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Assessment of genetic variability and molecular characterization of heat stress tolerant genes in *Arachis hypogaea* L. through qRT-PCR

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ABSTRACT

Temperature is the one of the most important environmental factor influencing plant growth and development limiting the crop productivity. Groundnut (*Arachis hypogaea* L.) is a major crop cultivated in tropical and subtropical regions where high temperature stress is a serious constraint for its production. In the present study, groundnut genotypes were screened for thermotolerance using temperature induction response (TIR) technique, where the seedlings were exposed to sub lethal temperature from 38°C-54 °C for 5 h followed by lethal temperature at 58 °C for 3 h. From the 100 diverse genotypes screened, 24 genotypes showed tolerance to high temperature with mean percent seedling survival of 92%, 30 genotypes showed moderate tolerance and 46 genotypes were identified as temperature susceptible compared with checks (100% survival). The expression patterns of selected six groundnut genotypes Kadiri 9, Narayani, Dharani, JL 24, TPT-3 and Kadiri 6 at seedling stage were analyzed under heat stress using Quantitative real-time reverse transcriptase PCR (qRT-PCR). qRT-PCR analysis revealed stress responsive nature of the selected genes. Stress responsive genes such as heat shock protein 90 (*HSP90*), dehydration responsive element binding-2A (*DREB2A*) and late embryogenesis abundant4-2 (*LEA4-2*) showed more than 20–50 fold increase in expression level in selected genotypes.

1. Introduction

Among the ever-changing components of the environment, the constantly rising ambient temperature is considered as one of the most detrimental stresses. The global air temperature is predicted to rise by 1.5 °C and heat waves are likely to occur more frequently by the end of the twenty-first century (IPCC, 2016). The increasing threat of global warming is already having a substantial impact on agricultural production worldwide as heat waves cause significantly yield losses with great risks for future global food security (Christensen and Christensen, 2007). High temperature stress has a wide range of effects on plants in terms of physiology, biochemistry and gene regulation pathways (Varshney et al., 2011; Bita and Gerats, 2013). The impact of heat stress is a complex function of intensity, duration, and rate of temperature change (Thakur et al., 2010). Reproductive phase is the most affected stage and the affected process is pollen grain development. In the tropical and subtropical regions, temperatures may even exceed the existing limit, which will further decline the process of land degradation (Battisti and Naylor, 2009).

To cope up with the changing environmental conditions, plants synthesize a set of specific stress responsive proteins and they are involved in altering specific biochemical processes necessary for adaptation. Plant heat shock factors (HSFs) are the terminal components of a signal transduction chain mediating the expression of genes responsive to various abiotic stresses (Nover et al., 2001). HSFs play a key role in plants response to various environmental stresses by regulating the expression of stress responsive genes such as heat shock proteins (*HSPs)*, dehydration responsive element binding proteins (*DREBs*), no apical meristem, ATAF1/2, cup-shaped cotyledon 2 (*NAC*), transcription factors containing highly conserved WRKY domain (*WRKY*), dehydrin (*DHN*), late embryogenesis abundant proteins *(LEA)* and abscicic acid response element binding proteins (*AREB*).

Groundnut (*Arachis hypogaea* L.), is an important oil, food, and feed legume crop grown in over 100 countries. It covered 26.71 million ha area worldwide with a total production of 44.86 million tons in 2016 (FAOSTAT, 2016). In the tropical and semi-arid tropical regions, about 90% of the world's groundnut production occurs. Much of the worlds groundnut production regions are characterized by high temperature.

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Groundnut is sensitive to temperature with an optimum for most processes being between 27 and 30 °C (Ntare and Williams, 1998). As the population is increasing geometrically resulting in increasing requirement for food security, the scientific community has to focus on developing plants with better tolerance to abiotic stresses and in particular heat stress.

Screening of groundnut genotypes for high temperature stresses in natural conditions which are highly variable is very difficult. Temperature induction response (TIR) is a robust and powerful technique to identify thermotolerant variants within cultivars or even in the inbred population. The advantage of TIR based screening method is that large number of seedlings can be screened in a short time. Studies on acquired thermotolerance in groundnut have been reported by several authors (senthil kumar et al., 2006; Gomez et al., 2011). One of the most widely studied aspects of thermotolerance is the enhanced expression of HSPs. Most of them have reported the central roles of HSFs in various abiotic stresses, including heat stress (Scharf et al., 2012). Therefore, transcription factors may have potential applications in improving biotic and abiotic stress tolerance in plants; however, knowledge of their function in heat stress responses remains limited. Expression and characterization analysis of transcription factors (TFs) under heat stress in groundnut is yet to be investigated. In present study, the genetic variability of widely grown groundnut genotypes was assessed for thermotolerance through TIR technique and the expression patterns of heat stress responsive genes at seedling stage through quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was discussed. With the aim to enrich the candidate heat responsive genes in groundnut at seedling stage, we selected nine genes that have not been characterized for their heat stress responsive nature in groundnut but characterized in other crops, so far and studied their expression pattern in the present study.

2. Materials and methods

2.1. Plant materials

The present work was conducted at the department of crop physiology, Institute of Frontier Technology, Regional Agricultural Research Station, ANGRAU, Tirupati, Andhra Pradesh. The Seed materials (100) were procured from Regional Agricultural Research Station, Tirupati, Kadiri, Andhra Pradesh and ICRISAT, Patancheru, Hyderabad, Telangana, India. About 2 day old seedlings germinated on filter paper in petri dishes were used for the experiment.

2.1.1. Temperature induction response

Groundnut seeds were surface sterilized by treating with 1 percent bavistin solution for 15 min and washed with the distilled water for 3–4 times and kept for germination at 30 °C and 60 percent relative humidity in the incubator. After 48 h, uniform seedlings were selected in each genotype and sown in aluminium trays filled with soil. These trays with seedlings were subjected to sub lethal temperatures (gradual temperatures increasing from 38 °C at time 0 to 54 °C at the rate of 0.5° raise in temperature per 10 min) for 5 h in the environmental chamber (WGC-450 programmable plant growth chamber). Consequently these seedlings were exposed to lethal temperatures (58 °C) for 3 h. Another set of seedlings not exposed to TIR, remains as control. Groundnut seedlings were allowed to recover at 30 °C with 60 percent relative humidity for 48 h.

A lethal temperature of 58 °C for 3 h and induction treatment from 38 to 54 °C for 5 h using TIR was considered as best lethal and induction temperatures for screening of groundnut seedlings for intrinsic heat tolerance at cellular level (Bharani et al., 2016). At the end of the recovery period, the number of seedlings that survived was recorded and percent reduction in growth over absolute control was calculated (Flow Chart 1).

Genetic variability for heat stress response in groundnut genotypes.

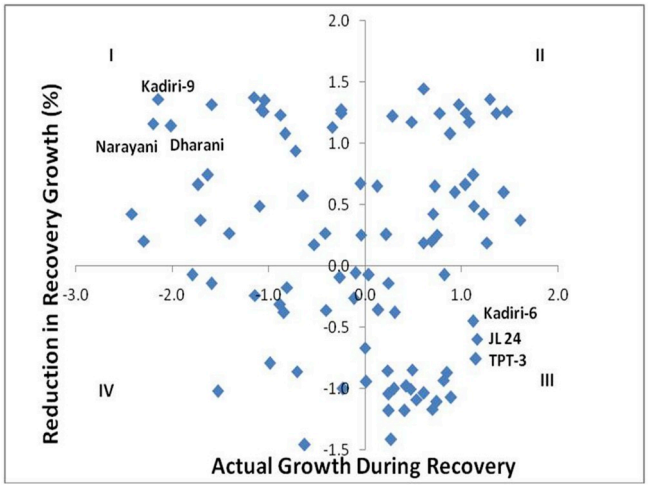


Fig. 1. Normal Z-distribution of Groundnut genotypes based on absolute growth after recovery period and percent reduction in recovery growth over control.

a) % survival of seedlings $= \frac{\text{No of seedlings survived at the end of the recovery}}{\text{total no of seeds sown}} \times 100$

b) % reduction in root growth $= \frac{\text{root growth of control seedlings} - \text{root growth of treated seedling}}{\text{root growth of control seedlings}} \times 100$

c) % reduction in shoot growth $= \frac{\text{shoot growth of control seedlings} - \text{shoot growth of treated seedling}}{\text{shoot growth of control seedlings}} \times 100$

By using normal Z distribution, the genotypes were classified into susceptible, moderately tolerant and tolerant based on percent survival and percent reduction in recovery growth over absolute control. Similar classification of tolerant, moderately tolerant and susceptible was followed in pea, rice and groundnut (Fig. 1) (Srikanthbabu et al., 2002; Vijayalakshmi et al., 2015; Rekha Rani et al., 2018).

2.2. Expression analysis

2.2.1. Total RNA isolation

Total RNA was extracted using phenol–chloroform method according to Sajeevan et al. (2014). RNA concentrations and purity were determined using a Nanodrop (GE health care, USA). RNA integrity was confirmed by electrophoresis using 1.2% agarose gel. Total RNA isolated from different tissues was diluted to 100 ng/μl concentrations for qRT-PCR experiments.

2.2.2. qRT-PCR primer designing

Nine candidate genes namely *NAC4* (Genbank accession no. HM776131), *WRKY* (Genbank accession no. EU853827), *HSP70* (Genbank accession no. EZ733089), *HSP90* (Genbank accession no. XM-025779528), *HSP60* (Genbank accession no. BW658281), *DREB2A* (Genbank accession no. DQ333948), *LEA4-2* (Genbank accession no. HM543585), *DHN1* (Genbank accession no. HM543578) and *AREB1Q* (Genbank accession no. NC-003074) were selected for characterization based on their role in abiotic stress tolerance from the available expressed sequence tags (EST) database and the sequences were retrieved from Genbank NCBI. The qRT-PCR primers were designed using the Primer 3 Plus software (Untergasser et al., 2007) considering the following parameters like product size of 90–120 base pairs, melting temperature (Tm) of 60–63 °C, length of 19–22 nucleotides and GC content of 45–55%.

2.2.3. Real-time PCR

5 μg of total RNA was reverse transcribed using 200 U M-MLV Reverse Transcriptase at 42 °C for 1 h with 30 pmol of oligo dT(18) primers and 10 mM dNTPs in 1X RT buffer. The synthesized cDNA was

used as a template for PCR amplification to see the specificity of the primers. For PCR, a 20 µl reaction was set containing 1 unit of Taq polymerase (Thermo Fisher Scientific, USA) in 1X reaction buffer, 2.5 mM MgCl2, 200 µM dNTP mix, 3 pmol of specific primers for each gene and template from RT reaction. The amplified products were separated on 1% agarose gel and documented. Further all the qRT-PCR reactions were carried out on Real Time PCR. Reactions were performed in a total volume of 10 µl, containing 1 µl of total RNA (100 ng), 400 nM of each primer, 5 µl of 2× one step SYBR RT-PCR buffer 4 (Takara) and 0.4 µl of prime script one step Enzyme Mix 2 (Takara) and made to 10 µl with RNase-free H2O. The qRT-PCR cycling conditions were as follows: 42 °C for 5 min and 95 °C for 10 s (reverse transcription) followed by 40 cycles of 15 s at 95 °C, 15 s at 61 °C with fluorescent signal recording and 15 s at 72 °C. The melting curve analysis was included after 40 cycles to verify the primer specificity by heating from 58 °C to 95 °C with fluorescence measured within 15 min. Elongation factor 1B (*ELF1B*) was used as internal control for normalization (Reddy et al., 2013). The expression of *NAC4, WRKY, HSP70, HSP90, HSP60, DREB2A, LEA4-2, DHN1*and *AREB1Q* genes was studied in control and selected tolerant and susceptible seedlings screened for thermotolerance. Relative expression levels of the genes in response to stress were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing with *ELF1B* reference gene (Reddy et al., 2013).

3. Results

Groundnut genotypes were classified into three different categories as tolerant, moderately tolerant and susceptible based on the percent reduction in recovery growth and percent survival after recovery period using TIR technique. The percent survival of seedlings varied from 10 to 100 percent with a mean survival of 70.6 percent of seedlings. The percent reduction in root growth varied from 2.07 to 100 percent with a mean of 37.48 percent and the percent reduction in shoot growth varied from 2.11 to 100 percent with a mean of 43.83 percent (Table S1). From the 100 diverse genotypes screened, 24 genotypes showed tolerance to high temperature, 30 genotypes showed moderate tolerance and 46 genotypes were identified as temperature susceptible compared with control (Table S2). Kadiri 9, Narayani, Dharani showed 9.81, 14.59, 18.58 percent reduction in root growth and 16.67, 13.45, 22.57 percent reduction in shoot growth. Jl 24, TPT-3, Kadiri 6 showed 31.04, 34.94, 36.70 percent reduction in root growth and 62.50, 21.47, 71.31 percent reduction in shoot growth. The normal z distribution of the genotypes studied showed that Kadiri 9, Narayani and Dharani were located in Quadrant I which is classified as highly tolerant, JL 24, TPT-3 and Kadiri 6 were grouped in Quadrant III, which is classified as susceptible (Fig. 1). The two popular varieties, Abhaya and Bheema were classified as moderately tolerant to temperature stresses as they fell in Quadrant II.

Using TIR, the contrasting genotypes selected for expression analysis are Kadiri 9, Narayani, Dharani which are highly thermotolerant and JL 24, TPT-3, Kadiri 6 highly susceptible. Specificity of the primers was confirmed by agarose gel electrophoresis using RT-PCR and melting curve analysis (Fig. S1). The melting temperatures of all amplification products are listed in Table 1. Real time PCR analysis for the genes *NAC4, WRKY, HSP70, DHN1* and *AREB1Q* were down regulated under stressful conditions. Three genes *HSP90, DREB2A* and *LEA4-2* were significantly up regulated to 20–50 fold increase in expression respectively when compared to control whereas only 10 fold increase was observed in *HSP60* (Fig. 2). In Kadiri 9, Narayani, JL 24 and TPT-3, *HSP90* indicated more than 20 fold increase in expression whereas less than 10 fold increase in expression in Dharani and Kadiri 6. *DREB2A* showed more than 15 fold increase in Kadiri 9 and JL 24 whereas less than 10 fold increase in Narayani, Dharani, TPT-3 and Kadiri 6. Three genes *HSP90*, *DREB2A* and *LEA4-2* showed increased expression in Kadiri 9 indicating their significance in imparting thermotolerance at seedling stage.

4. Discussion

Plants adapt to high temperatures by inherent cellular level tolerance as well as acquired tolerance to severe temperature stress. Developing thermotolerant genotypes is a challenging task as heat sensitivity is highly variable across various stages of plant growth. Screening of groundnut genotypes for high temperatures in natural conditions is very difficult. TIR technique is the best alternative to evaluate groundnut genotypes for thermo tolerance at seedling stage. By adapting the TIR technique, the existence of significant genetic variability across the genotypes of groundnut has been demonstrated and thermotolerant lines have been identified. Among all the genotypes, Kadiri 9, Narayani and Dharani showed the highest thermotolerance in terms of 100 percent seedlings survival and low reduction in root and shoot growth. These genotypes are able to survive even when they were exposed to lethal temperatures. Higher recovery growth was observed in tolerant genotypes is mainly because of altered metabolism in response to acclimation as reported by Larkindale et al. (2005) in Arabidopsis, SenthilKumar et al. (2003) in sunflower and Gangappa et al. (2006) in groundnut. Senthil Kumar et al. (2006) also reported that acclimated plants survive upon exposure to a severe stress and are considered as thermotolerant. The seedling survival, shoot and root growth were completely affected in the genotypes ICGV 02266 and ICGV 00351 despite of the recovery conditions maintained after exposing to sub lethal to lethal temperature.

Groundnut genotypes were selected for expression analysis based on the TIR results and their relative performance under field conditions to heat stress as reported by Bharani (2014).Our results showed enhanced expression of stress responsive genes in the selected induced seedlings. Variation in differential expression of stress responsive genes explains the stress responses nature of the plants. Molecular approaches will help in understanding the concept of heat stress tolerance apart from physiological and biochemical mechanisms (Barnabas et al., 2008). Validation of heat stress tolerant genes in plants could provide insight into their functions, which would facilitate their use in developing improved cultivars. According to Wahid et al., (2007), expression of HSPs and TFs is a strategy for adaptation to high temperatures, and *HSPs* synthesized during stress might have imparted thermotolerance. For understanding the biological processes in various developmental stages and several abiotic stresses, gene expression studies are important determinants. Quantification of gene expression levels has been done by numerous techniques like Northern blotting, semi-quantitative RT-PCR, and qRT-PCR. However, qRT-PCR is the most effective method for detection of PCR products with high sensitivity, specificity, rapidity, and accuracy (Park et al., 2008). Therefore, the expression pattern of known stress responsive genes *HSP90*, *HSP70*, *HSP60* and transcription factors like *AREB1Q, DREB2A, DHN1, NAC4* and *WRKY* genes was examined through Real time PCR at the end of induction in the selected genotypes.

In the present investigation, among all the genotypes Kadiri 9 showed more than 15 fold increase in expression with respect to *HSP90*, *DREB2A* and *LEA4-2* indicating tolerance to heat stress at seedling stage. Several other studies in different species demonstrated that upon acclimation in seedlings as well as plants, significant increase in HSPs expression (*HSP70* and *HSP90*) occurred (Senthil Kumar et al., 2006). Yamada et al. (2007) also showed that HSP70, HSP90 regulates heat shock response upon heat stress by regulating heat shock TFs in Arabidopsis. HSP90 and HSP70 are the predominant HSPs and they work individually or in HSP90-HSP70 complex and play an important role in nascent folding of aggregated polypeptide, hence providing stability. *HSP90* gene is up regulated several folds thereby playing a very important role in imparting stress tolerance. *HSP70* gene has crucial role in the control of plant response to multiple environmental stimuli and enhancing stress tolerance. Down regulation of *HSP70* in the seedlings recovered after induction challenge might be regulated by both the activation and the repression mechanisms under stress condition. The

Table 1
Sequence of the primers used for qRT-PCR amplification.

S.No	Gene	Primer sequence (F/R) 5'-3'	Length	Amplicon Length (bp)	Tm (˚C)
1	NAC4	GTTCGACCCTTGGGTTCTT	19	96	80.7
		CGCGATCCATTCGGATACTT	20		
2	WRKY	TCCAGCAAGGAAGCATGTAG	20	92	81.2
		CTGCAGTGAGAGAGTGGTTATG	22		
3	HSP70	GAAAGCCGAAGACAAACACC	20	97	81.6
		GTACTCGACCTCTTTGCTCG	20		
4	HSP90	ATCACAGGAGAGAGCAAAAAGG	22	96	78.1
		TGACTCTGCAAAGATCCTCAAA	22		
5	HSP60	TCGGATCCACTAGTAACGGC	20	92	90.1
		TGCCATCTCTTGCAAACAAC	20		
6	DREB2A	TTGAGACTCCTGGTTGTGTTC	21	113	88.4
		CATCTCTTCCGCACCATTCT	20		
7	LEA4-2	GGACAACATGGACATCCTACTG	22	94	84.1
		CTATCACTCCCTCAGTCACCT	21		
8	DHN1	AAGGTGGGAGGAGGAAGAA	19	112	83.6
		GAGGTGGTTGTCCCTTGATG	20		
9	AREB1Q	ACAAGGGCAACCAGCATTAGG	21	95	82.3
		TCACCACCACCATACCAACCA	21		
10	ELF 1B	AAGCTTCCCTGGCAAAGCTCAA	22	153	81.6
		TTCCTCAGCTGCCTTCTTATCC	22		

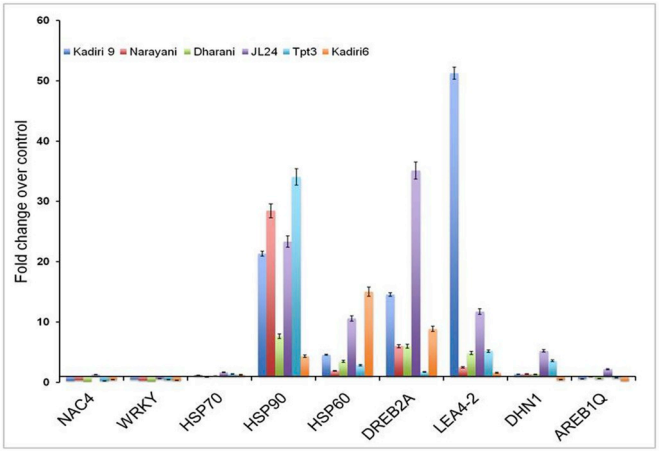
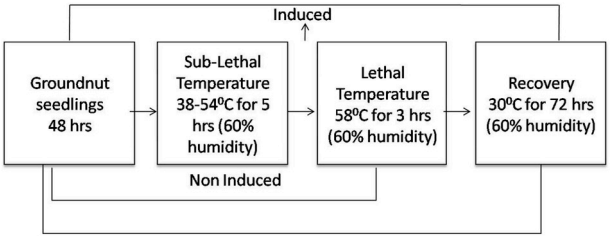


Fig. 2. Quantitative expression analysis of nine candidate genes for heat response at seedling stage.



Flow chart 1. Standardized temperature induction response (TIR) protocol to screen groundnut genotypes.

lower expression level of HSP70 might be due to the acquired thermotolerance induced by cellular tolerance that protected the groundnut seedling exposed to challenging temperatures. *HSP60* gene was up regulated indicating its possible putative role in overcoming the loss of mitochondrial function due to heat stress. *HSP60* gene was also up regulated in Arabidopsis and *Zea mays* under heat shock (Prasad and Stewart, 1992; Rikhvanov et al., 2007). This evidence indicates that several HSPs up regulated because of the efficiency of upstream regulatory mechanisms like signal perception and transduction leading to the expression of several TFs. Fragkostefanakis et al. (2015) reported that TFs not only play a major role in protection of plants from stress but also focus mainly in regulating gene expression and signal transduction in the stress response regulatory proteins. TFs such as *AREB1Q, DREB2A, NAC4, LEA4-2* and *WRKY* play a major role in regulating plant growth and metabolism (Lata and Prasad, 2011; Nakashima et al., 2012). Hence the expression of heat stress TFs was studied in the present investigation in selected genotypes. Sakuma et al. (2006) confirmed that *DREB2A* gene is strongly induced under heat stress along with up regulation heat shock protein 70. The up regulation of *DREB2A* gene suggests the key role of inducing several heat and drought tolerant genes thereby enhancing plant thermotolerance under stress. LEA4-2 is a member of the Late Embryogenesis Abundant (LEA) proteins which typically accumulate in response to low water availability conditions. Pruthvi et al. (2014) altered the expression of few downstream genes in Arabidopsis and showed higher expression of LEA4 in transgenics compared to wild type plants in groundnut under abiotic stress. There are few reports that the expression of dehydrins was confirmed in sugarcane and citrus under high temperature stress (Wahid and Close, 2007; Porat et al., 2004). Research has been carried out on *WRKY* TFs in the recent past in several crop plants such as rice (Ross et al., 2007), cucumber (Ling et al., 2011), maize (Wei et al., 2012) and tomato (Huang et al., 2012), due to their important role in various biological processes. Compared to the drought and heavy metal stress, there are few reports on response of *WRKY* genes to high temperature stress (Suchithra et al. 2017). *NAC4* TFs regulate immune responses in plants by pathogen induced cell death Lee et al., (2017). Knowledge of NAC4 function in the high temperature response of groundnut is limited and whereas, NAC4 was up regulated under salinity and drought in groundnut and horsegram (Banavat et al. 2018; Pandurangaiah et al., 2014). *LEA4-2, DHN1, NAC4* and *WRKY* genes were expressed under drought, salinity, cold and abscisic acid induced stress in groundnut (Su et al., 2011; Chen et al., 2014; Li et al., 2014) also showed increased expression under heat stress indicating their role in cross protection. In our study, the down regulation of *AREB1Q, DHN1, NAC4* and *WRKY* genes in response to heat stress in groundnut is not fully understood. However the mechanism and function of these genes under heat stress is yet to be identified. It is possible that heat sensitive plants may encounter a critical stress condition at higher temperatures that resulted in a large number of stress related genes and this might be the reason for differential expression of genes. In most plant species, heat stress tolerance is a complex trait, often controlled by various biosynthetic and signaling pathways including cross talks among multiple stress controlling pathways. Thus, comprehensive knowledge of the up regulated and down regulated genes under heat stress is necessary for

better understanding of the interactions among pathways in response to stress. From the present study it can be evident that the TIR technique is an effective method to screen groundnut genotypes for thermotolerance from large population. The identified genotypes showed to possess high level of thermotolerance at seedling stage. HSPs and TFs which are accumulated in the selected genotypes play an important role at cellular level imparting heat stress tolerance. Genes such as *HSP90*, *HSP60*, *DREB2A* and *LEA4-2* which are up regulated during heat stress at seedling stage should be further validated under field conditions to understand the molecular mechanism involved in abiotic stress tolerance. The genes which are highly up regulated at the seedling stage are further to be validated at whole plant level at multiple or combined environmental stresses to know the possible roles associated to heat stress tolerance.

5. Conclusion

Our study suggested that the thermotolerance of the selected genotypes was associated with a higher expression of heat shock proteins and the heat stress transcription factors. The data generated in this study partially resolve genes that play a key role in regulating the response to heat stress in groundnut. Further detailed study of these genes will facilitate an understanding of the molecular mechanisms to heat stress and other stresses, which will provide us the basis of effective genetic engineering strategies for improving the multiple stress tolerance of groundnut.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

RRK, PS and PL designed the experiments. RPV provided the seed material. RRK carried out all the TIR and qRT-PCRs. RU supervised the work. RRK and VH analyzed the data and RRK drafted the manuscript. All authors approve the final version of the draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101242.

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