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Full Length Research Paper

Effects of heat stress on gene expression in eggplant (*Solanum melongema* L.) seedlings

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In order to identify differentially expressed genes involved in heat shock response, cDNA amplified fragment length polymorphism (cDNA-AFLP) and quantitative real-time polymerase chain reaction (Q-PCR) were used to study gene expression of eggplant seedlings subjected to 0, 6 and 12 h at 43°C. A total of 53 of over 2400 bands amplified were differentially expressed between the control and heat treatment; 42 up-regulated and 11 corresponding to genes repressed by heat stress. 24 transcript-derived fragments (TDFs) were successfully isolated, cloned and sequenced. BLAST searching revealed that 15 heat stress response transcripts presented similarities with those that encoded small molecular shock protein, disease resistance protein, stress-related protein, enzymes related to photosynthesis as well as biosynthesis of protein, and so on. The expression patterns of five randomly selected TDFs were confirmed by using Q-PCR experiments. The direct and indirect relationships of genes corresponding to the 15 TDFs with stress tolerance are discussed.

Key words: Eggplant, heat stress, cDNA amplified fragment length polymorphism (cDNA-AFLP), transcript-derived fragment (TDF), quantitative real-time polymerase chain reaction (Q-PCR).

INTRODUCTION

Eggplant (*Solanum melongema* L.) is a well-known vegetable cultivated widely in the world. The optimal temperature for eggplant growth and development ranges from 22 to 30°C. With the global warming, temperature in subtropical and tropical regions is often above 35°C, which results in serious heat injuries in eggplant, concretely involving limited plant growth, reduced productivity and damaged quality. It has been estimated that over 50% of the yield potential of major crops is routinely lost due to the damages caused by environmental stresses including drought, cold, excess water, heat and so on (Boyer, 1982). Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought and other stress (Mittler, 2006). The plant response to heat stress is complex and depends on the signaling flow of information by which the plant can sense the changes in its surrounding environment and induces the changes of gene expressions (Kotak et al., 2007). Functional genomic tools can be applied for identifying and isolating the genes involved in plant abiotic stress tolerance(Langridge et al., 2006). Among the genome wide expression analysis techniques, the cDNA amplified fragment length polymorphism (cDNA-AFLP) method for global transcriptional analysis is an open architecture technology that is appropriate for gene expression studies in non-model species (Meyers et al., 2004). It was previously employed to identify and isolate differentially expressed transcript-derived fragments (TDFs)

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associated with abiotic stresses such as the response of cowpea nodules to heat stress (Simões-Araújo et al., 2002), wild barley to water stress (Suprunova et al., 2007), *Poncirus trifoliata* to cold acclimation (Meng et al., 2008) and pigeonpea to drought stress (Priyanka et al., 2010). In physiology, effects of calcium and calmodulin antagonist on antioxidant systems and heat resistance of eggplant seedlings had been reported under high temperature stress (Chen et al., 2004), whereas, the study of the expression of thermotolerance-relevant genes of eggplant has not been reported. So, the primary aim of this study was to isolate and identify the genes expressed under heat stress conditions by using cDNA-AFLP technology, and to validate the expression patterns of the selected TDFs by quantitative real-time PCR. This study on differentially regulated gene expression will help to find the molecular alteration induced by heat shock treatment in eggplant. At the same time, analysis and deduction of the functions of heat stress-responsive genes will help in elucidating the underlying mechanisms of heat stress tolerance in eggplant.

MATERIALS AND METHODS

Plant material and growth conditions

The heat tolerant eggplant inbred line 05-4, bred by Vegetable Research Institute, Guangdong Academy of Agriculture Sciences (VRI-GDAAS), was used in this study. The heat treatment and cDNA-AFLP analysis were conducted in key laboratory of VRI-GDAAS, Guangzhou, Guangdong province of P. R. China in 2009 and 2010. The seedlings of eggplant at the fourth or fifth leaf stage were displaced and preincubated in a growth chamber at 28℃ (day)/25℃ (night), and 12/12 h (light/dark) photoperiod with light supplied at an intensity of 72 µmol m^{-2} s^{-1} for three days. Then, a different set of plants were immediately exposed to 43℃ for 6 and 12 h as heat shock treatments, and the plants was cultivated at 28℃ as control (0 h). Each treatment (heat shock for 0, 6 and 12 h) was done with three replicates and five plants each for each replicate. The seedlings were uniformly watered under the controlled condition of approximately 80% humidity.

Total RNA extraction and cDNA-AFLP analysis

The third fully expanded leaves of plants treated at 43℃ for 0, 6, and 12 h were collected, respectively, immediately immersed in liquid nitrogen and stored at -80℃ for later use. RNA was extracted from 500 mg leaves according to Trizol extracted method (TaKaRa, Japan). The extracted RNA quality and integrity were determined by agarose gel electrophoresis. 1 µg of total RNA was used to synthesize double-stranded cDNA using SMART cDNA Library Construction Kit (Clontech, America) according to the manufacturer's instructions.

The template for cDNA-AFLP was prepared according to Bachem et al. (1996) and Vuylsteke et al. (2006) with minor modification. Double-strand cDNA of leaves were double-digested with *Taq* I and *Ase* I (MBI Fementas, America) as restriction enzymes to generate adapter ligation sites and the products were ligated to the adaptors. Pre-amplification was carried out in a volume of 25 µl containing: 1x PCR buffer, 2 mM $MgCl_2$, 50 ng of *Ase* I and *Taq* I primers, 0.16 mM dNTPs, 1.25 U of *Taq* polymerase, 2 µl of diluted 10-fold digesting and ligation mixture. The amplification was carried out in 7000 thermal cycler (ABI) with the following conditions: 94℃ for 3 min; 27 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 1 min; followed by an extension of 7 min at 72℃. The selective amplification was carried out in a total volume of 20 µl containing: 1x PCR buffer, 2 mM $MgCl_2$, 40 ng of *Taq* I NN and *Ase* I NN selective amplification primers (where N represents a selective nucleotide), 0.2 mM dNTPs, 1 U of *Taq* polymerase, and 1 µl of diluted 20-fold pre-amplification product. The selective amplification used 37 cycles including 12 touchdown cycles comprising a reduction of the annealing temperature form 65 to 56.6℃, in 0.7 steps, which was then maintained for 25 cycles at 56℃; followed by an extension of 7 min at 72℃. Amplified products were separated on a 6.25% denaturing polyacrylamide gel run at 60 W for 120 min, and visualized with the silver staining. All reactions were replicated twice.

TDF isolation, cloning and sequence analysis of DNA fragments

The polymorphic transcript-derived fragments (TDFs) based on presence, absence or differential intensity were cut from the gel with a sharp razor blade, with maximum care to avoid any contaminating fragments, and then eluted in 40 µl of double distilled water at 37℃ for 2 h or overnight. 6 µl of the aliquot was used for reamplification in a total volume of 25 µl, using the same set of corresponding selective primers and the same PCR conditions as for the selective amplification. PCR products were resolved by a 1.2% agarose gel electrophoresis, TDFs were called AXXTXX-n, while XX and n represented the randomly selected primer and the different TDF with the same primer combination, respectively.

Selected amplified DNA fragments were cloned into a pMD™-18 Vector (TaKaRa, Japan) and transformed into competent *Escherichia coli* (DH5α). As a result of the possibility of comigration associated with cDNA-AFLP PCR (Lang et al., 2005), three colonies were selected for each transformation event. Plasmid DNA was isolated and analyzed by restriction enzyme digestions (*Hind* III and *Mse* I), then the products were determined on a 1.5% agarose gel and the analysis of the nucleotide sequences of TDFs was submitted to the database (http://www.ncbi.nlm.nih.gov) using the BLAST algorithms.

Q-PCR

Q-PCR system was used to confirm the differential expression of cDNA fragments isolated from the control and heat treatments with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) and iCycler iQ (Bio-Rad) according to manufacturer's instruction. Each reaction contained 1 µl of diluted 10-fold cDNA as template. In contrast, water was used as a negative control. Gene specific primers were designed on the basis of 80 to 150 bp fragments using the Primer 5.0 program (PREMIER Biosoft International, Canada). Actin was used as an internal house keeping gene, which was used to ensure cDNA quantity and integrity. The PCR cycle program consisted of 1 cycle of 3 min at 94℃, 40 cycles of 20 s at 94℃, 20 s at 56℃ and 20 s at 72℃. Equal amounts of mRNA were taken for three independent PCR reactions. Each real time assay was then tested in a dissociation protocol to ensure that each amplicon was a single band. The formula used was as follows: Ratio = $2^{-\Delta\Delta Ct}$. The primers used for the Q-PCR are shown in Table 1.

RESULTS

cDNA-AFLP analysis

cDNA-AFLP patterns were generated from 05-4 line

Table 1. Primers used for Q-PCR analyses.

TDF	Forward primer	Reverse primer
A9T5-1	5’-AAGATCCACACCACCTGC-3’	5’-GAAATAATGCTTATCTATG-3’
A9T10-3	5’-ATTTGGAAGGTCCATGGTTAAAATG-3’	5’-AAATAGGACGAATATACG-3’
A10T6-1	5’-ATCCCCGAAACAAGGTGGAATCTC-3’	5’-GTGACTCTTGCCAAGTTG-3’
A10T8	5’-AGTTGGACAAGTTGTTTACCATAG-3’	5’-ATCCAGTGATTTTGCATAC-3’
A11T6-3	5'-CCAGACCGAGTGATGAGGC-3'	5'-CATTTCATTTATCCCGGCA-3'
Actin	5'-GCTAGTGGTCGTACAACTGG-3'	5'-CAGCAGTGGTGGTGAACATATAAC-3'

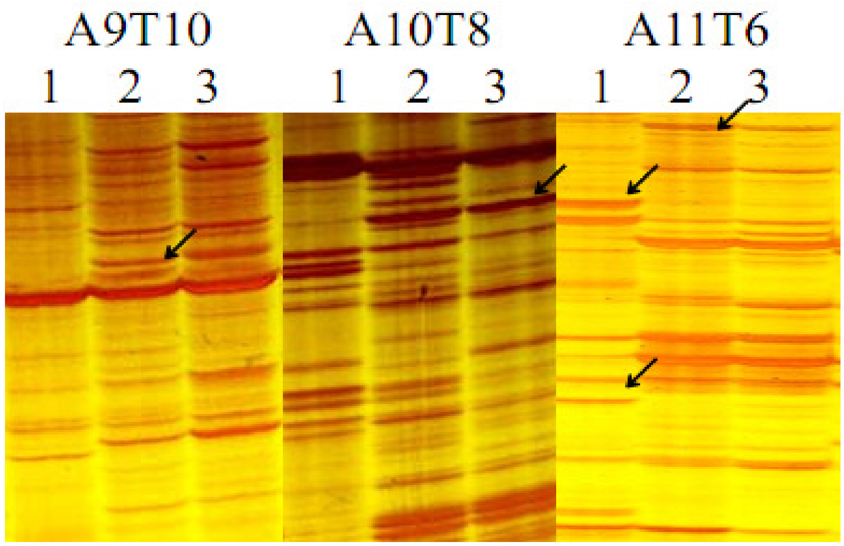

Figure 1. Partial detail of cDNA-AFLP showing the differential expression of the genes with primer combinations A9T10, A10T8 and A11T6 in eggplant. Lanes 1, 2 and 3 represent 0, 6 and 12 h of heat treatment, respectively.

subjected to 0, 6 and 12 h at 43°C. Example of cDNA-AFLP comparative expression profile after denaturing polyacrylamide gel electrophoresis and silver staining is shown in Figure 1. The expression profiles obtained from 64 primer combinations showed approximately 2400 bands; 42 up-regulated and 11 corresponding to genes repressed by heat stress. 24 transcript-derived fragments were isolated, cloned and sequenced. These sequences are envisaged to serve as a potential source of heat stress-inducible genes, and hence may be used for deciphering the mechanism of hot tolerance for the eggplant. Homology searches disclosed that 15 TDFs shared significant similarity with the known/putative proteins or ESTs available in the databases, and their length DNA, sequence identities and E-value are presented in Table 2. Six of the other nine TDFs encoded proteins with unknown function, and the remaining three TDFs showed no significant matches, which might represent several unreported novel genes related to heat shock reaction. 13 of the 15 TDFs were up-regulated, and only A8T5 and A10T7-2 were down-regulated.

Based on the putative function of the transcripts among the total 15 TDFs, we found that genes corresponding to several TDFs were thought to encode stress or defense-related protein, such as small molecular heat shock protein (A4T9), ribosomal protein rpl5 (A11T4), NBS-LRR resistance protein-like protein (A10T7-2), zinc finger protein (A11T6-3), nudix hydrolase (A10T8), cationic peroxidase (A9T10-3), RNA binding protein (A9T5-1), Chl a-b binding protein (A5T10-2) and so on.

Confirmation of differentially expressed transcripts using Q-PCR

In order to confirm the reliability of cDNA-AFLP analysis in expression profiles, five randomly selected TDFs from the 15 TDFs were used to examine their expressions after 0, 6, and 12 h in response to heat shock using Q-PCR with specific primers. Q-PCR analysis revealed that TDF A9T5-1, A9T10-3, A10T6-1, A10T8 and A11T6-3 had the same expression patterns with the results observed through cDNA-AFLP (Figure 2). In general, the expression data provided by Q-PCR were in good

Table 2. Homologies of TDF sequences isolated from heat treated eggplant by cDNA-AFLP analysis.

TDF name	Length (bp)	Origin	Sequence similarity	E-value
A4T9	143	1	Nelumbo nucifera small molecular heat shock protein 19 (HSP19) mRNA, complete cds (EF421195)	0.13
A4T11-1	108	1	Arabidopsis thaliana integral membrane family protein (AT5G19980) mRNA, complete cds, golgi nucleotide sugar transporter 3(NP_177760)	7e-18
A5T4	239	1	Solanum panduriforme tRNA-Thr *(trnT*) gene, partial (EU427552)	3e-32
A5T10-2	74	1	Solanum tuberosum clone 062G02 chlorophyll a-b binding protein 3C-like mRNA, complete cds (DQ252493)	0.60
A8T5	79	2	Solanum tuberosum soluble NSF attachment protein mRNA, complete cds (AF225512)	0.19
A9T5-1	129	1	Ricinus communis RNA binding protein, putative, mRNA(ref|XM_002532410.1|)	6e-12
A9T10-3	143	1	Lycopersicon esculentum *ep5 C* gene for cationic peroxidase, exons 1-3 (AJ634698)	7e-37
A10T6-1	139	1	Solanum lycopersicum NBS-LRR resistance protein-like protein (Mi-1C) gene,complete cds (DQ863290)	9e -29
A10T7-2	152	2	Entamoeba histolytica HM-1:IMSS auxin efflux carrier family protein, putative, mRNA (XM_648750)	0.49
A10T8-1	137	1	*Ricinus communis* mutt/nudix hydrolase, putative, mRNA (XM_002524414)	2e-30
A11T4	81	1	Solanum tuberosum ribosomal protein subunit 5 *(rpl5*) gene, partial cds(AF095274)	4e-04
A11T6-3	157	1	*Arabidopsis thaliana* zinc finger (C3HC4-type RING finger) family protein (AT3G19910)mRNA, complete cds (AC215442)	2e-11
A11T7	228	1	Nicotiana benthamiana calcium ATPase (NbCA1) mRNA, promoter region and complete cds (GU361620)	9e-88
A11T8-1	172	1	Arabidopsis thaliana binding (AT3G06670) mRNA, complete cds(ref|NM_111547.4|)	1e-08
A11T9	84	1	Nicotiana tabacum mitochondrial DNA, complete genome (BA000042)	9e-10

Sequences comparisions were performed using the BLASTN and BLASTX programe at NCBI worldwideweb server. 1 represents up-regulated in resistant genotype after 6 or 12 h heat treatment, 2 represents down-regulated in resistant genotype after 6 or 12 h heat treatment.

agreement with profiles detected by cDNA-AFLP at all time points. When compared with the control, the expression quantities of genes corresponding to TDF A9T5-1 and A9T10-3 were found to increase at 6 h, but

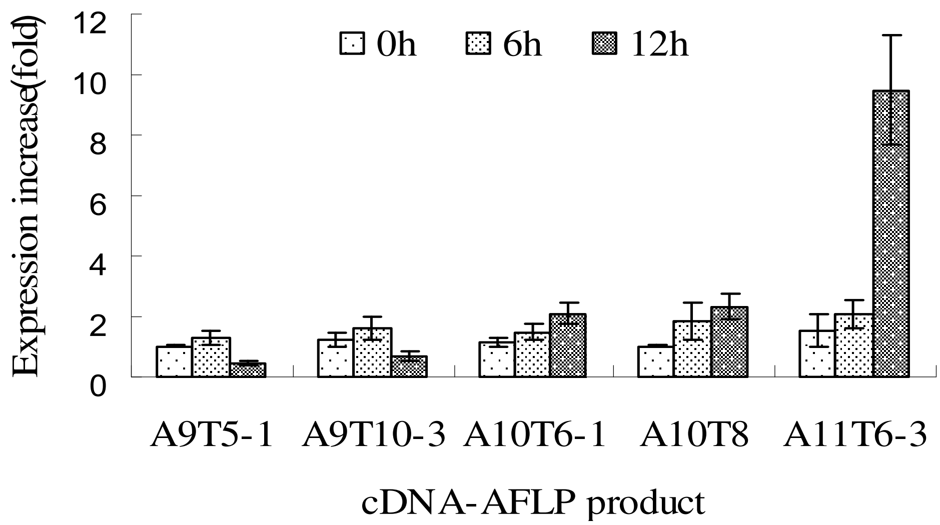

Figure 2. mRNA expression level comparision between different time points of heat treatment in eggplant. 0 h mRNA of each comparision was considered as 1 and each treatment was normalized by comparing △△Ct with 0 h, followed by 6 and 12 h of treatment.

decrease to 0.43, 0.55 fold at 12 h, respectively. Genes encoded NBS-LRR resistance protein-like protein (A10T6-1), zinc finger protein (A11T6-3), and nudix hydrolase (A10T8) involved in stress or defense increased by one to nine fold after been subjected to heat shock treatment for 6 or 12 h.

DISCUSSION

A4T9 induced by heat stress was homologous to the small molecular heat shock protein. Small molecular HSP (sHSPs) family acts as molecular chaperones to prevent other proteins against heat-induced denaturation and aggregation (Vierling, 1991). The sHSPs can form large multimeric structures and are responsible for a wide range of cellular functions; amongst these, they are able to increase thermotolerance *in vivo* (Water et al., 1996). The expression of plant class I sHSP in *E. coli* has been shown to be associated with the enhancement of resistance to high temperature stress (Soto et al., 1999). Simões-Araújo et al. (2002) also identified transcripts that present similarities with those that encode small molecular heat shock proteins in cowpea nodules subjected to heat stress.

Sequences analysis showed that A11T6-3 was homologous to zinc finger protein isolated from *Arabidopsis thaliana,* A10T6-1 was homologous to NBS-LRR resistance protein (Mi-1C) of tomato, and A10T8 was homologous to nudix hydrolase of *Ricinus communis*. There were significant functions against major abiotic stresses (salt, osmotic, stress, cold and dehydration) for zinc finger family protein (Mukhopadhyay et al., 2004), NBS-LRR protein (McHale et al., 2006), nudix hydrolase (Elżbieta, 2008) and so on. Like many signaling and regulatory genes that are stress specific, the zinc-finger protein responses to a large number of biotic and abiotic stresses, such as zinc-finger protein Zat12 is thought to be involved in cold and oxidative stress signaling in Arabidopsis (Sholpan et al., 2005). Most of the largest class of known R proteins in plant includes those that contain a nucleotide binding site and leucine-rich repeat domains (NBS-LRR proteins), and NBS-LRR proteins may recognize the presence of the pathogen directly or indirectly (Meyers et al., 2003). Plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defense responses, such as activation of an oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes, and the hypersensitive response (McHale et al., 2006). It has been postulated that the role of Nudix hydrolases is to sanitize or regulate the accumulation of toxic compounds, cell signaling molecules, or metabolic intermediates induced by oxidative stress (Bessman et al., 1996).

Gene corresponding to TDF A9T5-1 encoded putative RNA binding protein. The RNA-binding protein has emerged as an important regulatory factor in a variety of physiological processes, including stress resistance (Janne et al., 2004).

A9T10-3, which was homologous to gene for the enzymes involved in cationic peroxidase, was activated by heating treatments. Heat stress as well as plant–pathogen can increase the activated oxygen species (AOS) and H_2O_2 accumulation, and cationic peroxidase play an important role in eliminating H_2O_2 (Dowd and Johnson, 2005).

A11T4 was up-regulated in response to heat shock, and it showed close matches to ribosomal protein subunit

5 *(rpl5*) gene. Ribosomal protein (R-proteins) are classified into two groups based on their roles in metabolism: in the first group, many R-proteins are components of ribosomes that are assembled in the nucleus, and they are responsible for polypeptide synthesis in all eukaryotes, and in the second group, many R-proteins are involved in diverse stresses (Cheng et al., 2010). Their roles in aiding ribosomes in adapting to cold stress have been reported (McIntosh et al., 2005).

A5T10-2 was homologous to the chlorophyll a/b binding protein mRNA, which indicated that response to heat shock in eggplant seedlings was associated with photosynthesis. The chlorophyll a/b binding protein is a kind of integral membrane protein with a helix-loop-helix organization, which can bind chlorophyll a and b molecules. Roberto et al. (1997) showed that chlorophyll a/b binding protein CP24, CP26 and CP29 had a major regulatory role in the light-harvesting function and were important in environmental stress resistance.

A11T7 was a significant homologous to *Nicotiana benthamiana* calcium ATPase (NbCA1) mRNA. Ca^{2+}-ATPase is one kind of Ca^{2+} pump that drives Ca^{2+} flux from symplast to apoplast. The Ca^{2+}- ATPase plays vital role in plant adaptation to various stresses, and is normally considered to be a "defense system" that reestablish balance of calcium (Song et al., 2008). The plasma membrane Ca^{2+}-ATPase activity was increased by heat acclimation (HA, 38°C/10 h) or cold acclimation (CA, 8°C/2.5 d), which suggest that plasma membrane Ca^{2+}-ATPase was involved in the chilling resistance and the thermotolerance of grape plants (Jian et al., 1999; Zhang et al., 2006).

Gene corresponding to TDF A4T11-1 encoded Golgi nucleotide sugar transporter (NST) 3, which was a kind of integral membrane family protein. NST are involved in the transport of nucleotide sugars from the cytosol into the Golgi apparatus or *endoplasmic reticulum* (ER) lumen via an antiport system; this transport is temperature-dependent and in a saturable manner (Martinez-Duncker et al., 2003). The majority of integral membrane proteins including transporters, channels and pumps contain hydrophobic a-helices and can be selected based on TransMembrane Spanning (TMS) domain prediction (Ward, 2001). Glycosylation, sulfation and phosphorylation of proteins, proteoglycans and lipids occur in the lumen of the Golgi apparatus. The nucleotide substrates of these reactions must be first transported from the cytosol into the Golgi lumen by specific transporters, so, nucleotide sugar transporter are essential for the regulation of post-translational modification in eukaryote (Berninsone and Hirschberg, 2000).

Gene of the TDF A5T10-2, A11T8 and A11T9 were high homologous to tRNA-Thr gene, binding mRNA and mitochondrial DNA, respectively. It revealed that these genes might be involved in the regulation of physiological and biochemical process for plants responding to heat stresses. Although there was no direct proof to clarify their relationship with stress resistance, we deduced that they took part in the response to heat stress in eggplant.

Genes corresponding to A8T5 and A10T7-2, which were down regulated by heat stress, encoded protein corresponding to soluble NSF attachment protein and the putative auxin efflux carrier family protein, respectively. NSF is an essential protein for vesicular trafficking, which is associated with the membranes by binding soluble NSF attachment protein, and it is known to exist both in the cytosol and in the membrane of subcellular organelles (Han et al., 2000). Recent findings in plants showed that vesicle trafficking played an important role in stress responses (Mazel et al., 2004). Basipetal transmission of auxin is mediated by polar auxin transport machinery, which involves influx and efflux carriers (Chen et al., 1998). The polarity of auxin transport is probably established by a basal localization of the efflux carrier in transporting cells. This auxin may function in the stress response by helping to control stomatal opening (Dietrich et al., 2001) and by allocating resources under poor growth conditions (Palme and Gälweiler, 1999).

In summary, the expression of genes potentially involved in response to heat treatment was investigated with the cDNA-AFLP approach on the heat tolerant inbred line 05-4 in this study. Most of the TDFs characterized have homology to genes related to stress defense, suggesting that they might play a part in the thermotolerance mechanism. Our findings provide a starting point for identification of novel candidate genes related to the defense mechanism against heat stress in eggplant. However, the molecular response of plants to heat stress is complex and our understanding of the mechanisms is limited because our analysis was restricted to an incomplete figure. Therefore, to obtain more detailed picture of gene expression related to heat treatment of eggplant, we have much more work to concentrate on the characterization of the identified TDFs, clone the full length of the related genes, study the functions of these genes and clarify the important physiological action of the candidate genes through gain and loss of function in model plants.

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