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EXPRESSION OF ANTIOXIDANT GENES CAN INCREASE COLD RESISTANCE IN TOMATO (*SOLANUM LYCOPERSICUM* L.)

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Abstract

Tomato (*Solanum lycopersicum* L.) is one of the important vegetable crops worldwide. It is consumed in fresh as well as in processed form and is a rich source of antioxidants. There are many limiting factors to its production including biotic and abiotic stresses, among which, cold stress is the most important one. For example, tomato from tropical region has virtually no capacity to survive even the slightest freeze. This study focuses on the expression of two genes *i.e., Glutaredoxin S17* (*LOC101268063*) and *LEA 25 protein* (*NM_001309385*.1). The objective of this research was to study the expression of two genes in two commercially grown tomato varieties *i.e.,* Roma and Rio Grande and to check which variety performs well under cold stress. The current research was conducted on seedlings of two tomato varieties *i.e.,* Roma and Rio Grande. Three different cold stress treatments *i.e.,* 4, 10, and 16°C were applied for 10 hours. Treatment 4°C was applied by placing tomato seedlings in fridge while treatment 10 and 16°C were provided in incubators by adjusting the temperature. Gene expression was studied using RT PCR. Results of RT PCR showed that both genes were expressed in cold stress, but differential expression of *Glutaredoxin S17* gene was more pronounced at 4°C whereas expression of *LEA 25 protein* gene was very low in all the samples of different treatments. Furthermore, *Glutaredoxin S17* gene expression was more pronounced in Roma variety than Rio Grande. Our findings suggest that tomato plants expressing *Glutaredoxin S17* gene could enhance cold stress tolerance. In future, by insertion or overexpression of *Glutaredoxin S17* gene, cold stress tolerance could be improved in tomato crop.

Key words: Antioxidant genes, Cold stress, Gene expression, Rio Grande, Roma, *Solanum lycopersicum,* tomato.

Abbreviations: CTAB: Cetyl trimethylammonium bromide; LEA: Late embryogenesis abundant; NCBI: National Center for Biotechnology Information; PCR: Polymerase chain reaction; Rpm: Revolutions per minute

Introduction

Tomato is an important vegetable and contains phenolic compounds, minerals and antioxidants which are essential for human health by preventing and curing chronic diseases (Raiola *et al*., 2014). It is used as a compulsory element of daily life in the form of fresh salad, tomato ketchup, tomato sauces and tomato juice (Raiola *et al*., 2015). In Pakistan, 60307 hectares land is used to cultivate tomato with an annual production of 575923 metric tonnes (FAOSTAT, 2016). Tomato is a model species for genomic studies having (2n=24) chromosomes and has a modest genome size of 950 Mb/haploid genome (Sato *et al*., 2012).

Among the various abiotic stresses, cold stress severely reduces the agricultural output of plants in colder regions (Atkinson and Urwin, 2012; Pandey *et al*., 2017). By definition, both freezing temperatures (< 0°C) and chilling temperatures (< 20°C) are included in cold stress. It is one of the main stress affecting growth and production of tomato (Miura and Furumoto, 2013). Different processes of plant growth *i.e.,* from germination of seeds, flowering to fruit development and ripening are badly affected by the cold stress. Besides, it severely affects the cellular homeostasis of plant (Zhang *et al*., 2017). In addition, both direct and indirect reactions of cold stress hamper the expression of the complete genetic potential of plants. Directly by inhibiting the plant metabolic reactions and indirectly by cold-induced osmotic inhibition (inhibition encouraged by cooling of H_2O absorption besides cellular dehydration encouraged by freezing), oxidative stress and other stresses.

Various plant species can resist the cold stress to a considerable degree, which is mainly based on re-programming of the gene expression that alter their physiology, growth and metabolism (Smita *et al*., 2013). To become accustomed to cold stress in cold acclimation, expression of genes is re-programmed and the metabolism is altered as well (Sangwan *et al*., 2002). Cold response is a very complicated phenomenon. It contains numerous kinds of metabolic tracks and gene regulation (Maruyama *et al*., 2014).

The process of cold tolerance is called as cold acclimation. Gene expression and metabolism are modified and re-programmed to adapt to cold stress during cold acclimation (Smita *et al*., 2013). In cold response, different metabolic pathways, cell compartments and gene regulation are altered, thus making it a very complex set of traits. In the signaling of cold stress, different approaches like transcriptional and post-transcriptional gene expression are modified to confer tolerance to plants (Nakashima *et al*., 2014). Exposure to low temperature can cause numerous physiochemical disorders, which result in the growth reduction. Low temperature is perceived by the plants

through a signal transduction which result in the activation of cold stress related genes and transcription factors. These transcription factors and genes repair the damage which occur due to low temperature and help in giving tolerance to the plants (Fig. 1).

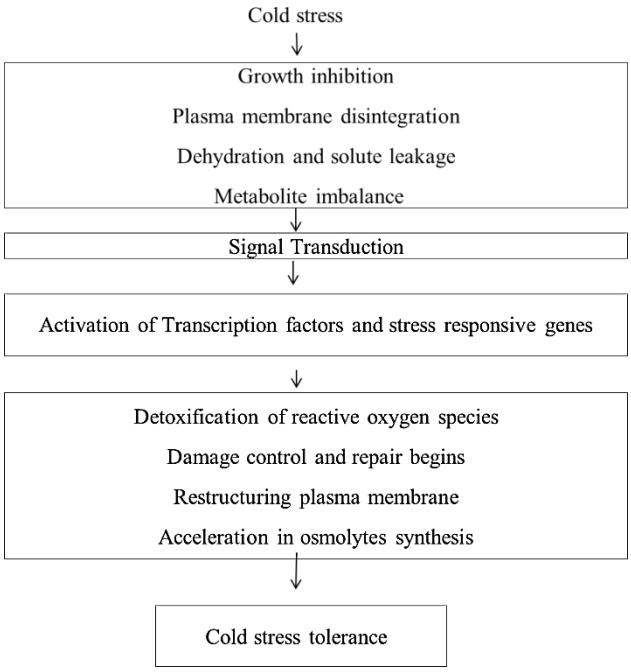

Fig. 1. Cold stress mechanism in plants. Important responses of the plants during exposure to low temperature. Source: (Yadav, 2009).

During the process of cold stress, various genes have been shown to be expressed (Deng *et al*., 2017; Hao *et al*., 2018; Kazemi-Shahandashti and Maali-Amiri, 2018; Zhuo *et al*., 2017). For example, Glutaredoxins are ubiquitous oxidoreductases, they utilize the reducing power of glutathione to reduce di-sulfide bonds of substrate proteins and adjust the cellular redox homeostasis. Tomato containing Arabidopsis *Glutaredoxin S17* gene induces chilling tolerance in the plants without adversely affecting the tomato plant's growth and development. When exposed to cold stress, a tomato plant containing *Glutaredoxin S17* gene indicated resistant to cold on account of low ion leakage, higher phytochemical efficiency of photosystem II and increased storage of soluble sugars than wild type tomato without the *Glutaredoxin S17* gene (Hu *et al*., 2015). Plants expressing *Glutaredoxin S17* gene are phenotypically different from others *i.e.,* they possess different growth and development rates before the onset of chilling stress (Hu *et al*., 2015).

When cytoplasm of cell become dehydrated due to cold stress then *LEA 25 protein* (late embryogenesis abundant protein) which are heat stable glycine rich protein plays important role in cold stress tolerance by protecting the proteins and prevent the protein aggregation as well as stabilizing the plasma membrane. Many of these cold proteins with recognized functions could help in tolerating the low temperature. The *LEA 25 protein* gene which is present in the barley contains a group-III LEA 25 protein which functions in low temperature during the process of cold acclimation. This gene is expressed in the aleurone layers at the end of embryogenesis, and also at seedling stage in a response to cold stress (Bentsinka and Koornneef, 2008). Though, there is no direct indication that *LEA 25 protein* expression during cold acclimation contributes to cold stress tolerance. Recent studies suggest that the *LEA 25 protein* gene gives tolerance to dehydration stress. It is also reported that *LEA 25 protein* gene in rice give tolerance to dehydration and salinity (Babu *et al*., 2004). The role of *LEA 25 protein* in inducing dehydration and freezing tolerance has been well established (Battaglia and Covarrubias, 2013). Recent studies showed that transformation of *LEA 25 protein* gene of tomato in yeast develop the tolerance against freezing and salinity (Gao and Lan, 2016). Interestingly, it has been found that this protein did not convey tolerance to the increased quantity of sorbitol, representing that the *LEA 25 protein* do not function under low water potential. Irrespective of all these, *LEA 25 protein* is considered as the best gene which can tolerate low temperature. Recent studies have shown that insertion of *LEA 25 protein* gene in wild tomato improve the activity of peroxidase and ascorbate peroxidase, alleviate lipid peroxidation and slow down the storage of hydrogen peroxide and chilling induced plasma membrane damage and thus improve overall cold tolerance (Sharma *et al*., 2016).

The main purpose of this study was to check the expression of two important genes *i.e., LEA 25 protein* and *Glutaredoxin S17*. The objectives of current research were: (1) To check the differential expression of two genes in tomato under cold stress, and find which gene perform well in cold stress and (2) to find which of the two tomato varieties is less prone to cold stress.

Materials and Methods

Study location: The present study was carried out at the Laboratory of Biotechnology Department, COMSATS University Islamabad, Abbottabad campus.

Tomato seeds collection and germination: Seeds of two tomato varieties *i.e.,* Roma and Rio Grande were collected from seed bank of National Agriculture Research Center, Islamabad. Seeds of both varieties were surface sterilized in laminar flow hood prior to sowing on MS medium (Murashige and Skoog, 1962) in magenta boxes. To break the seed dormancy, seeds were dipped in the distilled water in falcon tubes for 24 hours. Afterwards, seeds were washed with 70% ethanol in falcon tube for one minute and later with 7% (w/v) sodium hypochloride for approximately 5 minutes. After these handlings, seeds were washed five times with the same volume of doubled distilled water for five minutes. After this, sterilized filter paper was used for the drying of seeds before sowing on the MS medium. Seeds of both varieties *i.e.,* Roma and Rio Grande were germinated by placing five seeds in a magenta box containing MS medium and transferring to growth chamber with 25 ± 1°C temperature for 16/8 hours light and dark period. There were four boxes per each tomato variety. The germinated seeds were counted on weekly basis.

Cold stress treatments: After one month of seeds germination, cold stress was applied to the seedlings by subjecting them to three different temperature regimes *i.e.,* 15°C, 10°C, and 4°C for 10 hours. The refrigerator was used to apply 4°C treatment and other temperature treatments were applied via incubators by adjusting the required temperatures.

Molecular analysis

Leaves harvesting: Leaves of one-month old tomato seedlings were removed with the help of autoclaved scissor and forcep and directly froze in liquid nitrogen in order to stop the enzymatic activities. These leaves were placed into the zipper bags and were retained in -80°C freezer.

RNA extraction from leaves: CTAB (Cetyl trimethylammonium bromide) method of RNA extraction was used for the isolation of total RNA from the fresh leaf samples. About 250 mg of tomato leaves were collected in the sterilized pestle and mortar and ground into the fine powder in liquid nitrogen. Samples were placed in the sterile eppendorf tube in which 600 µl of CTAB extraction buffer was also added mixed slowly and the tube was then placed in the ice for 30 minutes at 65°C. Next to incubation, 600 µl of chloroform isoamyle alcohol was added into the tube and then centrifuged at 11000 rpm for 15 min at 4°C. After centrifugation, the supernatant was transferred to the fresh eppendorf and same volume of chloroform isoamyle alcohol was added and centrifuged again as done before. In the sterile eppendorf supernatant was collected and then equal volume of chilled lithium chloride was further added and placed in the -20°C for overnight. On next day, the samples were again centrifuged at 11000 rmp for 15 minutes at the constant temperature of 4°C. After centrifugation, the supernatant was discarded and pellet was washed with 70% ethanol and dried for half an hour. The pellet was dissolved in 50 µl DEPC water and placed at -20°C. After extraction RNA was stored at -80°C.

RNA quantification: After extraction, quality of the RNA was confirmed on 1% (w/v) agarose gel. 1% agarose gel was made for visualization of RNA by dissolving the 0.3 g of agarose in 30 ml 1xTBE. The solution of agarose was heated in an oven for about one minute so that it completely dissolves in buffer and become transparent. Later, it was left to cool down and, 6 µl ethidium bromide was added. Gel was poured in the gel electrophoretic tray and comb was introduced to make appropriate wells for filling RNA samples. RNA quantity in different samples was analyzed with the help nanodrop (Titertek berthold Colibri Spectrometer S/N 0638).

Designing of primers: The primers for two genes (*LEA 25 protein* and *Glutaredoxin S17)* were designed according to their sequences present on the NCBI data base. Software "Primer 3" was used to design the required primers (Table 1).

Complementary DNA (cDNA) synthesis: From RNA samples, cDNA was synthesized by using Topscript cDNA synthesis kit. From each sample total reaction volume of 20 µl was made. First, 2 µl of RNA along with 1 µl oligo (dT) primer and 11.5 µl nuclease free water was heated at 72°C for five minutes. Samples were placed on ice for chilling. After this, 2 µl 10x topscript RT buffer, 1 µl reverse transcriptase enzyme, 2 µl dNTPs mixture, and 0.5 µl RNAase inhibitor were added in each sample. RT reactions were placed at 50°C for 60 minutes and then heated at a temperature of 95°C for five minutes. The following reagents were contained in reaction mixture and the volume of total reaction was 5.5 µl. RT buffer =2 µl; dNTP=2 µl; Reverse Transcriptase =1 µl; RNase inhibitor =0.5 µl.

Polymerase Chain Reaction (PCR) and gel electrophoresis of PCR product: Total PCR reaction volume was 10 µl and reaction mixture contained 1µl cDNA, 1 µl of each forward and reverse primer, 5 µl master mix, and 2 µl of doubled distilled water. The PCR program was as denaturation (94°C, 5 min), 35 cycles of amplification, denaturation (94°C, 30 sec), annealing temperature (57.35°C, 45 sec), elongation (72°C, 60 sec) and final extension at (72°C for 10 min). To analyze the PCR products, 2 µl loading buffer was added to 8 µl of PCR product and was run on 1.5% gel. The agarose gel was run at 100 voltage for about 45 min. The amplicon bands were observed under UV light using gel documentation system.

Phylogenetic studies of selected genes: The phylogenetic studies were performed with "CLUSTAL Omega" software. For this purpose, first the protein sequences of different crops were retrieved using NCBI data base. Then these sequences were aligned with CLUSTAL Omega software and phylogenetic trees were constructed using the phylogeny.fr software.

Table 1. Primers used for the amplification of the target genes.

Gene	Forward primer	Reverse primer	Amplicon size (bp)
Lea 25 protein (*NM_001309385*.1)	5' CATGCAGCTGAAAAACAAGG 3'	5' TAAAAGCCAACACCCACACA '	200
Glutaredoxin S17 ((*LOC101268063*)	5' ATGCAAAAGAGCGGTGAGTT '	5' ATCCAAAATCAACCCCTTCC 3'	199

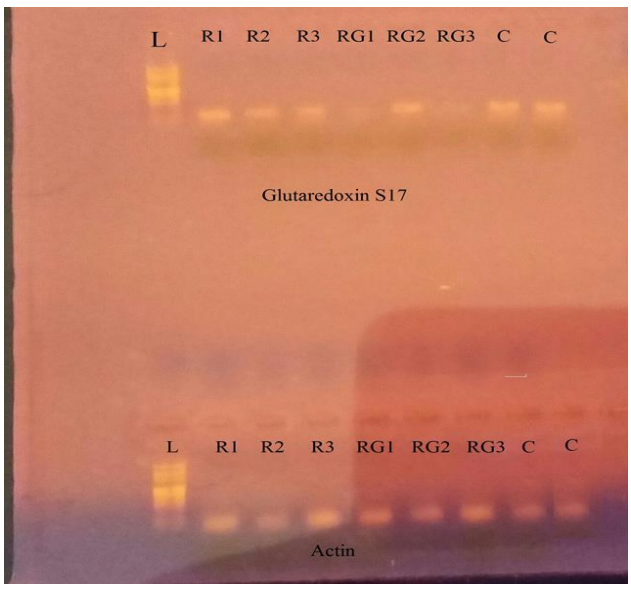

Fig. 2. Expression of *Glutaredoxin S17* gene under various cold treatments *i.e.,* 4ºC, 10ºC, 15°C of Roma and Rio Grande varieties. L: DNA ladder. In lower portion expression of *actin* is shown which was equally expressed in all samples. R1 is Roma variety at 4°C cold stress, R2 is Roma at 10°C, R3 is Roma at 15°C, whereas RG1, RG2 and RG3 are Rio Grande varieties at 4, 10, and 15°C respectively. C is a control.

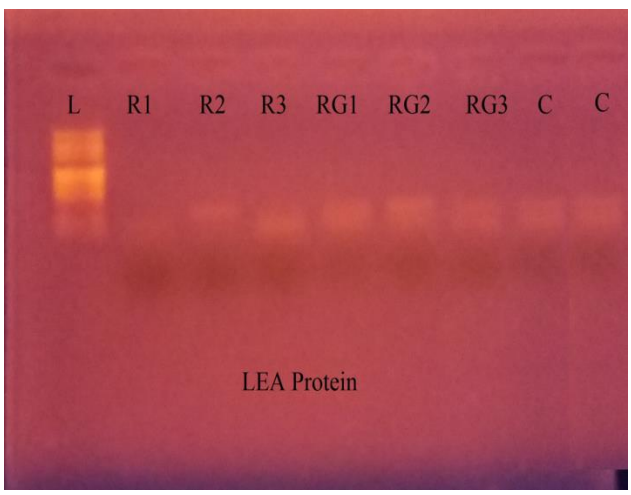

Fig. 3. Expression of *LEA 25 protein* gene in Roma and Rio Grande varieties under induced cold conditions *i.e.,* 4°C, 10°C and 15°C. L: DNA ladder. R1 is Roma variety at 4 °C cold stress, R2 is Roma at 10°C, R3 is Roma at 15°C, whereas RG1, RG2 and RG3 are Rio Grande varieties at 4°C, 10°C and 15°C respectively. C is a control.

Results

Molecular analysis

Quality and quantity of RNA: The total RNA from cold treated tomato plantlets was analyzed through gel electrophoresis. In every sample, two bands appeared. These two bands were of ribosomal RNA because it is 80% of total RNA, whereas mRNA and tRNA are less in quantity so their bands cannot be seen. The lower band which is lighter in weight is 18s subunit and upper band which is higher in weight is 28s subunit of rRNA. The clear bands of gel showed that RNA is of good quality without any degradation. RNA quantity was measured by nanodrop (Titertek Berthold Colibri Spectrometer S/N 0638).

Expression analysis of cold stress genes: The expression of two cold stress related genes *i.e., Glutaredoxin S17* and *LEA 25 protein* was analyzed through RT PCR using cDNA as a template.

Expression analysis of *Glutaredoxin S17*: RT PCR was done to find out the expression of *Glutaredoxin S17* gene under different cold stress conditions by using specific primers *i.e.,* 5' ATGCAAAAGAGCGGTGAGTT 3' (forward) and 5' ATCCAAAATCAACCCCTTCC 3' (reverse). As a control, the *actin* gene was also run. The expression pattern of *Glutaredoxin S17* and *Actin* gene is given in (Fig. 2.) From the results it is clear that expression of *Glutaredoxin S17* gene can be seen in all the samples but very low expression was found in RG_1(Rio Grande at 4 °C) and RG_3 (Rio Grande at 15°C) as shown in Fig. 2.

Expression analysis of *LEA 25 protein* gene: RT PCR was done to find out the expression of *LEA 25 protein* gene under different cold stress conditions by using specific primers *i.e.,* 5' CATGCAGCTGAAAAACAAGG 3' (forward) and 5' TAAAAGCCAACACCCACACA 3' (reverse). As a control, the *actin* gene was also run. The expression pattern of *LEA 25 protein* gene is given in (Fig. 3.) From the results it was found that *LEA 25 protein* gene is expressed in all the samples but the expression of this gene was very low as shown in Figure 3.

In silico characterization of selected genes

Multiple sequences alignment of *Glutaredoxin S17* gene: For this purpose, first of all sequences of *Glutaredoxin S17* gene from five different plants were aligned. The results of this alignment are given in supplementary File S1.

Phylogenetic tree of *Glutaredoxin S17* gene: Protein sequence of *Gutaredoxin s17* gene was blasted to check the similarity with related species *i.e., Arabidopsis thaliana*, *Brassica napus*, *Capsicum annum* and *Solanum tuberosum*. *Brasica napus* and *Arabidopsis thaliana* are in the cluster close to the tomato *Glutaredoxin S17*. While *Solanum tuberosum* and *capsicum annum* are least similar to tomato because they fall in a distant cluster (Fig. 4).

Multiple sequences alignment of *LEA 25 protein* gene: Multiple sequences alignment of *LEA* 25 *protein* gene was done with sequences from the same five different plants as performed for the *Glutaredoxin S17* gene (File S1).

Phylogenetic tree of *LEA 25 protein* gene: Protein sequence of *LEA 25 protein* gene was blasted, and phylogenetic tree was made. The phylogenetic tree showed three clusters (Fig. 5). *Solanum tuberosum* is in the middle cluster. *Arabidopsis thaliana* and *Brassica napus* fall in the same cluster while *Solanum lycopersicum* and *Capsicum annum* fall in the same cluster. The tomato *LEA 25 protein* showed high similarity with *Capsium annum* and *Solanum tuberosum* as compared to the *Brassica napus* and *Arabidopsis thaliana*.

Fig. 4. Phylogenetic tree of *Glutaredoxin S17* gene.

Fig. 5. Phylogenetic tree of *LEA* 25 *protein* gene.

Discussion

Various environmental stresses in nature affect the growth and productivity of the tomato plants. As a result, food production is decreasing day by day due to the effects of numerous abiotic constraints (Awais *et al.,* 2018; Arif *et al.,* 2019). Therefore, reducing yield losses is a chief concern for all over the world to manage with increased food needs. Cold stress is considered as one of the chief abiotic stress and badly affects the plant productivity and growth (Chen *et al*., 2014). Cold is the most important environmental stress for plants that confines the productivity and quality of economically significant plants like tomato. Plants which are grown in moderate areas are naturally tolerant to low temperature, and this is called as cold acclimation (Thomashow, 2010). Process of cold acclimation is related with improved antioxidative activity of enzymes, changes in composition of lipid membrane, changes in expression of genes, cellular organelles modifications and improved stiffness of cell walls (Byun *et al*., 2014). Though, some plants bear certain temperatures but cannot continue to survive because fluid nature of plasma membrane become condensed and changed the lipid membrane structure, which finally causes ion leakage from the membrane.

High quantity of reactive oxygen species in the cell, harm the lipids, proteins, and nucleic acids. Hence, cold stress tolerance must be developed by plants. Temperature requirements of each plant are unique, which are optimal for its appropriate development and growth. A unique set of temperatures, which are optimal for one plant, may not be suitable for other plants. Plants which are inherent of hot environment, when exposed to low temperature exhibit symptoms of injury. Maize (*Zea mays*), cotton (*Gossypium hirsutum*) and tomato (*Solanum lycopersicum*) are plants which exhibit chilling injury at low temperature. Temperature lower than 10–15°C is dangerous for these plants and they are disrupted physically and show symptoms of injury. Numerous physical symptoms which appear in plant just because of low temperature are wilting, necrosis and leaf expansion. The success of many crops is based on the capacity of plant to survive in the low temperature at the end of spring or early days of autumn. Thus, the ability of plants to survive in low temperature is very beneficial for the economy of country.

All over the world including Pakistan more area has been documented as unproductive due to cold stress. Genetic improvement of tomato is influenced by the background of germplasm and depends on the stable improvement of genotypes suitable to restricted environments (Gerszberg *et al*., 2015). Cold stress badly disturbs growth, and also causes changes in the morphology, physiology and alter biochemical process of plants. Significant crop losses are due to cold stress which disturb plant growth (Chinnusamy *et al*., 2007). Plants differ greatly in their ability to bear cold. Tomato which is from tropical origin is unable to bear low temperature and its metabolism is disrupted when it faces temperature of 0-12°C.

Gene expression analysis of two cold stress related genes *i.e*., *Glutaredoxin S17* and *LEA 25 protein* was carried out in under current study. This expression analysis was carried out under different cold temperature conditions. As *Glutaredoxin S17* gene belongs to global and conserved heat stress-responsive factor and it gives cold tolerance to both yeast and plant species. Hence, expression of *Glutaredoxin* S*17* in tomato modify and re-program a large number of cold-responsive components to reduce the damage caused by cold. *LEA* 25 *proteins* genes play pivotal role in membrane stabilization and also prevent the protein aggregation (Boucher *et al*., 2010). It has been seen that these genes are involved in freezing tolerance as they mitigate the damaging effects of dehydration which are due to freezing. In current results expression of *Glutaredoxin S17* was more pronounced than *LEA 25 protein* gene under induced temperature stress conditions. From the results it is also clear that expression of *Glutaredoxin S17* gene can be seen in all the samples but very low expression was found in RG_1 (Rio Grande at 4°C) and RG_3 (Rio Grande at 15°C) and in Roma variety it was equally expressed in all the samples under different stress conditions whereas expression of

LEA 25 protein gene was very low in both varieties under different temperature conditions. As low temperature stress increases the accumulation of reactive species which include hydrogen peroxide that result in severe damage to membrane, proteins and lipids, thus plants expressing *Glutaredoxin S17* gene showed less accumulation of hydrogen peroxide and as a result no damage related to membrane is detected in these plants. Also plants expressing *Glutaredoxin S17* gene affects the activity of ROS scavenging enzymes in tomato plant (Hu *et al*., 2015). So overexpression of this gene in tomato plants may results in enhanced tolerance to chilling stress (Zhang *et al*., 2004).

Conclusions

The expression study of *Glutaredoxin S17* and *LEA 25 protein* genes revealed that *Glutaredoxin S17* gene performed well compared to *LEA 25 protein* in cold stress. This gene expression was more pronounced at 4°C. However, further research, such as functional study, is needed to confirm the role of this *Glutaredoxin S17* gene in cold stress of tomato crop.

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