

Glycine betaine protects tomato (*Solanum lycopersicum*) plants at low temperature by inducing fatty acid desaturase7 and lipoxygenase gene expression

T. Karabudak · M. Bor · F. Özdemir ·
İ. Türkan

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Abstract Cold stress is among the environmental stressors limiting productivity, yield and quality of agricultural plants. Tolerance to cold stress is associated with the increased unsaturated fatty acids ratio in the plant membranes which are also known to be substrates of octadecanoid pathway for jasmonate and other oxylipins biosynthesis. Accumulation of osmoprotectant, glycine betaine (GB) is well known to be effective in the protecting membranes and mitigating cold stress effects but, the mode of action is poorly understood. We studied the role of GB in cold stress responses of two tomato cultivated varieties; *Gerry* (cold stress sensitive) and *T47657* (moderately cold stress tolerant) and compared the differences in lipoxygenase-13 (TomLOXF) and fatty acid desaturase 7 (FAD7) gene expression profiles and physiological parameters including relative growth rates, relative water content, osmotic potential, photosynthetic efficiency, membrane leakage, lipid peroxidation levels. Our results indicated that GB might have a role in inducing FAD7 and LOX expressions for providing protection against cold stress in tomato plants which could be related to the desaturation process of lipids leading to increased membrane stability and/or induction of other genes related to stress defense mechanisms via octadecanoid pathway or lipid peroxidation products.

Keywords Tomato · Chilling stress · FAD7 · LOX · Lipid peroxidation · PUFAs

Introduction

Low temperature is among the major environmental stressors which limit plant growth and productivity. Most of the crop plants including tomato are severely affected by chilling injury when growth temperature is below 10 °C and the development of unique mechanisms to cope with chilling and freezing temperatures is vital for plants [1]. Alteration of the lipid composition of the cell membrane is known to be associated with low temperature tolerance since cell membranes are the first sites of injury [2]. Chilling stress affects membranes due to the impairment of membrane permeability by the transition of lipids from liquid crystalline phase to gel phase. Chilling resistant plants have more unsaturated fatty acids in their membranes due to the increased activities of fatty acid desaturase enzymes [2]. Besides the well known structural roles of fatty acids in plant membranes; alleviated fatty acid composition of the membranes was found out to be a common response under different environmental stresses.

Polyunsaturated fatty acids (PUFAs) are important constituents of plant cell membranes, particularly chloroplastic membranes and alterations in their composition affect different cellular and physiological processes such as cold adaptation and survival, ion channel modifications, pathogen defense, pollen formation, chloroplast development [3, 4]. There are two different PUFA biosynthetic pathways; trienoic acids (18:3) are generally synthesized through the prokaryotic and hexa-decatrienoic (16:3) acids are synthesized through the eukaryotic pathways [2, 5]. Tolerance to low temperature stress is associated to increased unsaturated fatty acid composition of the membranes and tolerant plants have higher activities of desaturases when they are subjected to low temperature degrees [2, 6]. PUFAs are also the substrates for synthesis of

T. Karabudak · M. Bor (✉) · F. Özdemir · İ. Türkan
Department of Biology, Science Faculty, Ege University,
Bornova, 35100 Izmir, Turkey
e-mail: melike.bor@ege.edu.tr

jasmonate and other oxylipins which have unique roles in development and stress responses in plants [3, 7].

Plant membranes are the key components of stress responses not only by being the sites of signal perception but also by being the source of important signaling molecules [8, 9]. Alterations in the membrane lipid composition of plants in relation to octadecanoid pathway were also reported in response to various stresses such as wounding, drought, salt, irradiance, high and low temperature [2, 10, 11]. Fatty acids are believed to be targeted into the octadecanoid pathway where they are converted to active signaling molecules such as jasmonic acid (JA), which is an inducer of gene expression [9]. JA is synthesized from trienoic acids by the activity of lipoxygenases (LOXs) including 9- and 13-LOXs and LOX pathway products are speculated to be important regulators of pathogenesis and oxidative stress related genes in plants [8]. Shi et al. [10] reported that JA signaling via octadecanoid pathway participated in the cold-induced transcription of three fatty acid desaturase (FAD) encoding genes in *Arabidopsis thaliana*.

FADs catalyze the introduction of double bonds into the aliphatic tails of fatty acids which affects plant responses against a variety of stresses via the enhancement of membrane fluidity [12]. It has been postulated that FAD activities are also important for the fine-tuning of the balance between tolerance and susceptibility to abiotic and biotic stresses in plants [12–15]. Over-expression of FAD7 in rice led to enhanced JA levels and up-regulation of FAD7 were reported under the effects of different stresses including wounding [12, 16, 17]. Moreover, over-expression of FAD7 gene resulted in higher trienoic acid levels in tomato plants and increased their tolerance to chilling stress. Shi et al. [10] also reported that induction of three omega-3 FAD genes including FAD7 in relation to octadecanoid pathway in response to low temperature in *Arabidopsis*. JA biosynthesis has different aspects in model plant *A. thaliana* and in crop plant tomato as addressed by Sun et al. [17]. Identification of Spr2 gene encoding omega fatty acid desaturase which is involved in JA biosynthesis in tomato plant has provided a new site within this pathway. Loss of function of a FAD7 homolog in *Solanum lycopersicum* has increased the plant's susceptibility to biotic stresses in relation to inability of the plant to synthesize JA [14, 18]. However, to date the direct link between JA pathway and regulation of unsaturated fatty acid levels within the cell is still not clear and more work is required to understand the overlapping or correspondence of plant defense mechanisms under different environmental stressors.

Efficient enhancement of tolerance to a variety of stresses including cold stress is associated with the maintenance of stable leaf water status. Plants have evolved different strategies to provide and retain water under

unfavorable conditions and the accumulation of compatible solutes such as, polyols, sugars, aminoacids, betaine, is among the best known protective mechanisms [19, 20]. The compatible solute, quaternary ammonium compound, Glycine betaine (GB) is well characterized and accumulation of GB is correlated with the increased stress tolerance in several plant species. On the other hand, exogenous application of GB or introduction of genes associated with GB biosynthetic pathway to non-GB synthesizing plants have potential in enhancing the tolerance against various environmental stresses including cold stress [20]. It has been suggested that GB acts not only as an osmoprotectant but also as a membrane stabilizer contributing to the stabilization and integrity of cellular membranes when plants are subjected to low temperature conditions [20]. However, the mode of action of this protective mechanism on membranes remains to be unclear and GB induced expression of cold-regulated genes were hypothesized to be more related to the genes responsible for the reduction of reactive oxygen species formation under cold stress conditions [21, 22].

Maintenance of membrane integrity and increase in membrane fluidity is important for low temperature tolerance and the role of GB in improving cold stress responses in different agricultural plants even in non-GB synthesizing plants such as, tomato was found out to be dependent on the protection of membranes as emphasized by previous studies. We hypothesized that GB not solely but in a part can induce the expression of genes encoding enzymes responsible for membrane lipid re-organization and/or modification under low temperature stress conditions. Therefore we studied the role of GB in cold stress responses of two *S. lycopersicum* cultivated varieties; *Gerry* (cold stress sensitive) and *T47657* (moderately cold stress tolerant) and compared the differences in lipoxygenase (TomLOXF) and fatty acid desaturase 7 (FAD7) gene expression profiles. We used semiquantitative RT-PCR approach for the expression analysis of LOX and FAD7 genes in relation to other physiological parameters including relative growth rates, relative water content, osmotic potential, photosynthetic efficiency, membrane leakage, lipid peroxidation, regarding stress effects and mitigation of these effects in control, GB treated, cold stressed and GB + cold stressed groups.

Materials and methods

Plant material and growth conditions, GB treatment and cold stress application

Solanum lycopersicum L. cvs. *Gerry* (cold sensitive) and *T47657* (moderately cold tolerant) were provided from

Syngenta. Seeds were sterilized with 10 % chloride solution for 10 min and rinsed with sterile distilled water five times. Sterilized seeds were germinated for 4 days in dark at 25 °C on sterile filter paper in petri dishes. After germination seedlings were planted in 13.5 cm diameter plastic pots filled with perlite (1 plant per pot) and grown at 16/8 h light/dark photoperiod at 25/20 °C, relative humidity of 60–70 %, photosynthetic photon flux density of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 weeks and watered regularly with ½ strength Hoagland solution. Plants were divided into four groups (control, GB treated, cold stressed and GB + cold stressed) each contained 20 plants and repeated in 2 independent series. GB treatment was carried out according to [20] 10 mM GB solution containing 0.005 % (v/v) Tween-20 was sprayed on to the leaves of plants in the GB and GB + cold divided groups and control plants were sprayed only with water. Following the foliar applications plants were kept at dark for 24 h and then the groups divided for cold stress were transferred to a growth chamber which was set at 16/8 h light/dark photoperiod at 10/5 °C, relative humidity of 60 %, photosynthetic photon flux density of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for cold stress application for 5 days. Control and GB treated plants were grown at 16/8 h light/dark photoperiod at 25/20 °C, relative humidity of 60–70 %, photosynthetic photon flux density of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 days. Throughout the experiment all measurements were performed on the fourth leaf.

Determination of relative growth rate

Five plants for each group were used for growth analyses and sampled as shoot and root fractions on the 0, 1 and 5th days of cold stress treatment. Root, shoot fresh (FW) and dry weights (DW) were measured. For DW analysis, root and shoot samples were dried at 70 °C for 72 h. and then weighed. The relative growth rates (RGR) of the seedlings were calculated according to Hunt et al. [23].

Measurement of chlorophyll fluorescence

Leaves of seedlings from three individual plants per treatment were used for chlorophyll fluorescence analysis. Prior to fluorescence measurements, a 1 cm² circular surface of the upper face of an excised leaf was dark adapted for 30 min using a dark leaf clip. The basal non-variable chlorophyll fluorescence level (F_0), the maximal fluorescence induction (F_m), variable fluorescence (F_v) and the ratio of F_v/F_m were determined by a Plant Efficiency Analyser (HANSATECH Inst. Ltd., Norfolk, UK). Photochemical efficiency of PS-II (maximum quantum yield F_v/F_m) was figured.

Lipid peroxidation

Lipid peroxidation was determined by estimating the TBA-reactive substances (TBARS) content in 0.5 g leaf fresh weight according to Madhava and Sresty [24]. TBARS are products of lipid peroxidation by thiobarbituric acid reaction. The concentration of TBARS (nmol g FW⁻¹) was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM⁻¹ cm⁻¹.

Leaf osmotic potential (ψ_π)

Leaf osmotic potential was measured on the 1st and 5th days of the stress treatments by Vapro Vapor pressure Osmometer 5520. The data were collected from three leaves per replicate. These results were converted to MPa according to Santa-Cruz et al. [25], by multiplying coefficient of 2.408×10^{-3} .

Relative water content (RWC)

Leaf samples which were collected at the 1 and 5th days of cold stress were used for RWC assay. After fresh weight determination, these samples were immersed in ddH₂O for 5 h. The turgid leaf samples were quickly dried prior to the determination of turgid weights. Dry weights of leaves were determined after oven-drying at 70 °C for 72 h. RWC was calculated according to Smart and Bingham [26] using the following formula:

$$\text{RWC (\%)} = \frac{[(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})] \times 100.}$$

Membrane ion leakage

Membrane ion leakage was determined according to Scotti et al. [27] and the electrical conductivity of the samples was measured by WTW Inolab Conductivity Meter 720. Three leaf discs of 1 cm diameter were excised per sample and X_0 values were measured. Discs were immersed in vials containing 20 mL ddH₂O for 2 h and X_i values were determined. X_t values were measured after the leaf discs were autoclaved. The percentage of membrane ion leakage was calculated using the given formula:

$$\text{Membrane ion leakage (\%)} = \frac{[(X_i - X_0) / (X_t - X_0)] \times 100.}$$

Glycine betaine determination

GB contents were determined by HPLC (Shimadzu-VP101, Japan). GB extraction from plant tissues and HPLC

analysis were performed according to Subbarao et al. [28] and Bessieres et al. [29] with slight modifications. Leaves (0.5 g) were frozen in liquid nitrogen, homogenized with 4 mL water:chloroform:methanol (3:5:12 v/v/v) solution and incubated at +4 °C overnight. Extraction mixture was kept at 4 °C overnight; 1 mL of the upper methanol phase was taken and purified by using BioRad AG1-X8 ion exchange resin. Ion exchange resin was removed by centrifugation (5,000×g, 10 min) and supernatant filtered through a 0.45 µm membrane filter before subjection to the HPLC system. Nucleogel RP column (100-8, 300 × 7.7 mm) preceded by a guard column was used and mobile phase (15 mM KH₂PO₄) was delivered by an analytical isocratic pump at a flow rate of 0.8 ml min⁻¹ at 70 °C. GB content of the samples was detected by UV detector at 195 nm and quantification was done by comparing the peak surface areas with those obtained with pure GB standards.

LOX and FAD gene expression analysis

Total RNA was isolated from the leaves (100 mg) of the plants using Purelink RNA Mini Kit (Ambion) following the manufacturer's protocol. Quantity, quality and integrity of each RNA sample was assessed spectrophotometrically with Biophotometer (Eppendorf) and by visualizing RNA on an ethidium bromide-stained agarose gel. Samples were treated by DNaseI and 10 µg RNA was reverse transcribed using Qscript cDNA synthesis kit (Quanta). PCR amplifications of cDNAs with LeFAD7 (GenBank accession no: AY248742) F: 5'-ATTGTTGGGCATTGAGGGTA-3' R: 5'-TACTTCTATTTCAAGGAACC-3'; TomLOXF (GenBank accession no: FJ617476) F: 5'-TCATCACAATCCACAAAAACCC-3' R: 5'-AGGACCGCGTAAATGGTGTT-3'; LeAC (GenBank accession no: U60482.1) F: 5'-GATGCTCCAAGGGCTGCTT-3' R: 5'-AGTCCAGCACAAATACCTGGC-3' were performed at 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min respectively for 35 cycles. RT-PCR products were loaded on 1 % (w/v) agarose gel with 0.5 µg mL⁻¹ EtBr. Stained gels were photographed with Vilber Lourmat gel imaging system and then analyzed with Bio-Profil Bio-1D Windows Application V11.9 software package (Vilber Lourmat, Marne la Vallée, France).

Statistical analysis

All analyses were done on a completely randomized design and data obtained were subjected to analysis of variance (ANOVA) and the mean differences were compared by lowest standard deviations (LSD) test. Each data point represented in the figures was the mean of six replicates ($n = 6$) for relative water content, osmotic potential, photosynthetic efficiency, membrane ion leakage, lipid peroxidation and glycine betaine contents; ten replicates

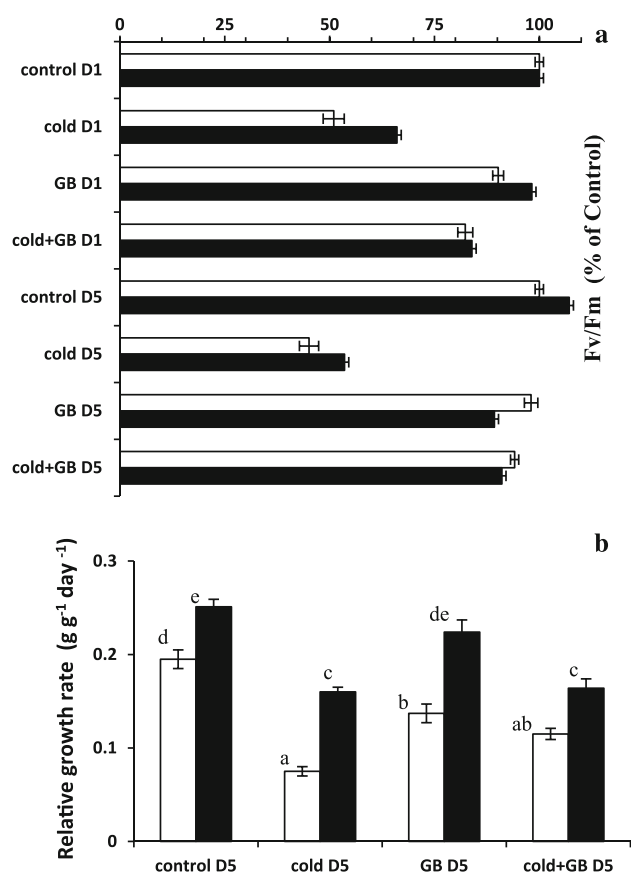


Fig. 1 The effect of cold stress treatment on F_v/F_m (a) and relative growth rate (RGR) (b) of *Solanum lycopersicum* L. cv Gerry (open bars) and T47657 (dark bars) during the experimental period D1 (day 1) and D5 (day 5). Mean \pm SD based on six replicates ($n = 6$) for F_v/F_m and ten replicates ($n = 10$) for RGR are presented

($n = 10$) for relative growth rates. Comparisons with P values < 0.05 were considered significantly different. In all the figures the spread of values is shown as error bars representing standard errors of the means.

Results

Photosynthetic efficiency was measured as the F_v/F_m values and compared by the % of difference in the cold stressed, GB treated and GB + cold stressed groups' F_v/F_m values to that of the control groups' values. Photosynthetic efficiency was affected in both varieties by cold stress treatment on days 1 and 5, and the cold sensitive variety (Gerry) exhibited a higher degree of decline as compared to the cold tolerant variety (T47657) (Fig. 1a) on both days. GB treatment alleviated the inhibitory effects of cold stress on photosynthetic efficiency significantly in both varieties and as F_v/F_m values recorded were close to that of the control values in GB + cold stressed Gerry and T47657

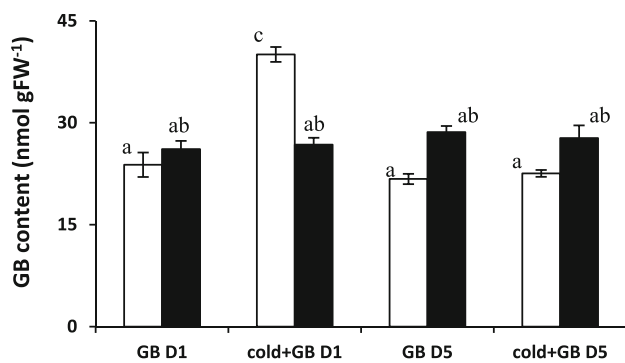


Fig. 2 GB content in the leaves of *Solanum lycopersicum L. cv Gerry* (open bars) and *T47657* (dark bars) plants after exogenous GB treatment during the experimental period D1 (day 1) and D5 (day 5). Mean \pm SD based on six replicates ($n = 6$) are presented

plants. Cold stress inhibited growth of both varieties as indicated by the decrease in relative growth rates (Fig. 1b) and the decline was significantly remarkable on *Gerry* variety as compared to that of *T47657*. RGR values decreased 2.6 and 1.6-fold in cold stressed groups on day 5 in *Gerry* and *T47657* varieties respectively. When GB treated groups were subjected to cold stress better RGR values were recorded as compared to that of cold stressed group in *Gerry* variety. On the other hand RGR values were almost the same in cold and GB + cold stressed groups of *T47657* variety. Decline in RGR values were up to 1.7 and 1.5-fold in *Gerry* and *T47657* varieties respectively (Fig. 1b).

In order to confirm the uptake of foliar GB by tomato plants we have conducted an HPLC analysis to measure the endogenous levels of GB and these results were presented in Fig. 2. We have detected similar GB levels almost in all groups which were not significantly different in *Gerry* and *T47657* varieties on days 1 and 5. Endogenous GB levels were 21.73–23.82 and 26.13–28.63 nmol g⁻¹ FW in *Gerry* and *T47657* respectively. A significant difference in GB content was detected only in GB + cold stressed group of *Gerry* on day 1, which accumulated 40.06 nmol g⁻¹ FW GB.

Leaf osmotic potential and RWC were measured in order to provide information about the leaf water status of the plants subjected to cold stress and GB + cold stress. Leaf osmotic potential values were decreased in cold stressed groups of both varieties (Fig. 3a). GB treatment restored the leaf osmotic potential values almost to that of the control levels in cold stressed groups of *Gerry* and *T47657* varieties on days 1 and 5. Decline in the relative water contents of the plants under the effect of cold stress were recorded in both varieties during the experimental period (Fig. 3c). The RWC levels were almost the same in cold stressed and GB + cold stressed groups of *Gerry* and

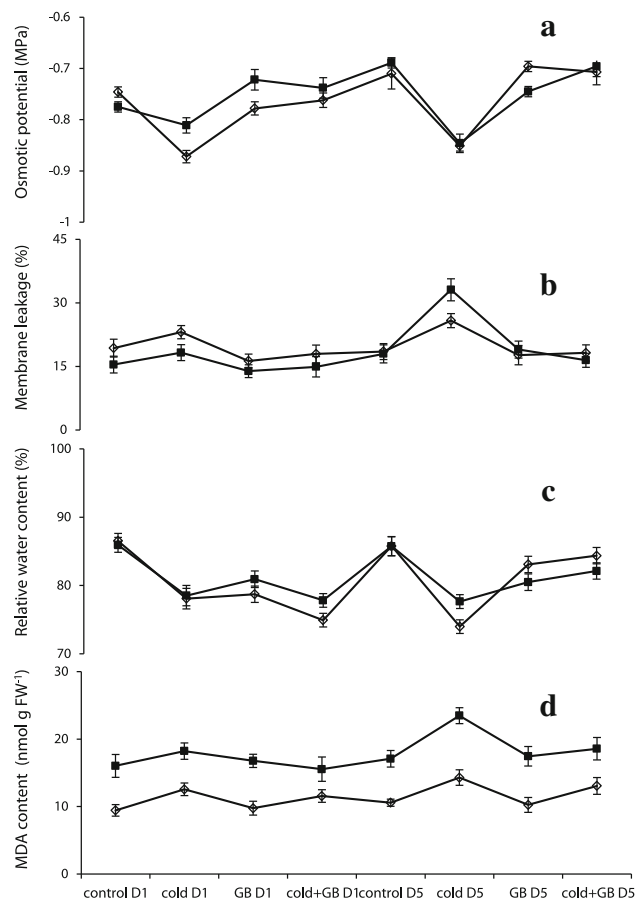


Fig. 3 The effect of cold stress treatment on leaf osmotic potential (a), membrane leakage (b), relative water content (RWC) (c) and lipid peroxidation as MDA content (d) in the leaves of *Solanum lycopersicum L. cv Gerry* (open diamond) and *T47657* (filled square) plants during the experimental period D1 (day 1) and D5 (day 5). Mean \pm SD based on ten replicates ($n = 10$) for osmotic potential, membrane leakage, RWC and MDA content are presented

T47657 varieties on day 1. However, GB treatment restored RWC values up to control levels in cold stressed groups of both varieties in the long term which was more remarkable in *Gerry* variety. Membrane leakage was increased as a consequence of cold stress in both varieties and *T47657* variety had higher membrane leakage values as compared to that of *Gerry* variety which were more prominent on day 5. GB treatment reversed membrane leakage levels to control groups' levels on cold stressed plants and this ameliorative effect was more obvious in *T47657* variety on both days (Fig. 3b).

In plant cells, membranes are the first sites of cold stress injury therefore it is important to determine the differences in the lipid peroxidation levels of the two tomato varieties under cold stress with or without GB treatment (Fig. 3d). In general lipid peroxidation levels were in good agreement with the membrane leakage, RWC and osmotic pressure results of both varieties. Lipid peroxidation levels were

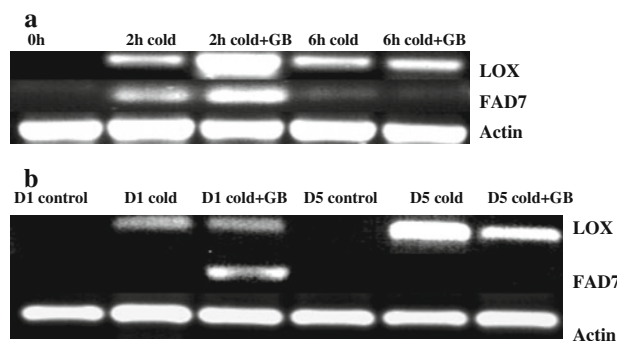


Fig. 4 Semi-quantitative analysis of TomLOXF and FAD7 gene expression by RT-PCR in the leaves of control, cold and GB + cold stressed *Solanum lycopersicum L.cv Gerry* plants. Actin gene transcripts were used as the control of RT-PCR products. Results presented are; **a** in short term; 0, 2 and 6 h and **b** in long term; 1 and 5 days respectively

increased in cold stressed groups of *Gerry* and *T47657* varieties on day 1. *Gerry* had higher MDA content as compared to the other variety and there was a significant increase in the lipid peroxidation levels (1.33-fold) as compared to control group. However, the affect of cold stress was more prominent on day 5 where the highest lipid peroxidation levels were recorded in both varieties (Fig. 3d). MDA levels increased 1.35 and 1.37-fold in cold stressed *Gerry* and *T47657* plants respectively. GB treatment improved membrane damage in *T47657* variety in response to cold stress which was more remarkable on day 5 with the MDA levels which were similar to that of control group's MDA levels.

Semi-quantitative RT-PCR was performed using primers designed for tomato LOX gene in cold stressed *Gerry* and *T47657* plants. The induction patterns were different in both plants within the short (2–6 h) and long terms (days 1 and 5) of the stress treatment. Different expression patterns were obtained in cold stress and cold stress + GB treated plants as compared to control groups where no gene induction was observed (Figs. 4a, b, 5a, b). In *Gerry* variety within the 2nd and 6th h of cold treatment, both GB treated and untreated plants showed LOX gene induction and the highest LOX expression was induced by GB treatment in the 2nd h of cold stress. On days 1 and 5, cold stress induced LOX gene expression and relative intensities were detected in GB treated and untreated plants on both days but, the latter ones had higher induction patterns (Fig. 4a, b). LOX gene expression was affected differently in *T47657* variety within the experimental period. In the short term, when *T47657* plants were subjected to cold stress, LOX gene expression was only induced in the GB treated plants within the 2nd and 6th h of the cold stress and the induction was higher in the latter one. On day 1, LOX expression was induced both in cold and GB + cold

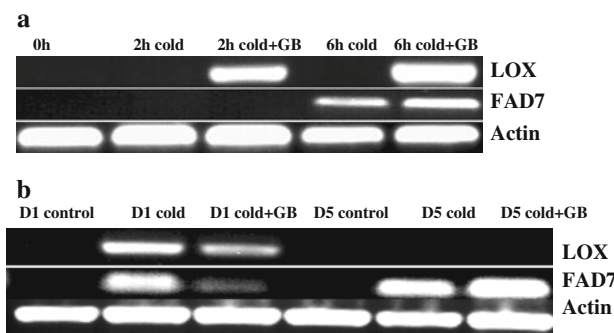


Fig. 5 Semi-quantitative analysis of TomLOXF and FAD7 gene expression by RT-PCR in the leaves of control, cold and GB + cold stressed *Solanum lycopersicum L.cv T47657* plants. Actin gene transcripts were used as the control of RT-PCR products. Results presented are; **(a)** in short term; 0, 2 and 6 h and **(b)** in long term; 1 and 5 days respectively

stressed groups however, there were no induction of LOX gene in these groups on day 5 (Fig. 5a, b).

Like LOX gene, FAD7 gene expression was differentially regulated in tomato varieties *Gerry* and *T47657* under the effect of cold stress in the short and long term (Figs. 4a, b, 5a, b). In *Gerry* variety, FAD7 gene expression was induced within the 2nd h of the cold stress and increased to a higher extent in the GB + cold stressed group (Fig. 3a). When plants were subjected to cold stress for a longer period (1–5 days), FAD7 expression was detected only in the GB treated group on day 1 (Fig. 4b). In *T47657* variety FAD7 gene expression exhibited a different pattern as compared to that of *Gerry* variety. At the 2nd h of cold stress, FAD7 expression was not detected in any group but, it was induced both in the cold and GB + cold stressed groups of *T47657* variety at the 6th h (Fig. 5a, b). The rate of induction was higher on the GB treated group at the 6th h of the cold stress. On day 1, FAD7 expression was seen only in the cold stressed group and on day 5 both cold and GB + cold stressed groups had FAD7 expression, the latter one had higher transcript intensity as compared to the others (Fig. 5b).

Discussion

Tomato is an important agricultural plant that has high sensitivity to chilling and cold stress especially at the early stages of growth. Different studies have increased our understanding about the molecular and physiological characteristics of cold stress tolerance and sensitivity and it is widely accepted that membrane fatty acids are the key components of this phenomenon as membranes are the first sites of stress injury. Expression and regulation of the genes encoding fatty acid desaturases are important for

manipulation of the fatty acids levels and turnover rates. To date, there were several reports regarding the species and genotypic variations in the fatty acid compositions and contents of plants not only for cold stress tolerance but also for other environmental stressors [1, 5, 12]. Genetically manipulated tobacco [30], *Arabidopsis* [31], and rice [32] plants for altered levels of cis-unsaturated fatty acids exhibited higher chilling tolerance. On the other hand, different degrees of chilling tolerance can be found among plant genotypes possessing same levels of unsaturated fatty acids which confirm that other components of the plant metabolism are also necessary for whole cold stress resistance in plants [33, 34].

We performed RGR analysis in order to compare the effects of cold stress in tomato varieties *Gerry* and *T47657*. Decline in RGR values were recorded as a consequence of decreased total dry weights which was to a greater extent in the sensitive variety *Gerry*. Our findings are in good agreement with earlier studies indicated the inhibition of growth under low temperatures in chickpea [35], rice [34] and tobacco [36]. Venema et al. [37] reported that relative shoot growth rate decreased in five *Lycopersicon* species during 14 days of chilling at 10 °C. GB treatment improved RGR values in variety *Gerry* however it was not effective for variety *T47657*. Same RGR values were recorded in cold and GB + cold stressed groups of this variety. The ameliorative effects of exogenous GB application on growth rates were indicated in numerous studies done with several crop species including tomato [20, 38–40]. Considering the other parameters especially the higher abundance of FAD7 transcripts and better membrane integrity in the long term we can propose that moderate cold tolerant variety had an active and effective defense against cold stress regardless of GB treatment which also provides a good way to see genotypic differences between these two varieties. This might indicate the contribution of FAD enzyme to membrane organization leading to better growth performance and photosynthetic efficiency under cold stress conditions. Since, cold stress tolerance is proposed to be associated with increased expression of several FAD genes as indicated by previous studies [13–15]. Moreover, as tomato is not a GB synthesizing plant in its nature it should have other efficient and stable ways of mitigating cold stress.

Photosynthetic efficiency is among the first parameters affected by cold stress as the maintenance of chloroplast function via thylakoid membranes and renewal and turnover rates of D1 protein are highly susceptible to low temperatures [1]. Cold stress affected F_v/F_m values of both cultivars and the rate of decrease was more remarkable on *Gerry* during the experimental period. GB treatment before cold stress alleviated this inhibitory effect equally and F_v/F_m close control groups' values were recorded in *Gerry* and *T47657*. For instance, genetically engineered rice (*Oryza*

sativa L.) with the ability to synthesize GB had more efficient photosynthesis under low temperature conditions and the authors claimed that GB protected photosystem II and decreased the level of its inactivation caused by the stress [40]. Similarly, Ariizumi et al. [34] reported that increase in the unsaturation of fatty acids improved photosynthesis rates and growth in transgenic rice plants at low temperatures. Transgenic rice plants with higher levels of cis-unsaturated fatty acids exhibited less impaired photosynthetic rates when subjected to chilling temperatures however, there was no improvement of photosynthesis at lower degrees [34]. Yu et al. [1] reported that over expression of another FAD gene; FAD3 alleviated inhibition of the maximal photochemical efficiency of tomato plants under chilling stress as compared to wild type plants.

Park et al. [20] reported that foliar GB application improved chilling tolerance in tomato plants. Even in the lowest GB concentration (1.0 mM GB) plants exhibited chilling tolerance and they proposed a threshold value of $0.09 \mu\text{mol g}^{-1} \text{FW}$ for endogenous GB level in order to provide sufficient protection against low temperature. Interestingly, we have detected lower endogenous GB levels after GB treatment as compared to their proposed threshold value. An explanation to this discrepancy would be the differences between the ages and genotypes of the plants used in both studies because the GB treatment method and concentration we have used were the same with that study. Such low levels of GB accumulation may be sufficient for mitigating the negative effects of cold stress in tomato plants as we have recorded better performance in most of the parameters we have measured in GB + cold stressed groups, even in the sensitive variety. Park et al. [20] also emphasized that lower concentrations of GB translocated to the chloroplasts were effective in providing chilling tolerance in tomato plants. Considering and comparing overall results, particularly the transcript profiles in our study it may be proposed that, maintenance of low levels of GB might be important in triggering short term cold stress responses. However, for prolonged periods of cold stress and recovery, higher accumulation of GB is needed in order to facilitate other desired effects such as, osmotic adjustment and elevation of ROS [20, 38, 41].

Membrane integrity is a good indicator of plants' tolerance or sensitivity to chilling or low temperature stress and susceptible crop plants have increased levels of membrane leakage when subjected to low temperatures [30, 35]. Our results showed that cold stress increased membrane leakage in the leaves of *Gerry* and *T47657*. However, moderate leakage levels over control groups were recorded in these plants when they were GB treated before subjected to cold stress. Significant increase in membrane leakage was recorded in chick pea plants exposed to low temperature [35]. Yu et al. [1] reported

better leaf and root membrane leakage levels during chilling stress in transgenic tomato plants over-expressing FAD3. In *Medicago sativa* L. exogenous treatment of GB has improved relative electric conductivity of plants when plants were exposed to a range of low temperatures [42]. Park et al. [20], measured ion leakage in order to assess membrane damage and reported that exogenous GB application reduced the damage remarkably in tomato plants subjected to chilling stress.

Stable leaf water status is important not only in cold stress tolerance but also in other environmental stresses affecting agricultural plants' development, growth and survival. In this study, cold stress negatively affected leaf water status of *Gerry* and *T47657* varieties and similar levels of osmotic potential, membrane leakage and RWC were detected in cold stressed groups of both varieties. Chilling tolerant maize genotypes have higher leaf water potentials and better leaf water status than chilling sensitive genotypes when exposed to low temperatures [43]. GB treatment has improved osmotic potential, membrane leakage and RWC values of GB + cold stressed plants were not significantly different than that of control groups. This was an expected result of GB accumulation in plants which were proved to be the case in different plants. For instance, Naidu et al. [44] reported that GB accumulation to higher extend in wheat plants subjected to +4 °C for 5 days has contributed to the maintenance of leaf water status at control levels.

Lipid peroxidation of the membranes and detection of degradation products such as MDA is a general concept for considering the degree of stress effect in response to different environmental stressors in plants. On the other hand, MDA is one of the electrophile species derived from fatty acid degradation of the membranes and believed to have an activity in plant defense gene expression [45, 46]. In this study, despite the fact that MDA levels increased in response to cold stress in both varieties there were significant differences between the rates of increment on daily basis. MDA levels increased to a higher extend in *Gerry* on both days while we recorded increase in MDA level on day 5 in *T47657*. Weber et al. [47] emphasized the difference between MDA and the other electrophile species-small vinyl ketones in contributing to the activation of defense gene expression in plants; the former has a role in response to abiotic stresses while the latter one strongly acts in response to biotic stress. GB treatment alleviated inhibitory effects of cold stress on membranes and as a consequence lower MDA levels were recorded in these groups of both varieties. This remedial effect of GB treatment was more apparent on *T47657* parallel to other parameters related to membrane integrity. Parallel to our results, the presence of GB and proline in maize seedlings have increased chilling stress tolerance and reduced lipid peroxidation levels [21].

FAD7 and LOX genes were not constitutively expressed in leaf tissues of control plants whereas their expression levels were changed both in cold stressed and GB + cold stressed plants in the short and long term, which indicates these genes' contribution to cold stress responses in these plants. Accordingly, the up and down regulation of these genes were different between the varieties and GB treatment was significantly effective in regulating these genes' expression. This effect was more remarkable for LOX, especially at the first 6th h of the cold stress and there was a good correlation of this gene's expression with FAD7's expression in moderately cold tolerant variety *T47657*. Comparing the transcript intensities it was clear that FAD7 expression was also induced by GB in both varieties but according to long term results in *T47657* we could say that FAD7 expression was induced in response to cold stress. Avila et al. [12] proposed a functional relation between FAD7 and JA synthesis in tomato and *Arabidopsis* plants leading to susceptibility or tolerance to aphids and other pathogens. Up-regulation of FAD genes was found out to be responsible for enhancing JA levels and inhibiting SA accumulation in *Arabidopsis* and potato [12]. FAD7 could potentially influence SA signaling indirectly by influencing the accumulation of reactive oxygen species; Mene-Saffrane et al. [48] proposed that trienoic fatty acids can have a role as antioxidants where in *Arabidopsis fad3fad7fad8* mutant had high levels of ROS. This could also be the reason for different expression patterns in LOX and FAD7 transcripts of moderately cold stress tolerant variety *T47657* and cold stress sensitive variety *Gerry* especially on the long term. There were several studies indicating the contribution of FAD7 gene expression to cold stress tolerance. Liu et al. [2] has reported that over expression of LeFAD7 gene in tomato has increased trienoic fatty acid content and transgenic plants exhibited higher tolerance to chilling stress as compared to that of wild type plants. Tobacco plants over-expressing FAD3 and FAD7 had increased the levels of fatty acid 18:3 (linolenic acid) which led to better membrane fluidity characteristics and increased cold and chilling tolerance [49]. LOX expression patterns could be related to JA biosynthesis and JA induced gene expression which was reported to be positively affected by different molecules including ABA, H₂O₂ and fatty acids conjugates [4]. Holley et al. [50] postulated that different environmental stimulus such as wounding, UV and fatty acids can activate different signaling molecules and pathways which are merged at the level of MAP kinases, and this could also be the case for cold stress affects in tomato plants.

In conclusion, GB might have a role in inducing the expression of FAD7 and LOX genes under the effect of cold stress in tomato plants and the induction of these genes can facilitate a better protection against cold stress in

relation to the desaturation process of lipids, increased membrane stability and/or induction of other genes related to stress defense mechanisms via octadecanoid pathway or lipid peroxidation products. Although the multigenic characteristics of responses and defense strategies against environmental stresses vary to a great extent in plants, the involvement of well known genes and signaling pathways could play a common role or interact for at least triggering the first phases of abiotic and biotic stress responses.

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