Research Article

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Expression profiling of stress responsive genes in cell suspension of *Elettaria cardamomum* (L.) Maton under abiotic stress

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Abstract

Cardamom is an economically important spice, valued for its multiple utility from culinary to medical purposes. The plant is highly susceptible to both abiotic and biotic stresses. Despite the fact that several studies focusing on stress in cardamom have been conducted; a molecular analysis at the cellular level has not been reported. This study highlights the molecular response of homogenous population of cardamom cell suspension following the temperature and drought stresses for a short period of time (20 mins). Temperature stress at 30, 35, and 40°C caused a significant increase in the transient expression of genes, sHSP 17.8 and sHSP 17.9, which are molecular chaperones involved in protein folding coping with the heat stress response of plants. Drought stress with various concentrations of PEG 6000 has demonstrated only a little increase in the expression of transcription factors, WRKY 35 and WRKY 71. The study implies that sHSP 17.8 and sHSP 17.9 play a crucial role during heat stress, which is a major limiting factor for the cultivation of cardamom in lower altitudes where atmospheric temperature is usually high. But WRKY 35 and WRKY 71 genes are found not to have a high impact at the cellular level in response to drought stress in cardamom when it is exposed to a brief duration of drought. Understanding the molecular mechanism underlying abiotic stress response in cardamom will aid in developing elite varieties adaptable to lower altitudes and to cope with the frequent climatic variations.

Keywords: Gene expression, temperature, drought, sHSP, WRKY, callus culture

Introduction

A vast majority of spices serve as active components in age-old culinary, pharmaceutical and cosmetic industries. The multiple utilities of spices in various industries offer them a supreme position in the global economy. *Elettaria cardamomum* (L.) Maton, also called small cardamom for its inimitable flavor, aroma and for its versatile utilities from food adjuncts to indigenous medicine, is rightly acclaimed as the "Queen of spices". It is considered as the intensely valuable spice in the world after saffron and vanilla (Ashokkumar *et al*., 2020). Small cardamom, native to the southern region of India is a rhizomatous perennial herbaceous monocot belonging to the family Zingiberaceae. The largest cardamom producers in the world include Guatemala, India, Sri Lanka and Nepal, and also happens to found in Tanzania, Indonesia, Vietnam, Thailand, Papua New Guinea, and El Salvado in small scale (Vijayan, 2018).

Cardamom is a sciophyte that thrives in elevations up to 600-1200m above msl under an average annual rainfall of 1500-4000 mm and a temperature ranging from 10–35°C (Madhusoodhanan *et al*., 2002). Cardamom plantations are prominent in humid tropical climate with soil rich in organic contents (Mohammedsani, 2019). Abiotic stress is a major obstacle in growing cardamom as the

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plant is extremely vulnerable to climate change; and crop production is heavily climate-dependent despite the technological advancements. The agricultural yield in the Indian Cardamom Hills taking into consideration the climate change was discussed in detail by Murugan *et al*. in 2012. Heat and drought stresses are thought to be the two main abiotic constrains in the cultivation of cardamom. Plants display a distinctive set of cellular and metabolic responses when subjected to excessive heat stress, at least 5°C above the ideal growing conditions and these adaptive responses are necessary for the plants to survive in the high temperature setting (Guy, 1999).

Plant heat shock proteins (HSPs), which act as molecular chaperones, are vital cellular components in promoting stress tolerance and facilitating plants to develop survival mechanisms against a variety of biotic and abiotic challenges (UlHaq *et al*., 2019). In plants, WRKY Transcription Factors have been studied in detail for their role in regulating drought stress responses. Phukan *et al*. (2016) provided a comprehensive review on the role of WRKY transcription factors in the molecular mechanism of plant stress responses. The response of cardamom to external stress at the cellular level has not been evaluated yet but it could be a highly interesting simulation model for comprehending the basis of adaptability. Plant cell suspension cultures are frequently used to explore a number of molecular phenomena without having to deal with the structural complexity of the tissue organization (Zagorskaya and Deineko, 2017). Cell suspension culture experimental setup is ideal for examining a wide range of topics, including ion transport, the generation of secondary metabolites, gene expression analysis and stress response reactions (Moscatiello *et al*., 2006; Menges *et al*., 2003; Chandran *et al*., 2020). Suspension-cultured cells are well suited for the cellular and molecular investigation of complicated physiological processes due to the homogeneity of *in vitro* cell population, abundance of material, high pace of cell development, and good reproducibility of conditions (Li, 2019). The present study has developed a cell suspension system of cardamom for evaluating the expression pattern of certain stress related genes following the induction of abiotic stress under controlled environment, which can shed light on the molecular basis of adaptation at the cellular level of cardamom.

Materials and methods

Surface sterilization of explant

Rhizomes of cardamom collected from the cardamom conservatory of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) Palode, Thiruvananthapuram, Kerala were used as the source material for micropropagation. Samples were thoroughly washed under running tap water for about 10-15 mins followed by rinsing with Tween 20 and further repeated washing in distilled water. Then the explants were treated in a solution of distilled water with 4 drops of Tween 20 and a pinch of Bavistin, kept on shaking for 15 mins followed by multiple washing steps with distilled water and kept on the same for 20 mins. In the laminar air flow chamber, rhizomes were descaled into leaf bases and core sucker and antibiotics treatment was done with 0.1 % ampicillin /streptomycin sodium sulphate solution for half an hour followed by surface sterilization with 0.1 % mercury chloride for 8 mins, each treatment was followed by minimum 4 washing steps with sterile distilled water. The inner leaf base of 0.5-1.0 cm size was kept on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with different combination of plant growth regulators for callus induction.

Cardamom cell suspension

For the purpose of callus induction, MS medium enhanced with various combinations of cytokinins, 6-benzylaminopurine (BAP) and kinetin (Kn), coupled with auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), was kept at

25±2°C room temperature under both dark and 12-hour photoperiod conditions. The acquired friable callus was further subcultured in liquid media with the same PGR composition to produce a cardamom cell suspension culture, which was subsequently employed for molecular studies after four subcultures resulting a fine cell suspension. Cultures were maintained in 250 mL flasks at 25±2°C with uninterrupted shaking at 125 rpm for a 12 hour photoperiod. Subculturing was performed in every 20 days by transferring 0.5 gm of cell culture into 60 mL of fresh nutrient medium. Cell growth was monitored by obtaining the fresh weight and dry weight of the cells at 5 days interval for a period of over 30 days, by harvesting the cells using Whatman filter paper and oven drying at 60°C for 6hr.

Induction of abiotic stress

Twenty day old cultures of cardamom cell suspension with maximum growth rate was exposed to different temperatures of 30, 35 and 40°C for a period of 20 min to induce heat stress, while keeping the control at 25°C (Wu and Wallner, 1984). Similarly PEG 6000, a water-soluble and nonionic natural polymer found to mimic drought stress (Musculo *et al*., 2014), was used at different concentrations of 20, 30 and 40% incubated for the same time period in another set of cultures to investigate the impact of drought stress in cardamom cells. After the course of experiment cells were harvested and subjected to RNA isolation using modified CTAB method (Nadiya *et al*., 2015) and concentration was determined using a Nano photometer (IMPLEN), and the integrity of RNA was confirmed with 1.0% agarose gel electrophoresis by means of ethidium bromide staining.

Selection of candidate reference gene

cDNA was synthesized by reverse transcribing 500 ng total RNA with Takara PrimeScript™ RT Reagent kit following the manufacturer's instructions. The cDNA templates were diluted in 1:5 ratios with nuclease free water and used as the template for RT-qPCR amplification. Reference gene standardization was performed for identifying the endogenous control. Reference genes are supposed to maintain their expression throughout the experimental circumstance, which in turn is necessary for a consistent transcriptional quantification (Kozera and Rapacz, 2013). Based on the relative stability of their expressions and prior use as internal reference genes for RT-qPCR investigations in other plant species, five prospective reference genes were chosen. The reaction was setup in QuantStudio™ 5 System (Applied Biosystems) with SYBR®Green chemistry and all the reactions were performed in triplicate for a single biological replicate in 40 universal temperature cycles. By comparative analysis of Ct values and standard deviation and by analyzing the stability based on the SD of Cq values, along with the coefficient of variance (CV), correlation coefficient (r) and p-value (p) using the software BestKeeper the potential reference gene as an endogenous control was determined (Pfaffle et al., 2004).

Primer design and RT-qPCR amplification

MEGA X software was used to align the gene sequences that were retrieved from the NCBI GenBank (Kumar *et al*., 2018). The following criteria were employed during the designing of primers via IDT primer quest: Tm ranging from 59 and 65°C with an optimum Tm of 62°C; length 18 to 25 base pairs, with 22 bp being the ideal length; GC content of 45 to 65%; and amplicon size between 100 and 150 bp. With the assistance of an online tool Net Primer (https://www.premierbiosoft.com/netprimer), the efficiency of the newly designed primers were evaluated and those sets of primers with values higher than 80 were chosen. Using NCBI Primer-BLAST (Ye *et al*., 2012), the proposed primers were analyzed for their specificity, and those primers with non-specific results were excluded. Two primers WRKY 35 and WRKY 71 specific for osmotic stress genes were synthesized and the rest of the primers including reference gene primers and two sHSP primers for evaluating genes under temperature stress available in the research

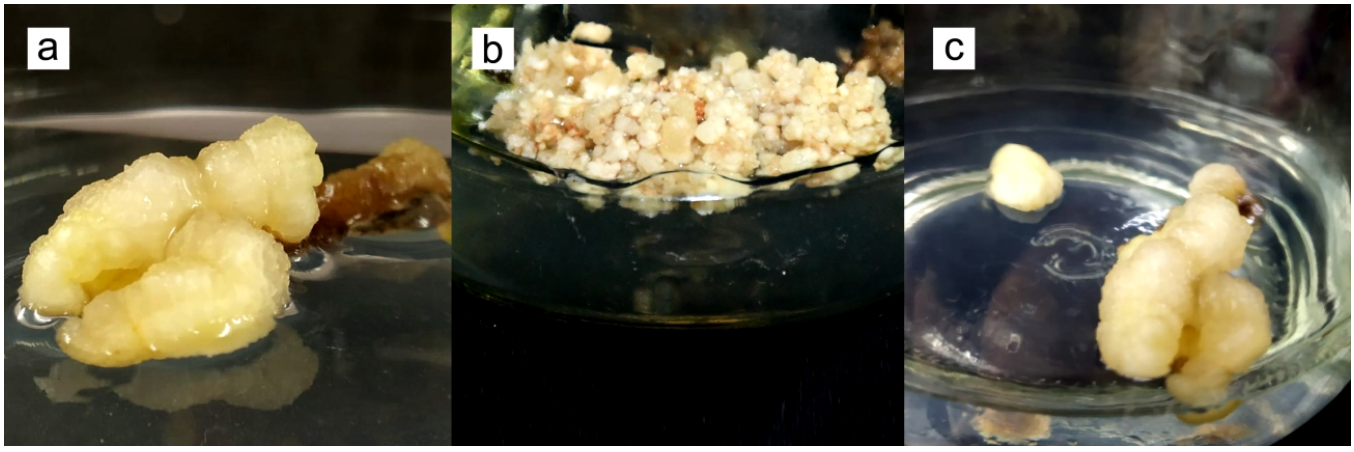

Fig. 1. (a) White compact callus (b) Creamish white friable callus and (c) Creamish white compact callus

laboratory were used for the study. The samples were subjected to RT-qPCR reactions for gene expression analysis, with the specific primers under universal PCR conditions at 60°C annealing temperature for 40 cycles.

Results and discussion

Callus initiation and cell suspension growth curve analysis

Regardless of the hormone combination, callus initiation occurred in MS medium within four weeks. Depending on the supplemented PGR strength, callus progressed to both compact and friable callus in another two to four weeks (Fig. 1). In MS media, treated with 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (Kn), the maximum response with an enhanced callus fresh weight was observed. The majority of the media combinations produced soft, cream-colored, friable calluses; Table 1 presents the type, fresh weight and percentage of response of the callus to various hormone combinations and photoperiods. In order to produce a cell suspension with an active rate of proliferation, the maximum friable callus obtained from the aforementioned hormone combination was subsequently subcultured in the same medium without Phytagel (Fig. 2). Twenty-day-old suspension cultures were utilized for the study because analysis of the growth pattern of cells over a period of thirty-days with five days interval, measuring the fresh weight and dried weight revealed that growth peaked at day 20 and since then it remained either stationary or declined (Fig. 3).

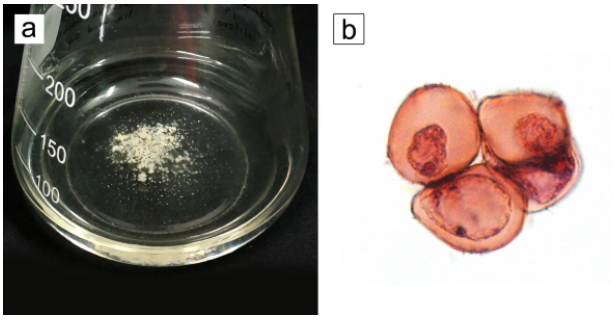

Fig. 2. (a) Cardamom cells in suspension (b) Microscopic imaging of safranin stained cells in suspension

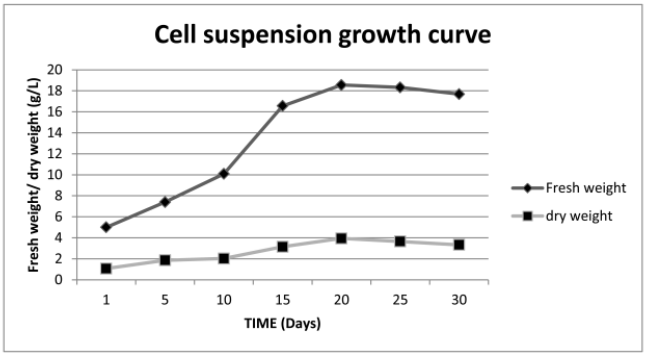

Fig. 3. Cardamom cell suspension growth curve depicting the fresh weight and dry weight of cells.

Abiotic stress induction and standardization of endogenous control

Following treatments at various temperatures and PEG concentrations, no morphological changes in the colour or texture of the cells were found. High-quality RNA with acceptable concentration and purity values was produced by the modified CTAB technique (Nadiya *et al*., 2015), which was subsequently reverse transcribed into cDNA. Electrophoresis revealed that the primers yielded single band on the PCR, and a

Table 1. Fresh weight, percentage response nature and nature of the callus under various hormone combinations and photoperiods

Growth Conditions	Concentration			Fresh weight	Percentage Response (%)	Features
	2,4-D	Kn	BAP			
Light	0.5	-	-	0.61±0.04[b]	70	White compact callus
	1	-	-	0.77±0.07[b]	70	Cream white friable callus
	2	-	-	0.68±0.03[b]	50	Cream white friable callus
Dark	0.5	-	-	0.70±0.03[b]	70	White compact callus
	1	-	-	0.97±0.06[a]	90	Cream white friable callus
	2	-	-	0.95±0.07[a]	70	Cream white friable callus
Light	1	0.5	-	1.11±0.09[a]	70	Cream white friable callus
	2	0.5	-	1.04±0.07[a]	60	Cream white friable callus
Dark	1	0.5	-	1.36±0.15[a]	90	Cream white friable callus
	2	0.5	-	1.21±0.12[a]	80	Cream white compact callus
Light	1	-	0.5	0.53±0.04[b]	60	Cream white compact callus
	2	-	0.5	0.52±0.04[b]	60	Cream white compact callus
Dark	1	-	0.5	0.68±0.05[a]	90	Cream white compact callus
	2	-	0.5	0.64±0.03[ab]	90	Cream white compact callus

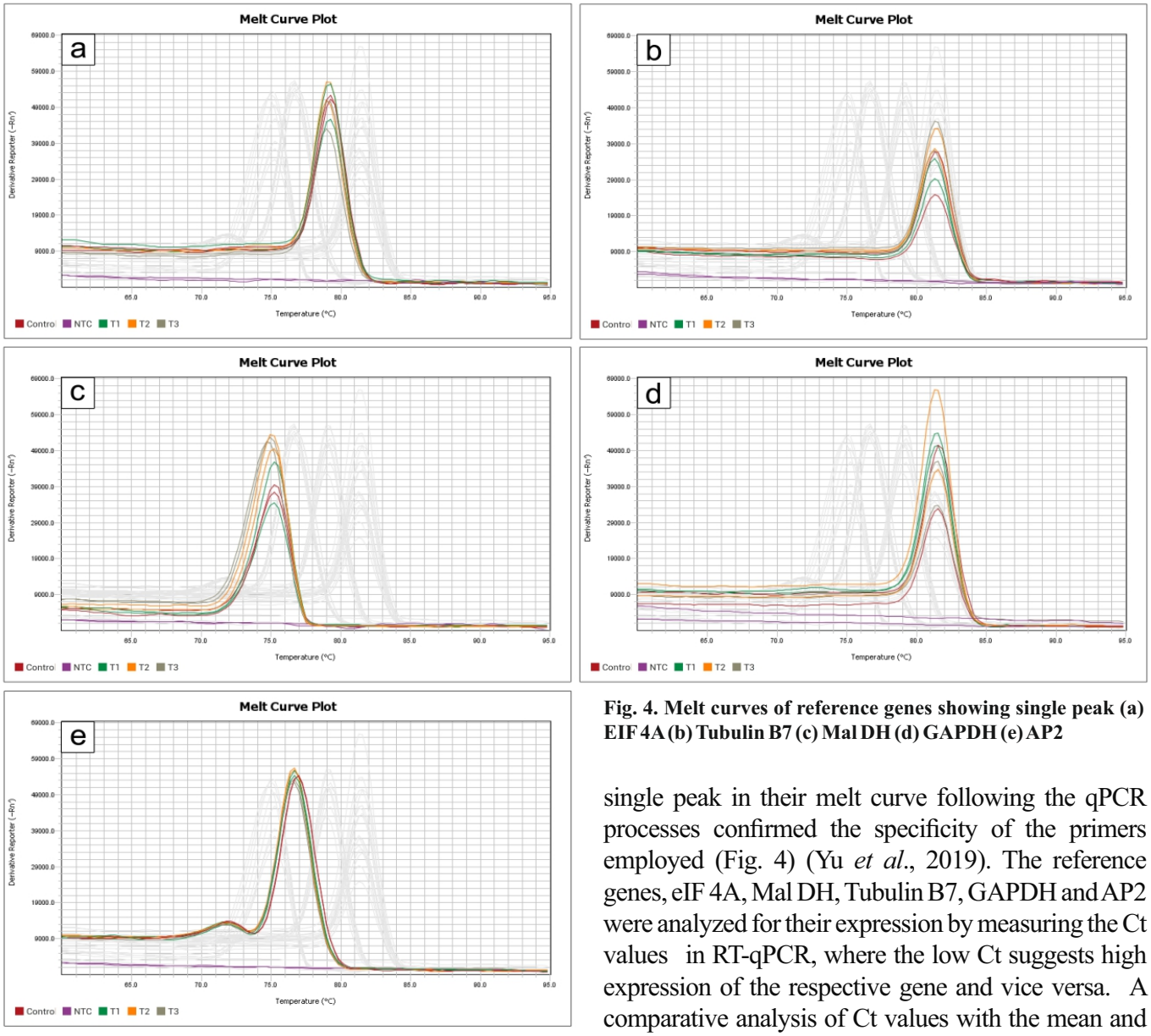

Fig. 4. Melt curves of reference genes showing single peak (a) EIF 4A (b) Tubulin B7 (c) Mal DH (d) GAPDH (e) AP2

single peak in their melt curve following the qPCR processes confirmed the specificity of the primers employed (Fig. 4) (Yu *et al*., 2019). The reference genes, eIF 4A, Mal DH, Tubulin B7, GAPDH and AP2 were analyzed for their expression by measuring the Ct values in RT-qPCR, where the low Ct suggests high expression of the respective gene and vice versa. A comparative analysis of Ct values with the mean and

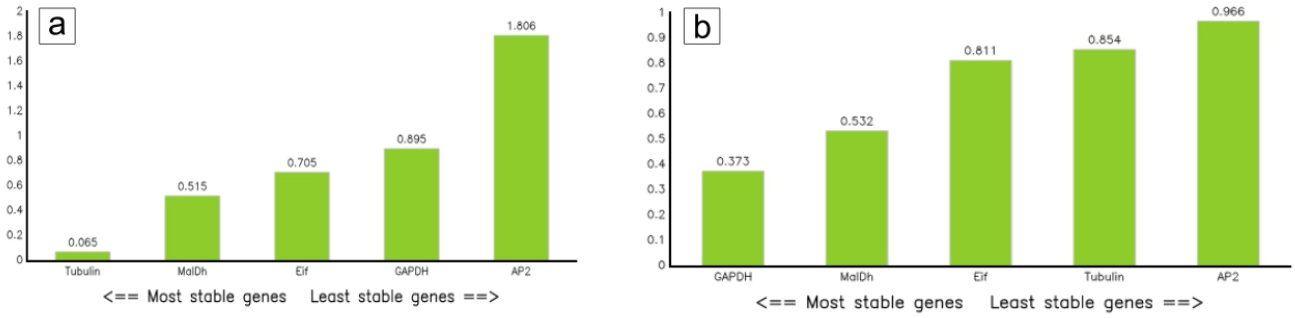

Fig. 5. Gene stability analysis using BestKeeper,where the x-axis represent different reference genes and y-axis represents Standard Deviation(a) Tubulin B7 the most stable endogenous control for osmotic stress (b) GAPDH the most stable endogenous control for temperature stress.

standard deviation, and the gene stability calculation based on BestKeeper index (Pfaffl *et al*., 2004) were utilized for normalization of endogenous control in both temperature and osmotic stress. Analysis of Comparative Ct and BestKeeper outputs revealed that GAPDH was the most stable candidate gene across temperature treatments, while Tubulin B7 was the best candidate reference gene for osmotic stress. With the lowest Ct and Standard Deviation values, GAPDH and Tubulin B7 have both demonstrated the highest levels of stability. Fig. 5 presents a summary of the expression profile of genes.

Expression analysis of stress related genes

Employing quantitative real-time PCR, the expressions of sHSPgenes and WRKY genes were examined in order to assess the effects of temperature and drought stress on cardamom cell suspension, respectively Details of primers used for gene expression analysis are represented in Table 2. WRKY 35 gene expression appeared to have increased by two fold at 40% concentration during a short spell of water stress induced by PEG 6000, whereas WRKY 71 gene expression exhibited only 1.5 fold increase at the same concentration, and there was no discernible variation in expression when exposed to 20 and 30% PEG concentration. In response to heat stress sHSP 17.8 gene has shown a gradual upregulation in its expression pattern from 2 fold at 30°C to 12 fold at 35°C and 55 fold times during 40°C. In addition, sHSP 17.9 displayed a significant upregulation from 33 fold at 30°C to 371 fold at 40°C with an intermediate value of 136 fold changes at 35°C when compared to the control (Fig. 6).

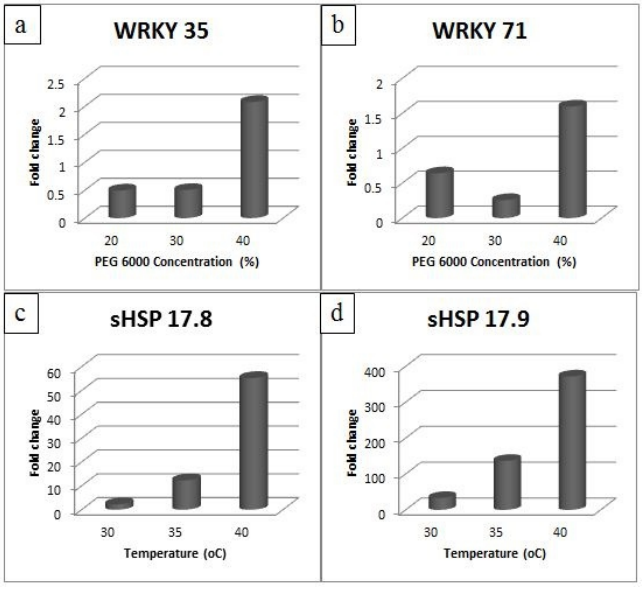

Fig. 6. Representation of gene expression of sHSP and WRKY genes in cardamom under temperature and drought stress respectively (a) WRKY 35 (b)WRKY 71 (c) sHSP 17.8 and (d) sHSP 17.9.p

A change in the external environment can cause a shift in internal cellular signals. In response to that cells will exhibit differential gene expression, and the amount of transcript accumulated will vary depending on the genotype and the duration of exposure (Milioni *et al*., 2001). Experiments by Sacchs *et al*.(1986) supported the fact that an increase in temperature by 2.5°C per hour can induce the expression of several sHsps in 20 min among which some tend to induce even in 3 to 5 min. The involvement of HSP in thermotolerance of rice and its usage as biomarkers for identifying heat-sustainable rice cultivars using Hsp 26.7, Hsp 23.2, Hsp 17.9A, Hsp 17.4, Hsp 16.9A, sHsp 17.7, and Hsp 18.6 have been discussed (Murakami *et al*., 2004; Wang *et al*., 2015; Chen *et al*., 2014).

According to Pavli *et al*.(2011) the early expression of heat shock proteins in sorghum enabled the cells to recover and maintain its growth when exposed to temperature stress. Similarly, sHSP 17.8 and sHSP 17.9 play a crucial part in coping with the heat stress response in cardamom, which is a major factor restricting cardamom cultivation only to the cool higher elevations. Similar to temperature, drought stress is also detrimental to plants that hampers productivity and numerous transcription factors that govern the expression of genes associated to stress are responsible for regulating plants response to drought stress (Zhu, 2002; Xiong *et al*., 2002). According to He *et al*. (2016), TaWRKY1 and TaWRKY33 transcription factors derived from wheat demonstrated activation of several stress-related downstream genes that promote root growth and germination rates in *Arabidopsis* under a variety of stresses by reducing water loss and increasing heat tolerance. In response to cold, dehydration, salt, ABA, H_2O_2, ethylene, salicylic acid and methyl jasmonate treatment *Musa* WRKY71 gene expression was found to be up-regulated suggesting its involvement in different stress condition (Shekhawat *et al*., 2011). However, WRKY 35 and WRKY 71 genes have shown least impact on drought stress response in cardamom at cellular level.

Table 2. Details of primers used for gene expression analysis.

Sl No.	Primers	Sequence (5' to 3')	L	Tm
1	WRKY-35 (F)	TATGGGCTTGGAGGAAGTATGG	22	60.25
2	WRKY-35 (R)	CTGCGCTCCACTTGCTTC	18	58.24
3	WRKY-71 (F)	ATCATCTCGACGACGGCTATC	21	59.82
4	WRKY-71 (R)	ATCTCTCCACCCTCTTCTTCAC	22	60.25
5	sHSP 17.8 (F)	TCGTGTTCGGTGAATGACTAC	21	57.87
6	sHSP 17.8 (R)	TCAAGAGCTGACGGTTTAAGG	21	57.87
7	sHSP 17.9 (F)	GGCATCGCGGATGTACG	17	57.60
8	sHSP 17.9 (R)	TGTGAGGATGTTTAACCTGGAC	22	58.39

F: Forward primer; R: Reverse primer; Tm: Melting temperature; L: Length;

Conclusion

Although no reports of stress-related studies at the cellular level in cardamom have been produced to date, this cell suspension study could be considered as a preliminary attempt to comprehend the molecular basis of adaptability. This work is a prelude into the abiotic stress response of cardamom at the cellular level, in-depth research involving *in vitro* and *in vivo* cardamom plants will help to better understand how the plant adapts to unpredictable climate change which could ultimately lead to the creation of elite varieties with improved abiotic stress tolerance.

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