397	Not annotated	AK322988.1
14	EX149646.1	257	Expressed protein	AK325580.1
15	EX149649.1	404	Not annotated	AK319191.1
Unknown				
1	EX149622.1	321	No significant similarity	AK322988.1
2	EX149606.1	412	No significant similarity	No hit
3	EX149645.1	186	No significant similarity	AC239738.5
4	EX149574.1	300	Expressed protein	No hit

et al. 1998) and suppression subtractive hybridization (SSH; Diatchenko et al. 1996) have been developed. Suppression subtractive hybridization (SSH) technique is an efficient method for detecting genes that are differentially expressed amongst different cell types or among cells

under modified conditions (Diatchenko et al. 1996). Further, SSH has also been successfully employed in identifying developmentally important genes like isolating differentially expressed genes in several developmental stages in melon fruit (Choi et al. 2004), during ripening

Table 3 List of ESTs from temporal library, putative gene product and their possible function

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
Development				
1	EX149670.1	404	tetratricopeptide repeat (TPR)-containing protein	AC232775.1
2	EX149675.1	431	elongation factor 1-alpha / EF-1-alpha	AK326450.1
3	EX149680.1		oxygenase family similar to flavanone3-hydroxylase	AK329740.1
4	EX149681.1		RNA recognition motif (RRM)-	No hit
5	EX149692.1	206	putative 21 kDa protein precursor	AK323328.1
6	EX149693.1	290	RNA recognition motif (RRM)-containing protein	AK322263.1
7	EX149698.1	404	F-box family protein, similar to SKP1 interacting partner2	AP009522.1
Stress				
1	EX149660.1	254	HSC70	BT013586.1
2	EX149661.1	404	Heat shock - DNA-Jlike	AK323027.1
3	EX149664.1	221	polymyxin resistance protein amA	AK322154.1
4	EX149665.1	321	Superoxide dismutase	AK246450.1
5	EX149679.1	255	cold-stress inducible protein	AK319970.1
6	EX149694.1	380	Le Proteinase inhibitor NDK I	No hit
Translation				
1	EX149688.1	394	60S ribosomal protein L24	AK321022.1
2	EX149672.1	416	P40-like protein	AK324984.1
3	EX149669.1	428	Nucleosomal assembly protein	AK319864.1
4	EX149690.1	419	ribosomal protein L24-like protein	AK321022.1
Transcription				
1	EX149682.1	405	Lysine-specific histone demethylase	AK320498.1
2	EX149685.1	402	TGACG-sequence-specific DNA-binding protein	AK319272.1
3	EX149691.1	348	WRKY	AK322575.1
4	EX149697.1	348	WRKY transcription factor	AK326455.1
Metabolism				
1	EX149667.1	199	3-oxoacyl-[acyl-carrier-protein] reductase	AK325714.1
2	EX149683.1	397	NADPH-cytochrome p450 reductase	No hit
3	EX149686.1	164	cytochrome P450 like T BP	AC215459.2
Signal Transduction				
1	EX149666.1	397	Calmodulin 7	AC233130.7
2	EX149676.1	240	calcium-binding protein	AK321186.1
3	EX149677.1	430	calcium-dependent protein kinase,	AC215487.4
Transport				
1	EX149673.1	306	ABC transporter family involved in resistant to lead	BT013314.1
2	EX149696.1	428	ATP carrier protein 1, mitochondrial	BT013912.1
Unknown				
1	EX149662.1	294	Unknown	No hit
2	EX149678.1	401	<i>S. lycopersicum</i> chromosome 12 clone	AC234113.1
3	EX149700.1	186	<i>S. lycopersicum</i> chromosome 1	AC239738.5
Uncharacterized				
1	EX149663.1	396	Not annotated	AC215492.2
2	EX149668.1	347	zinc finger (CCCH-type) family protein	AK327303.1
3	EX149671.1	404	Uncharacterized protein	AK322835.1
4	EX149674.1	410	Not annotated	AK246899.1
5	EX149684.1	280	Expressed protein	No hit
6	EX149687.1	171	Expressed protein	No hit
7	EX149688.1	321	Expressed protein -similar to leaf senescence related	CU928193.6
8	EX149689.1	149	Not annotated	No hit

Table 3 (continued)

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
9	EX149695.1	405	Not annotated	AC226514.1
10	EX149699.1	349	zinc finger FYVE type protein	AK247418.1

Table 4 List of ESTs from lateral root library, putative gene product and their possible function

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
Development				
1	EX149541	402	flavanone 3-hydroxylase	AK329740.1
2	EX149544	405	Pectinmethyl esterase inhibitor	AK323328.1
3	EX149545	204	pollen-specific BP10 protein multi-copper oxidase type-1	BT014417.1
4	EX149552	339	Rubber elongation factor	AK321019.1
5	EX149558	403	Embryo abundant protein	AK326022.1
6	EX149559	212	RNA recognition motif (RRM)-containing protein	AK320374.1
7	EX149560	324	Alpha tubulin	AK322065.1
8	EX149561	370	nodulin 12B precursor	AC232704.1
9	EX149549	254	<i>S.lycopersicum</i> clone 132340F HSP 70 like	BT013586.1
10	EX149550	245	Ethylene-responsive late embryogenesis-like protein	AK324230.1
11	EX149568	264	<i>L. esculentum</i> Hsc70	L41253.2
Stress				
1	EX149543	406	glutathione peroxidase	AK322392.1
2	EX149555	141	ubiquitin carrier-like protein	AK329553.1
3	EX149556	427	cinnamic acid 4-hydroxylase	AK323427.1
4	EX149557	402	Low temperature induced protein	AK319970.1
5	EX149572	296	ADC	AK319876.1
Translation				
1	EX149547	163	L3 Ribosomal Protein	AK319840.1
2	EX149562	195	L19 Ribosomal Protein	AK329713.1
3	EX149565	332	60S ribosomal protein	AK325399.1
4	EX149570	404	translationally controlled tumor family protein	AK324459.1
5	EX149571	405	P40 like protein (ribosomal associated)	AK324984.1
Transcription				
1	EX149546	404	transcription factor JERF1	AK319828.1
Metabolism				
1	EX149540	341	3-oxoacyl-[acyl-carrier-protein] reductase	AK325714.1
2	EX149553	370	S-adenosyl-L-methionine synthetase	AK320008.1
3	EX149563	439	glutathione S-transferase	AK323682.1
4	EX149564	405	ketol-acid eductoisomerase	No hit
5	EX149566	217	ATP synthase delta' chain, mitochondrial precursor	AC212275.3
6	EX149567	404	ADP-glucose pyrophosphorylase	BT014088.1
7	EX149569	368	arabinogalactan-protein precursor	AK329153.1
Signal Transduction				
1	EX149542	401	<i>B.vulgaris</i> mRNA for small G protein	AK328636.1
Transport				
1	EX149548	425	ABC transporter	No hit
Uncharacterized				
1	EX149554	417	Not annotated	No hit

Table 5 List of ESTs from profuse rooting library, putative gene product and their possible function

S.No.	EST Accession No.	Size (bp)	Putative function	NCBI hit
Stress				
1	EX149657.1	406	Le LEACO2 ACC oxidase	AC209585.1
Metabolism				
1	EX149655.1	248	Le 133393F, Adenosine kinase	AK320801.1
Structural proteins				
1	EX149654.1	365	Le L136C6, complete 60S Ribosomal protein L34	AK321389.1
Unknown				
1	EX149658.1	113	No significant similarity	No hit
Uncharacterized				
1	EX149656.1	248	Le Heinz, chr 5 BAC RCD1 like, no WWE	AK325207.1
2	EX1496591.1	180	Hypothetical protein	No hit

of banana fruit (Manrique-Trujillo et al. 2007) or at an early developmental stage of banana fruit undergoing changes in ethylene responsiveness, genes involved in carnation flower maturation (Kim et al. 1999) and related to fruit quality in tomato (Stevens et al. 2008). In this endeavour, an attempt was made to understand the molecular

basis of root development in tomato, using functional genomics tool SSH. We profiled gene expression during root development program, to attain a global perspective of genes whose expression is modulated in root specific manner or temporally or in lateral root formation or during profuse rooting.

Fig. 2 Expression analysis of ESTs from Root specific library. 0.2 µl and 0.5 µl of cDNA template made from RNA of 8d old shoot (lane 1), 8d old root (lane 2) and water control (lane 3) was subjected to PCR using different ESTs specific primers (a–n). The PCR products were resolved on 1.5 % agarose gel. Endogenous GAPDH amplification served as control (o)

Fig. 3 Expression analysis of ESTs from temporal library. 0.5 μ l of cDNA template made from RNA of 8d old root (*lane 1*), 40d old root (*lane 2*) and water control (*lane 3*) was subjected to PCR using different ESTs specific primers (**a–l**). The PCR products were resolved on 1.5 % agarose gel. Endogenous GAPDH amplification served as control (**m**)

Most of the ESTs found in root library are part of house-keeping pathways like transcription, translation, metabolism etc., which account for 64.65 % (104 ESTs) of total ESTs identified. However, some of the genes belongs to WRKY and myb gene family, known to take part in root development, were found in root specific library. WRKY is required for the growth of roots by stimulating synthesis of flavonols, an endogenous regulators of auxin transport (Grunewald et al.

2012) and later has a role in root growth of legumes (Volpe et al. 2013). In contrast, a gene encoding for Lysine-specific histone demethylase (KDM1), which is involved in transcriptional repression of root genes via demethylation of histones was also identified in ESTs library probably required for tight regulation of temporal genes (Krichevsky et al. 2011).

There are reports indicating direct or indirect involvement of stress related genes in root development (Chen

Fig. 4 Expression analysis of ESTs from lateral root library. 0.5 μ l of cDNA template made from RNA of 40d old *S. peruvianum* (*lane 1*), 40d old root of *S. lycopersicum* (*lane 2*) and water control (*lane 3*) was subjected to PCR using different ESTs specific primers (**a–j**). The PCR products were resolved on 1.5 % agarose gel. Endogenous GAPDH amplification served as control (**j**)

Fig. 5 Expression analysis of EST from profuse root library. 0.5 μ l of cDNA template made from RNA of 40d old *S. peruvianum* (lane 1), 40d old root of *S. lycopersicum* (lane 2) and water control (lane 3) was subjected to PCR using different ESTs specific primers (upper panel). The PCR products were resolved on 1.5 % agarose gel. Endogenous GAPDH amplification served as control (lower panel)

and Xiong 2005). Many stress inducible genes were detected in our studies. Arginine decarboxylase, hsp70, hsp 81-2, superoxide dismutase, glutathione peroxidase, cinnamic acid 4-hydroxylase and ACC oxidase are some of the examples of stress related genes. Arginine decarboxylase (ADC) is not only capable of enhancing ability of scavenging reactive oxygen species (ROS) but also has been shown to be involved in root growth as transgenic with ADC had longer roots compared to wild type (Wang et al. 2013). Similarly, heat shock proteins are well known stress proteins but also shown to perform root specific functions. Hsp70 was involved in instrumenting root-shoot communication in cucumber plants (Li et al. 2014) whereas expression of hsp81-2 was found high in roots of *Arabidopsis thaliana* (Yabe et al. 1994). In addition, a gene encoding for superoxide dismutase (SOD) is major component of plant defence system but decrease in manganese superoxide dismutase leads to reduced root growth (Morgan et al. 2008). Other identified genes like glutathione peroxidase, cinnamic acid 4-hydroxylase and ACC oxidase have been involved in root metabolism and growth (Passaia et al. 2013; Dennis and Kennedy 1986). Other than genes with known role in root growth, a large number of ESTs belongs to the category of uncharacterized and unknown proteins. 22.36 % (36 ESTs) of total genes identified could not be assigned to any function.

In conclusion, five EST libraries were constructed using SSH approach to identify root-specific genes. One hundred sixty-one unique ESTs from various libraries were identified and deposited in NCBI-dbEST database. Thirty-six ESTs were selected and validated for their expression and 17 ESTs showed differential expression. This information may lead to identification of novel pathways involved in tomato root development in particular and plants in general.

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Contribution of authors KN performed all the experiments. DJ and TKB prepared the manuscript and AKG was involved in conceptualization and overall monitoring of the work.

Conflict of interest The authors declare that there are no conflicts of interest.

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