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Biochemical and molecular responses of hot pepper (*Capsicum annuum*) to cold stress

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ABSTRACT

Four cold tolerant hot pepper (*Capsicum annuum* L.) genotypes were identified at Division of Vegetable Science, ICAR-IARI, New Delhi which showed significantly better agronomic performance during the cold winter conditions of Delhi during 2019–20 when most of other genotypes succumbed. Present investigations were carried out to understand the biochemical and molecular basis of cold tolerance in these genotypes for which they were compared with cold sensitive genotypes for various biochemical parameters as well as for expression of some cold inducible genes. The results indicated that the cold tolerant genotypes had inherent biochemical and molecular mechanism which helped them in cold acclimation. These genotypes had strong antioxidant defense and ROS (Reactive Oxygen Species) scavenging system as displayed by significantly high activities of guaiacol-peroxidase and superoxide dismutase and lower levels of lipid peroxidation in response to cold stress. They also accumulated higher concentration of proline to maintain their membrane fluidity and H_2O_2. Twelve of the fourteen cold inducible genes studied in the present investigations had significantly high expression in cold tolerant genotypes. However the expression profile of these genes differed in different genotypes indicating that mechanism of cold tolerance was not identical in all the genotypes. The confirmation of existence of innate cold tolerance mechanism in our test genotypes can pave a way for future utilization of these genotypes in cold stress breeding.

Keywords: Cold inducible genes, Cold stress, Hot pepper, Guaiacol-peroxidase, Lipid peroxidation, Superoxide dismutase

Plants are exposed to various environmental stresses during their life cycle. Abiotic stresses such as heat, cold, drought, salinity and heavy metals severely impair the growth and development of plants in addition to inducing a series of morphological, physiological, biochemical and molecular changes in plants (Bray *et al.* 2000). Cold stress in particular, is a serious threat to crop productivity and yields. Although, hot pepper cultivation is meant for summer season but by developing cold tolerant/resistant genotypes, the growing season will get prolonged bringing higher remuneration to farmers and availability of fresh fruits to them year round. In order to tolerate cold stress, plants change the carbohydrate metabolism (Frankow-Lindberg 2001), boost the radical scavenging potential of the cells (Baek and Skinner 2003), alter the lipid composition of the biomembranes with respect to the maintenance of their fluidity (Welti *et al.* 2002) and synthesize and accumulate compatible solutes as well as cold acclimation induced proteins. In addition, up/down-regulation of gene expression following exposure to cold has been reported in many studies (Shinozaki and Yamaguchi Shinozaki 2000).

Hot pepper (*Capsicum annuum* L.) is a solanaceous species and one of the most economically important crops which is cultivated widely for its hot-tasting fruits. ICAR-IARI, New Delhi–India has a strong Chilli breeding programme and efforts in this direction have lead to the identification of few cold tolerant genotypes which have shown significantly better performance during the cold winter conditions of Delhi when most genotypes died or had become stunted when temperature dipped below 4°C. Thus, the study was carried out to elucidate the adaptive responses (particularly biochemical and molecular) of susceptible and tolerant hot pepper genotypes in response to cold stress. The study is important to understand the mechanism of cold stress tolerance and identify genes involved in the cold stress signalling in hot pepper.

MATERIALS AND METHODS

Experimental material: Eight chilli genotypes varying in their tolerance to cold stress namely DLS-C-1R-2 , DLS-E-3R, DLS-O-3R-2, CS-1R2, DLS-CT-IR2-1, DLS-P-1R-3,

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DLS-L-2R-5 , DLS-CT-IR2-2 were used in the study The cold tolerant (CT) genotypes selected were DLS-CT-IR2-1, DLS-P-1R-3 , DLS-L-2R-5 , DLS-CT-IR2-2 while the cold susceptible genotypes (CS) were DLS-C-1R-2 , DLS-E-3R, DLS-O-3R-2 ,CS-1R-2. The present experiments were conducted at the experimental farm of Division of Vegetable science, ICAR-IARI, New Delhi during the month of December and January 2019–20 whose geographical location lies at 28°38'23"N latitude, 77°09'27"E longitude and at an altitude of 228.6 m above MSL.

Biochemical parameters: Activity of guaiacol-peroxidase (GP) was determined following the method of Evers *et al.* (1994). The level of lipid peroxidation (Malondialdehyde assay) was measured in terms of TBARS content as per Heath and Packer (1968). Superoxide dismutase (SOD) activity was determined in crude extract by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light as described by Giannopolitis and Ries (1977). One unit of enzyme activity was determined as the amount of the enzyme needed for the inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer. Proline content was determined according to method of Bates *et al.* (1973) with modification. The amount of H_2O_2 was quantified as described by Loreto and Velikova (2001). H_2O_2 concentration was expressed as µmol/g fresh weight (FW).

Expression analysis of cold responsive genes: Expression of 14 different genes, viz. CaXTH1, CaXTH2, CaXTH3, CaCBF1B, HDZip, CaBZ1, CaWRKY1, Zn finger, CaTPP1, CaDIL1, CaPUB1, CaNAC2*,* CaEREBP-C2 and CaEREBP-C3 were studied in the tolerant and susceptible genotypes under study. The sequence of the primers used for qPCR analysis has been mentioned in Table 1. Leaf samples for expression analysis were collected from eight chilli genotypes used in the study on January 20, 2020 in liquid N_2 and stored in deep freezer (-80°C) until RNA isolation. Three biological replicates were collected from each line.

RNA Extraction: Total RNA from the chilli leaf samples was extracted using Tri-Xtract (G Biosciences, Geno Technology, Inc. St. Louis USA) following manufacturer's guidelines and quantified with a nanospectrophotometer. 1 µl of isolated RNA was loaded on a denaturing agarose gel to check its concentration and integrity. 2 µg of total RNA was reverse transcribed using Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc.) following manufacturer's instructions. 10-fold dilution of cDNA was made and 1µl of diluted cDNA was used as template for each qPCR reaction. For a 10 µl qRT-PCR reaction, 5 µl of 2XSYBR green mastermix (Applied Biosystem, CA,USA), 2 µl nuclease free water, 1 µl each of forward and reverse primer of desired gene (100 nm) and 1µl of template DNA was used. qRT-PCR was performed on *LightCycler® 96 Real-Time PCR* (Roche Life Sciences). The Q-PCR programe

Table 1 Primer sequences of the genes used for Gene expression studies

Gene	Primer sequence	Reference
CaXTH1	5'-ATCCCATTTCATCTTCAAATTAAAGC-3'	Cho *et al*. 2006
	5'-GGGGAAATGATTTATTGTTATTTCG-3'	
CaXTH2	5'-CTATGCCCGGCAGCTTGGGCTGAA-3'	
	5'-GACAACATTAGTAAACTCAATCC-3'	
CaXTH3	5'-GTGGGCTGAGAATTTTTACCAAGAT-3'	
	5'-GGCAAGAAAACCATTCATTGTTATTTTCTA-3'	
CaCBF1B	5′-GGATCCATGAACATCTTTAAAGC-3′	Kim *et al*. 2004
	5′-CTCGAGTTAAATGGAATAGCTCCA-3′;	
HDZip)	5′-TCTTGGGAGAGAGAACCCAAA-3′	
	5′-ACAGACAGGTGATGCTCCT-3	
CaDIL1	5' TCCACTATTCTGCTTCTCACTGAT 5'GAAGTCCTGTCCTTTGCTTCTTGA	Lim *et al*. 2018
CaPUB1	5′-CAATCCCTTACGAGCATAGAG-3′	Min *et al*. 2016
	5′-TTGGTAGTGTGGATGCTTCT-3′	
CaNAC2	CCGACCTCTGACGTTTGTTTG AGTTTCCTCAAGTCCTCGTTC	Guo *et al*. 2015
CaEREBP-C2	5'TTTTTGAGGTGCCTCCTTTG	Hwang *et al*. 2005
	5'TCTCAGTGGATCGTGACAGC	
CaEREBP-C3	5'AATTCCTGCTTTTCCGATCA3'	
	5'GGGAAATCGGAGTGGAGATT3'	
Zn finger	5'CAGGTTTCCGTGAGCAACTT3'	
	5'CCCATGTCGTCAATGTCAAG3'	
CaWRKY1	5'TCACTGGATTGAGGGGAGAC3'	
	5'CCACACCATTTTGTGTGCTC3'	
CaBZ1)	5'ATGGATGCCAACAACAACAA3'	
	5'GAAATTCATTGGGCCGACTA3'	
CaTPP1	5'TTGCTTCTCCAACTGACACG3'	
	5'ACTCTTCGCCGCTATTTTCA3'	

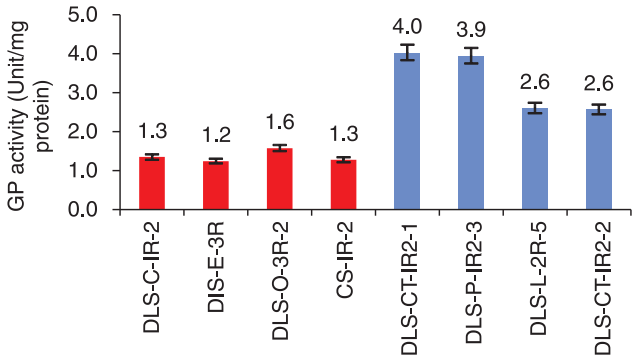

Fig 1 Variation in GP Activity in response to cold stress in different chilli genotypes under study.

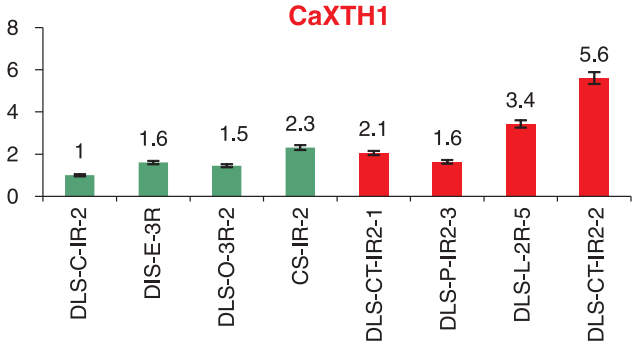

Fig 2 Variation in relative fold change in expression of Ca XTH1 in response to cold stress in different chilli genotypes under study.

comprised of initial denaturation at 95°C for 120 sec, 40 cycles of denaturation at 95°C for 30 sec annealing at 55°C for 60 sec and extension at 72°C for 30 sec. For Q-PCR two technical replicates were used per biological replicate of each genotype. The ΔCt of each target gene was normalized with internal control Ubiquitin (Hongjian *et al.* 2011). The ΔΔCt values were calculated taking ΔCt of DLS-C-1R-2 as calibrator and were used to plot graph to study the relative expression of each gene in the genotypes under study.

RESULTS AND DISCUSSION

Hot pepper is an important Solanaceous vegetable. Being of tropical origin, the optimal temperature for its growth ranges between 21–27°C (Deng *et al.* 2009). Low temperature severely affects the growth and reproduction of pepper plants, resulting in severe economic losses. Plants evolve many physiological, biochemical and molecular mechanisms to adapt to various stress condition including cold stress (Shinozaki *et al.* 2003). During the present investigations, eight hot pepper genotypes with varying cold tolerance were studied for their biochemical and molecular responses to cold. During the study period, the temperature dipped to a minimum of 10°C during day and 3°C during night on December 30, 2020 while in January, lowest night temperature (3°C) was observed on January 1, 2020 whereas lowest day temperature (13°C) was observed on January 9, 2020. Samples were collected for analysis from all the genotypes on 20 January 2020 when the max/min temperature in New Delhi was 15°C/8°C.

Biochemical Parameters: As a result of cold stress, reactive oxygen species (ROS) or free radicals are produced as a by-product in various cellular compartments, especially mitochondria and chloroplasts in association with different kinds of oxidases (Van Breusegem and Dat 2006). These ROS are important for signaling in several growth and developmental processes and in comprehending stresses along with programmed cell death (Bailey-Serres and Mittler 2006). But when ROS are present in excess amounts, they bring about a severe damage to cellular structure and macromolecules. Superoxide dismutase (SOD) and guaiacol peroxidase (GP) are two important antioxidant enzymes which help in scavenging of ROS (Pandey *et al.* 2017).

Fig 1 and Supplementary Fig 1(A-D) represent the biochemical responses of different chilli genotypes to cold stress. In all the biochemical parameters, the CS genotypes are represented by red bars while the CT ones by blue bars. Guiacol peroxidise (GP) activity was in the range of 1.2–1.6 unit/mg protein (Fig 1A) in all the CS genotypes while it was in the range of 2.6–4.0 unit/mg protein in all the CT chilli genotypes under study (which was almost more than double as compared to CS) thereby indicating, that the ROS scavenging system was more active in cold tolerant genotypes than the susceptible ones. The same phenomenon was observed in case of SOD activity (Supplementary Fig 1A) which also confirmed the occurrence of stronger ROS scavenging system in CT genotypes as SOD activity was much higher in tolerant chilli genotypes as compared to the susceptible ones. SOD activity ranged between 3.1–3.6 units/mg protein in the CS genotypes while the same ranged from 5.4–7.0 in the CT genotypes. Highest SOD activity was observed in the genotype DLS-L-2R-S (7.0 units/mg protein) while it was lowest in DLS-E-3R and DLS-O-3R-2 (3.1 units/mg protein).

According to Zhou *et al.* (2012), cold acclimation increases plant tolerance to a more-severe chilling and in this process, an accumulation of H_2O_2 in plants is often observed and abolishment of H_2O_2 using antioxidant scavenger before the plants subjected to the cold acclimation abolished the cold acclimation-induced beneficial effects on photosynthesis and antioxidant metabolism, leading to a loss of the cold acclimation-induced tolerance against chilling. In line with these findings, we also observed increased accumulation of H_2O_2 in cold tolerant hot pepper plants (4.7–6.5 µ mole/g FW) as compared to CS plants (6.1–13.0 µ mole/gFW) (Supplementary Fig 1B).

Melondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids is a useful index of general lipid peroxidation. An important prerequisite for cold acclimation in plants is the maintenance of cell membrane functional activity under low temperatures (Uemura *et al.* 2006). Since cold induced lipid peroxidation results in the destruction of polyunsaturated fatty acids and changes membrane physical and chemical properties, therefore studying lipid peroxidation is of importance for the

assessment of plant's capacity to adapt to low temperature. During the present study, the level of lipid peroxidation was observed to be comparatively low in the CT genotypes (Supplementary Fig 1C) which was manifested by low MDA activity that ranged between 3.3–4.1 mMole/g FW in the four cold tolerant genotypes while all the CS genotypes had high MDA activity (ranging from 5.0–6.7 mMole/g FW).

The cold adaptive process involves a number of biochemical and physiological changes, including increased levels of proline and H_2O_2 concentration etc (Holaday *et al.* 1992). Proline not only facilitates osmoregulation, but also protects plants from dehydration resulting from cold stress by reducing water potential of plant cells. It also functions as a molecular chaperone to stabilize the structure of proteins and plays a role in regulation of the antioxidant system (Armengaud *et al.* 2004). As shown in Supplementary Fig 1D, our observations are also in line with the earlier workers which suggest that proline helps in cold acclimation of plants, as in the present study all the CS genotypes had very low proline content (0.7–1.1 µg/FW) which was almost half of the value (except in DLS-E-3R) observed in the CT genotypes (1.2–1.6 µg/FW).

Expression of cold inducible genes: Under stress conditions, a series of signalling pathways regulate gene expression to produce various kinds of defensive proteins and molecules. Based on the extensive literature study, 14 cold inducible genes were selected for understanding their role in cold acclimatization in chilli.

Xyloglucan endotransglucosylase/hydrolase (XTH) has been recognized as a cell wall-modifying enzyme that is involved in diverse physiological processes in plants. In hot pepper, CaXTH homologs have been reported to have a role in the early events in the abiotic-related defense response including cold stress (Cho *et al.* 2006).

In the present investigation, expression of three homologs of Xyloglucan endotransglucosylase/hydrolase (XTH) gene of Chilli, viz. CaXTH1, CaXTH2 and CaXTH3 was studied. As shown in Fig 2 and Supplementary Fig 2 (A-B) and, highest expression of CaXTH1 (5.6 folds) was observed in DLS-CT-1R2-2 followed by DLS-2R-5 (3.4 folds) while in DLS-CT-1R2-1 (2.1 folds) and DLS-P-1R-3 (1.6 folds) the level of CaXTH1 expression was comparable to the expression levels observed in four cold susceptible (CS) genotypes (1–2.3 folds). Both CaXTH1 and CaXTH2 were observed to be induced at a higher level in all the CT genotypes as compared to CS genotypes. However an entirely different pattern of CaXTH3 expression was observed among the CT genotypes as the expression reached to 4.7 and 5.0 folds, respectively in DLS-CT-1R2-1 and DLS-P-1R-3 while it remained at 2.1 and 1.9 fold, respectively, in DLS-L-2R-5 and DLS-CT-1R2-2.

An important insight into the cold acclimation process has been provided by the discovery of the Arabidopsis CBF (CRT binding factor) cold-response pathway (Shinozaki and Yamaguchi-Shinozaki 2000). DREB1b/CBF1 is thought to function in cold-responsive gene expression, whereas DREB2s are involved in the drought-response (Yamaguchi-Shinozaki and Shinozaki 1994). In the present study, CaCBF1B (Supplementary Fig 2C) was observed to be induced at a significantly higher level in response to cold stress in all the four CT genotypes as compared to CS genotypes, although its highest expression was observed in DLS-CT-1R2-2 (5.5 folds). Overall its expression ranged between 2.3–5.5 folds in the CT genotypes against 1.0–2.1 folds in CS genotypes suggesting, thereby, its role in cold acclimation of tolerant genotypes used in the study. The CBF regulon includes genes that encode LEA or LEA-like hydrophilic polypeptides thought to play roles in freezing tolerance. LEA proteins have been reported to play a role in a large spectrum of cellular processes, from growth to stress response (Campos *et al.* 2013, Pathak and Ikeno 2017). In addition, homeodomain leucine zipper (HD-Zip) protein has also been found to interact with CaCBF1B, whose expression is also elevated by low temperature and drought (Kim *et al.* 2004). Therefore, during the present investigation, we also tried to study the expression of some of the CBF regulons including *Capsicum annuum* Drought Induced Late embryogenesis abundant protein 1 (CaDIL1) which is a critical regulator of transpirational water loss in pepper as also HD-Zip. The expression of CaDIL1 as well as HD Zip in pepper leaves was upregulated significantly in all the CT genotypes as compared to CS. Further the expression pattern in three of the four CT genotypes, viz. DLS-CT-1R2-1, DLS-P-1R-3 and DLS-L-2R-5 appeared to be same in both CaCBF1B and CaDIL1). Present results indicate that CaDIL1, HD Zip and their potential regulator CaCBF1B are also involved in cold tolerance in Chilli (Supplementary Fig 2D, E respectively).

A number of studies have revealed that plant NAC proteins are involved in transcriptional regulation in various processes such as stress signalling, lateral root formation, senescence and secondary wall formation as well as in response to various abiotic stresses, including abscisic acid (ABA), ethylene, drought, high salt, and low temperature (Hu *et al.* 2008). Our observation also indicates that CaNAC2 regulates cold tolerance in hot pepper as all the four CT genotypes used in the study were found to express CaNAC2 gene at a significantly higher level than the CS genotypes (Supplementary Fig 2F).

CaPUB1 has been identified as an abiotic stress-induced gene that encodes a U-box E3 Ub ligase in hot pepper (Cho *et al.* 2006). In our study (Fig. S2G) all the CT genotypes showed higher expression of CaPUB1 in response to cold stress which suggests that this gene is a positive regulator of cold stress.

In an attempt to determine a cold defense mechanism in plants, Hwang *et al.* (2005) identified a variety of transcription factors namely a bZIP protein (CaBZ1), WRKY (CaWRKY1), Zn finger, protein phosphatase (CaTPP1) and ethylene-responsive element binding protein (designated as CaEREBP-C2 and CaEREBP-C3) which were upregulated in response to cold stress. These genes were chosen for validation in the present study. We observed that all these transcription factors were induced at a higher level in

all the CT genotypes as compared to the CS genotypes, although some CS genotypes also showed higher expression of CaEREBP-C2 and CaEREBP-C3 (Supplementary Fig 2(H-M).

Cold tolerance in plants is a complex process. Chilling or freezing temperatures can result in the formation of ice in plant tissues, which causes cellular dehydration. In order to protect themselves, plants produce osmoprotectants to prevent ice formation. As a complex process, cold tolerance involves a number of physiological, biochemical and molecular mechanisms. Our study confirms the involvement of a number of biochemical factors such as activities of guaiacol peroxidase and superoxide dismutase enzymes, level of lipid peroxidation, accumulation of proline and H_2O_2 as well as expression of a number of genes/ transcription factors and their regulons in cold adaptation in chilli.

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