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Cold stress-induced biochemical and molecular responses in Safflower (*Carthamus tinctorious* L.)

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Abstract

Cold stress causes substantial losses in global agricultural productivity. When exposed to cold stress, plants usually exhibit a variety of cold-induced physiological, biochemical, and molecular responses. Safflower is one of the oldest, marginalized, neglected and domesticated multipurpose oilseed crops which is uniquely affected by cold stress at different growth stages. Because of lack of systematic research on cold signalling pathways and gene regulatory networks, the underlying biochemical and molecular mechanisms of cold signal transduction in different developmental stages of safflower are poorly understood. Therefore, this study sought to detect biochemical response and identify cold responsive safflower genes expressed at the rosette and bolling stages to provide novel insight about the growth stage's differential responses to cold stress. The results demonstrated that pseudo-marker snap genes CtAH10T0001700.1 and CtAH10T0001500.1 are the E3 ligase genes with RING finger domain while CtAH10T0000500 matched the GRAS family genes. These genes successfully exhibited similar cold responses as per their respective family. Overexpression of some E3 genes like CIP8 and HOS 1 could be associated with the cold susceptibility of plants in the bolling stage of safflower. Additionally, CtAH10T0001700.1 expression was found to be positively correlated with electrolyte leakage and proline content at the rosette stage. Meanwhile CtAH10T0001500.1, a negative cold regulator significantly correlated with electrolyte leakage and proline content. While CtAH10T0000500, a positive cold regulator significantly correlated with electrolyte leakage and proline at the rosette stage, but negatively associated with the two parameters at bolling stage. Overall, the genes were found to play a significant role in cold responsiveness of safflower plants at different growth stages.

Keywords

cold responsiveness; differential gene expression; Safflower

Introduction

Low temperature stress is one of the major limiting abiotic factors affecting agricultural crop production worldwide. It not only affects the overall yield of crops, but also the seed and plant quality (1). Recently, the global human population has been reported to have reached 8 billion people and it is estimated that it could increase to 8.5 billion by 2030 and 9.7 billion by 2050 (2). Meanwhile the cost of climate change for crops is estimated to increase up to $80 billion at 2 °C global warming per annum (3). Maximization and intensification of agricultural production is the last expedient to increase food

production. Identification of cold tolerant crop varieties could help to maintain sustainable food production, hence mitigate the challenges arising from increasing global human population growth and climatic changes.

Safflower (*Carthamus tinctorious* L.) is a multipurpose crop which is tolerant to many abiotic stresses such as drought, heat, cold and salinity (4). From prehistoric times, safflower has received a significant amount of attention as an oilseed and medicinal plant. The crop belongs to the Asteraceae family of flowering plants. Safflower has four distinctive growth stages which are affected by low temperature differently (5). The growth stage, immediately after seedling emergence is referred to as the rosette stage (6, 7). At this stage plants are regarded as cold hardy as they can withstand lower temperatures ranging from -7 ^{0}C to -26 ^{0}C (8–10). From the stem elongation phase through maturity, the susceptibility of the plants to stress increases, with high mortality at the flowering phase (6, 7). Plants in the elongation phase can recover after cold stress, although yield might be compromised (11).

The mechanism of cold tolerance in plants has been widely used to aid in development of cold tolerant varieties. As of today, several cold-responsive (COR) genes and their corresponding regulatory networks have been identified and characterised in different plant species (12, 13). Some of these genes code for proteins which function as chaperones, cryoprotectants and ice-binding proteins to provide defense mechanism in cold stressed plants. The regulatory genes which are also transcription factors, control expression level of many other genes. They achieve that by directly binding the cis-acting elements in the promoter regions of the target genes (13, 14). One of the most studied regulons involved in cold response is the C-repeat binding factor (CBF), also known as dehydration-responsive element binding factor 1 transcription factors (DREB1s). This regulon is driven by CBF transcription factors which induce the expression of COR genes (13).

In plant cells, the repercussion of cold stress include slowed metabolism, loss of membrane function, leakage of solutes (14, 15), increased production of reactive oxygen species (ROS) and hormonal imbalance due to denaturing of vital enzymes and proteins (14). To maintain continuous existence, plants have developed sophisticated and complex set of mechanisms (16). Some of the survival mechanisms to counteract against cold stress include production of metabolites such as amino acids (proline), carbohydrates and soluble proteins, which stabilize cell membranes against cold (17, 18).

In safflower, the biochemical and molecular mechanisms involved in cold tolerance that cause differential growth stage response to cold stress has not yet been reported. The current study aimed to determine the influence of cold stress on safflower plants at rosette and bolling stages using electrolyte leakage and proline assays. In addition, it identified and characterized COR genes expressed at crucial growth stages of safflower to provide novel insights into differential response of the rosette and bolling stages.

Materials and Methods

Study site and plant materials

The research work was carried out at Botswana University of Agriculture and Natural Resources (BUAN), in a net shade and Tissue Culture Laboratory. The safflower genotype Turkey was grown in the winter seasons of 2022 and 2023 (March to July). The plants were grown in a stepwise manner to reach two distinct growth stages (rosette and bolting) at which they were subjected to cold stress.

Experimental design and cold stress treatment

Application of cold stress in safflower plants was performed following a 2 × 3 × 2 factorial experiment replicated three times. The treatments were safflower growth stages (rosette and bolling), temperature (0 °C, 4 °C and 23 °C) and duration of exposure (8 h and 12 h). Initiation of cold stress was done by placing the plants in refrigerators with temperature set as per the temperature treatments stated above.

Determination of relative leaf electrolyte leakage (REL)

Immediately before removing the plants from a cold environment, three leaves from each plant were harvested and cut into 1 cm segments. The samples were weighed to 100 mg, put into 25 mL distilled water, and mixed with a gyratory shaker (200 rpm) at room temperature (25 °C) for 2 h. The initial conductivity reading was taken using a conductivity meter and recorded as conductivity 1 (C1). Thereafter, the samples were heated at 121 °C for 10 min to stimulate the highest leaf leakage (19). The contents were allowed to cool at room temperature and the second reading of electrolyte conductivity (C2) was taken. The relative electrical conductivity (C %) was calculated using the formula (C1/C2) × 100 (Eqn. 1) (20).

Proline content determination

Proline was extracted by grinding 0.5 g of freshly harvested leaves in liquid nitrogen. The powdered leaf samples were homogenized with 10 mL of 3 % sulphosalicylic acid in a test tube and centrifuged at 5000 rpm for 5 min. The supernatant was transferred into a 5 mL test tube and mixed with 2 mL of acidic ninhydrin and 2 mL of glacial acetic acid. The reaction mixture was heated at 95 °C in block heater for 1 h and cooled in an ice bath for 30 min (21). To the reaction mixture, 4 mL of toluene was added and vortexed for a min, then centrifuged at 1000 rpm for 5 min. The optical density of the samples was read at 520 nm using a spectrophotometer and the content of proline was calculated using standard curve. Proline content was calculated as: PC = [(µg proline/mL) × mL toluene)/115.5 µg/µmole] / [(g sample)/5] (Eqn. 2) where; 115.5 is the molecular weight of Proline (22).

Expression analysis of cold stress genes

Sample collection, total RNA extraction and cDNA synthesis

After induction of cold stress, three leaf samples were randomly cut from one plant in each pot to represent one biological replicate and quickly snap frozen in liquid nitrogen and stored at -80 °C until mRNA extraction. Total RNA was extracted from the previously frozen leaf tissues using

Quick-RNA™ Plant Miniprep kit (Zymo Research, Irvine, CA, USA). The integrity of the extracted RNA was confirmed using 0.8 % agarose gel electrophoresis. The purity and concentration of RNA were determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA extracted was reverse-transcribed to cDNA as a template for qPCR using ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs Inc., Ipswich, MA, USA) with oligo DT primers. Translation initiation factor 5A (EIF 5A) and ubiquitin-conjugating enzyme E2 (UBCE2), internal reference markers (Table 1), were used to test the success of cDNA synthesis.

COR genes were identified from literature and blasted using the NCBI Blastn. However, the output of the BLAST did not match any sequence of safflower. Safflower pseudo-marker snap genes were extracted from The Genome Database of *Carthamus tinctorius* (23) and blasted using NCBI Blastn (National Center for Biotechnology) obtained from https://www.ncbi.nih.gov to obtain mRNA sequences similar to that of safflower pseudo-maker snap genes. Confirmation of sequence similarities was done using Clustal Omega Pairwise Sequence Alignment (EMBL-EBI-www.ebi.ac.uk>msa). Genes with high percentage similarities were checked for cold responsiveness by studying their gene annotation: biological processes, functions and conserved domain sequences utilizing the PUBMED, Reverse position specific BLAST (RPS-BLAST) and Conserved Domain Database (CDD) (24, 25). COR homologs were then used to design specific primers using Primer3Plus tool obtained from https://www.bioinformatics.nl/cg-bin/primer3plus/primer3plus.cgi/. Details of the specific primers used are presented in Table 2.

Table 1. The primer description of reference genes used for normalization of safflower (*Carthamus tinctorious L.*) target genes

Unigene number	Gene symbol	Gene description	Sequence (5'–3')	Amplicon length (bp)	Tm of primer (°C)
comp40996_c0	EIF-5A	Translation Initiation Factor 5A	F 5'-TGTCCCTCATGTCAACCGTA-3'	120	59.96
			R 5'-GCATCATCAGTTGGGAGCTT-3'		60.23
comp31883_c1	UBCE2	Ubiquitin-Conjugating Enzyme E2	F 5'-GAGATGGCACCGTGAGTTATG-3'	102	60.53
			R 5'-GCCCTTCATGTACAGAGTTGTG-3'		59.66

The qRT-PCR was performed in a template containing 3 µl of cDNA, 10 µL of Luna qPCR master mix, 2 µM of the forward and reverse primers and topped up to 20 µL using DNase water. The reactions were performed on a Bio-Rad CFX 96 Connect real-time PCR detection system (BioRad Laboratories, Inc., Hercules, CA, USA) with each sample having 3 independent biological replicates. Translation initiation factor 5A (EIF 5A) and ubiquitin-conjugating enzyme E2 (UBCE2) were used as reference genes (Table 1) and no template control (NTC) as a negative control. The qPCR test was run with the following thermal cycling conditions: initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C denaturation for 15 sec, annealing at the melting temperature of the primers for 15 sec and extension at 72 °C for 30 sec. The relative expression level of the target gene was estimated using $2^{-\Delta\Delta CT}$ method (26).

Table 2. Primers used in qRT-PCR for amplification of safflower (*Carthamus tinctorious* L.) target genes

Name	Sequence (5'–3')	Sequence length (bp)	Tm of the primer (°C)
CtAH1700–1	F 5'-ATGGTGAATTGGCATTGGTT-3'	20	47.68
	R 5'-AAGGTTCAACGGCTATGGTG-3'		51.78
CtAH1500	F 5'-CATGCTCCCTCCGAAAAATA-3'	20	49.73
	R 5'-AGCAATGCACGATACAGCAG-3'		51.78
CtAH500	F 5'-GTTAGGGTTGCAAGGGTGAA-3'	20	51.78
	R 5'-GGATGTCCTCCTTCCTGACA-3'		53.83

bp-base pair, **tm**-melting temperature

Statistical analysis

Data collected was subjected to analysis of variance using R-program version 4.3.3 (Doebioresearch package, 2024). Treatment means were separated using Fisher's Least Significant Difference (LSD) at $P \leq 0.05$.

Results

Relative electrolyte leakage (REL)

Analysis of variance showed that safflower leaf relative electrolyte leakage (REL) significantly ($P < 0.001$) varied depending on the interaction of growth stage, temperature and duration of exposure. Control plants had the lowest REL value (9.9 %) in all growth stages. Application of cold resulted in insignificant increase in REL in plants at the rosette stage, except for plants cold stressed at 0 °C for 12 h which had notably lower REL relative to control plants (9.9 % control against 12.0 %). Conversely, exposure of plants in bolling to 4°C significantly increased the leaf's REL from 9.9 % to 14.5 % and 17.1 % in 8 h and 12 h of exposures, respectively. On lowering the temperature to 0 °C REL was increased to 36.5 % and 65.7 % in 8 h and 12 h of exposures, respectively. Thus, it can be inferred that plants in bolling stage lost more electrolytes than in rosette stage (Fig. 1).

Proline content

Under cold stress, plants in both growth stages underwent significantly ($P < 0.01$) high proline content increment due to the interactive effect of temperature and duration of exposure (Fig. 2). Cold stress of 4 °C for 8 h increased proline content from 5.57 to 6.93 µmol/g fresh weight (FW) at the rosette stage. Prolongation of cold stress to 12 h further increased proline content to 7.27 µmol/g FW but was not significantly different from 8 h exposure. Further lowering of chilling temperature to 0 °C did not notably increase proline content in 8 h of exposure. Nevertheless, 12 h of cold exposure considerably inflated proline content to 8.84 µmol/g FW. In comparison of the growth stages,

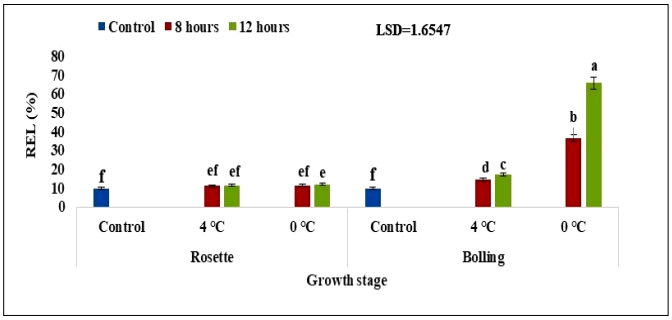

Fig. 1. Effects of growth stage, temperature and duration of exposure on relative electrolyte leakage (**REL**) of safflower. Means separated using the Least Significant Difference (**LSD**) at P ≤ 0.05.

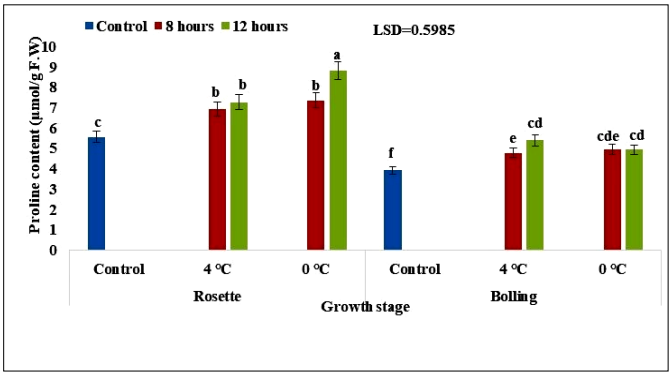

Fig. 2. Effects of growth stage, temperature and duration of exposure on proline content of safflower (*Carthamus tinctorious* L.). Means separated using the Least Significant Difference (LSD) at P ≤ 0.05.

plants in the rosette stage accumulated notably higher amount of proline in all the chilling conditions. Plants cold stressed at 4 °C for 8 h had 4.79 µmol/g FW in bolling against 6.93 µmol/g FW in rosette, while plants at 4 °C for 12 h had 5.40 µmol/g FW in bolling against 7.27 µmol/g FW in rosette. Meanwhile plant at 0 °C for both 8 h and 12 h had significantly lower values of 4.98 µmol/g FW and 4.94 µmol/g FW, respectively (Fig. 2).

Structural analyses of conserved domain of *Cynara cardunculus* var. *scolymus*

The RPS blast, NCBI BLASTn and CDD analyses revealed that all the three COR genes that showed similarities to safflower pseudo-marker snap genes are from *Cynara cardunculus var. scolymus* organism (Table 3). Excluding protein SCARESCROW, these genes encode RING proteins domain and belong to RING_U Box super family. The E3 ubiquitin protein ligase CIP8-like are identified by the cross-brace motif (RING-H2) while the RING finger and transmembrane containing protein 2-like are characterized by a unique cross-brace motif RING-HC_RNFT1-like with zinc binding site. SCARECROW is a GRAS member characterized by a lengthy GRAS domain (Fig. 3).

Sequence analyses of safflower and *Cynara cardunculus* var. *scolymus pairwise* alignment

Clustal omega pairwise sequence alignment results demonstrated that E3 ubiquitin ligase, RING finger & transmembrane domain-containing protein 2-like and protein Scarecrow, these three COR genes originating from *Cynara cardunculus var. scolymus* organism were 84, 94 and 82 (%) identical to CtAH10T0001700.1, CtAH10T0001500.1 and CtAH10T0000500 safflower pseudo-marker snap genes, respectively (Fig. 4).

Relative gene expression

The expression pattern of all the three safflower genes showed variability depending on either the individual or interaction effects of treatments under study. Upregulation of CtAH0001700.1 gene was significantly ($P < 0.01$) influenced by the individual effects of growth stage and temperature. Higher expression level was observed at bolling stage as compared to rosette and notably increased with decreasing temperature. Control plants (at 23 °C) had the least gene expression. Exposing the plants to cold stress of 4 °C tripled (from 1 to 3 fold change (FC)) the gene expression, while lower temperature of 0 °C resulted in 2.5-fold increase in gene expression against the control (Fig. 5).

Similarly, CtAH10T0001500.1 was highly ($P < 0.1$) expressed in bolling stage. However, unlike CtAH0001700.1, the gene was highly expressed in plants cold stressed at 4 °C for 8 h. For both growth stages, plants in warmer temperatures (23 °C) exhibited similar gene expression (1.3 FC bolling against 1.1 FC rosette). Exposure of plants to lower temperature of 4 °C for 8 h significantly reduced gene expression by 46.2 % in bolling stage, while it doubled (from 1.1 to 2.2 FC) in the rosette stage. Prolongation of cold stress to 12 h resulted in insignificant decrease (from 1.3 to 0.97 FC) in bolling stage and almost similar gene expression in rosette stage (1.11 against 1.27 FC) relative to the controls. Meanwhile, cold exposure of 0 °C did not significantly ($P < 0.05$) influence CtAH10T0001500.1 expression in both growth stages at 8 h and 12 h of cold exposure (Fig. 6).

Table 3. Description of homologs used in identifying safflower (*Carthamus tinctorious* L.) cold responsive genes

Safflower gene	Gene ID	Organism name	Gene description	Biological process	Sequence length (bp)	Citation
CtAH10T10001700.1	112512063	*Cynara cardunculus* var. *scolymus* (Artichoke/ globe arichoke)	E3 ubiquitin-protein ligase CIP8-like	- Protein ubiquination and degradation -Cold stress resistance	1381	(35)
CtAH10T10001500.1	112512836	*Cynara cardunculus* var. *scolymus* (Artichoke/ globe arichoke)	RING finger and transmembrane domain-containing protein 2-like	- Protein ubiquination and degradation -Cold stress resistance	1407	(34)
CtAH10T10000500.1	112513693	*Cynara cardunculus* var. *scolymus* (Artichoke/globe arichoke)	Protein Scarecrow	Gibberellin signal transduction, axillary meristem formation, root radial pattern formation and biotic and abiotic stress resistance	2393	(41)

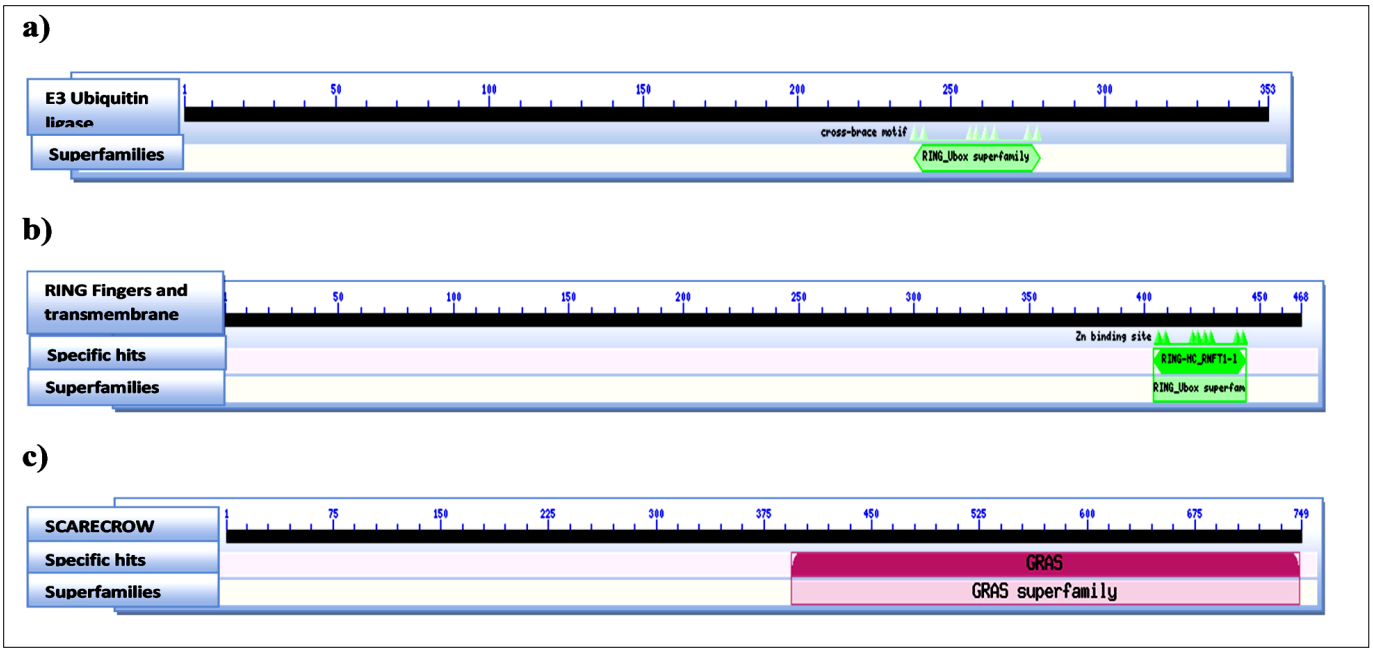

Fig. 3. Simplified graphical representation of the conserved domain sequence (CDS) showing superfamilies, domain motifs and zinc binding sites of cold responsive *Cynara cardunculus* var. *scolymus* genes.

Fig. 4. Partial pairwise sequence alignment of Safflower (*Carthamus tinctorious* L.) and Cynara cardunculus var. scolymus showing regions of similarities. ***** indicating stronger nucleotide similarities, *, **, * indicating weaker similarities/mismatch.

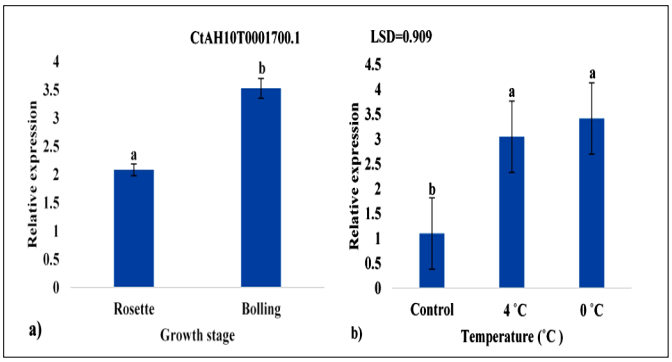

Fig. 5. Relative expression level of CtAH10T0001700.1 safflower (*Carthamus tinctorious* L.) gene under cold treatment. The relative expression level results were presented as (**a**) influenced by safflower growth stages, and (**b**) temperature at 0 and 4 °C. Multiple mean comparisons were performed using Fisher's Least Significant Difference (**LSD**) at P ≤ 0.05. Means with the same letters were not significantly different.

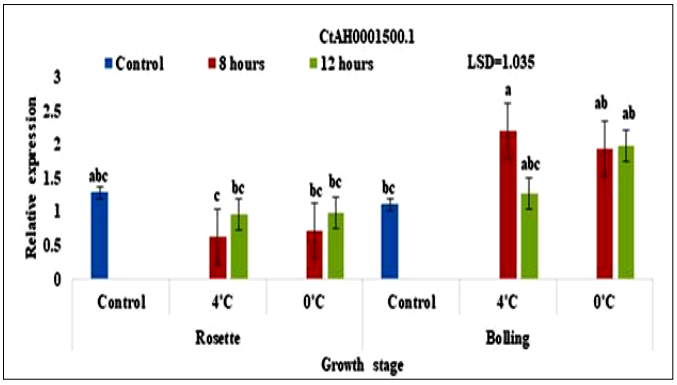

Fig. 6. Relative expression level of CtAH10T0001500.1 safflower (*Carthamus tinctorious* L.) gene under cold treatment of 0 and 4 °C for the duration of 8 and 12 hrs. Multiple mean comparisons were performed using Fisher's Least Significant Difference (**LSD**) at P ≤ 0.05. Means with the same letters were not significantly different.

Contrary to the other genes, CtAH10T0000500.1 gene expression gradually increased with lower temperature and longer duration of exposure at the rosette stage. Plants at 23 °C had a relative expression of 1, which increased 2-fold and 4-fold at 4 °C and 0 °C, respectively. A similar trend was observed in duration of exposure as relative gene expression increased from 1, 3 and 4-fold with prolongation of cold from 0 h, 8 h and 12 h, respectively. In comparison to the rosette stage, the gene was significantly downregulated in all cold treatment, regardless of the chilling temperature and duration of exposure in the bolling stage (Fig. 7).

Correlation analysis

Correlation analysis between safflower COR genes and REL showed a significant positive correlation at the rosette stage (0.48: P<0.05 for CtAH10T0001700.1 and 0.43: P<0.05 for CtAH10T0000500.1), except for CtAH10T0001500.1 (-0.47: P< 0.05) gene with negative correlation. At the bolling stage, a non-significant inverse relationship was noted between CtAH10T0001500.1 (-0.17: P<0.05) and

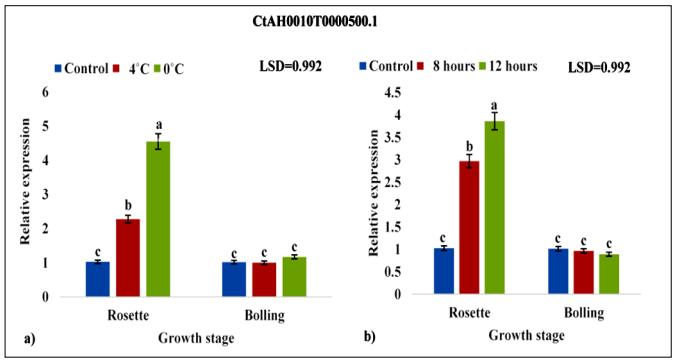

Fig. 7. Relative expression level of CtAH10T0000500.1 safflower (*Carthamus tinctorious* L.) gene under cold treatment. The relative expression level results were presented as (**a**) influenced by interaction of safflower growth stages and temperature at 0 and 4 °C, and (**b**) interaction of growth stage and duration of cold exposure at 8 and hours. Multiple mean comparisons were performed using Fisher's Least Significant Difference (**LSD**) at P ≤ 0.05. Means with the same letters were not significantly different.

CtAH10T0000500.1 (-0.92: P<0.05) with REL, while CtAH10T0001700.1 (0.49: P<0.05) had non-significant positive correlation (Table 4). A similar trend of relationship was observed between safflower COR genes and proline content as of REL at the rosette stage. CtAH10T0001700.1 and CtAH10T0000500.1 still had positive significant correlation of 0.41 and 0.32 (P< 0.05), respectively. Whereas CtAH10T0000500.1 (-0.41: P<0.05) negatively correlated with proline content in the rosette stage. Contrary to correlation results between REL and COR safflower genes, the genes had a significant positive relationship with proline content (0.96: P< 0.05 for CtAH10T0001700.1 and 0.68: P<0.05 for CtAH10T0001500.1), excluding CtAH10T0000500.1 (-0.19: P<0.05) with a significant inverse correlation (Table 4).

Discussion

Electrolyte leakage is an important indicator of the injury that occurs in the cell plasma membranes after exposure to either biotic or abiotic stress. When a cell is stressed, electrolytes such as K^+ ions are leaked out, resulting in loss of cell membrane integrity. The number of electrolytes lost is usually used as a measure of cell viability and death after stress exposure (27). In the present study, a significant number of solutes were lost at bolling stage relative to the rosette stage at all the chilling conditions. However, lower temperature of 0 °C resulted in higher solutes leakage with severe damage at 12 h (65.7% REL). High electrolyte leakage values at bolling stage suggested high solutes leakage and susceptibility of the stage to cold stress. In support of the current results, (28) other research also reported a cold susceptible African marigold (*Tagetes erecta*) genotype to have leaked 86.08 % of solutes after 3 months of cultivation in winter season while the cold tolerant genotype leaked 62.45 %. Longevity in cold stress was also found to

Table 4. Pearson correlation analysis of cold responsive safflower (*Carthamus tinctorious* L.) genes with relative electrolyte leakage and proline content.

		CtAH10T0001700.1	CtAH10T0001500.1	CtAH10T0000500.1
Relative electrolyte leakage (REL)	Rosette	0.48***	-0.47***	0.43***
	Bolling	0.49ns	-0.17ns	-0.92ns
Proline	Rosette	0.41***	-0.41***	0.32**
	Bolling	0.96*	0.68***	-0.19***

Correlation values are significant at P< 0.05; *0.05, **0.01 and ***0.001.

play a significant role in loss of electrolytes. At the bolling stage, plants chilled for 12 h at 4 °C and 0 °C had higher REL as compared to plants exposed to the same temperature but for shorter duration of 8 h. These findings are consistent with the results reported by other research (20, 29).

Proline is regarded as one of the most important biomarkers and signalling molecule associated with stress tolerance under stress conditions. Many plants species accumulate high concentrations of proline as a defense mechanism against any stress. Similarly in cold stress, more proline is accumulated to avoid dehydration induced by chilling temperatures, as a result maintaining the osmotic potential equilibrium and stabilizing proteins and membrane structures in plants (30). The results of the current study demonstrated a higher accumulation of proline in plants at the rosette stage which was at its peak at 0 °C and 12 h chilling exposure. Although after cold stress exposure, there was an increase in proline level at the bolling stage, the build-up of proline levels was relatively low. Some researchers investigated chickpea genotypes which produced significantly low levels of proline with being cold sensitive (31). Their argument was that cold sensitive plants respond slowly when it comes to proline accumulation, hence yielding relatively low levels of proline which is inadequate to provide a strong defense mechanism. Consequently, leading to osmotic pressure imbalance in the plant cells and ultimately occurrence of chilling injury in plants as evidenced at the bolling stage of safflower.

The findings of this study indicate that safflower's physiological adaptability to low temperatures relies on its ability to accumulate proline and maintain low electrolyte leakage. These factors are essential for its climate resilience, as they preserve cellular integrity and photosynthetic function under cold stress (27, 30). In arid and semi-arid climates, such as those prevalent in Southern Africa, safflower adaptability may be particularly challenging during the bolling stage, where low night temperatures can occasionally reach near or below zero (32). Insufficient proline accumulation and high electrolyte leakage can compromise cellular stability, leading to reduced photosynthetic efficiency, weakened plant health, and ultimately, reduced yield (27, 30). Cold stress at this critical bolling stage can negatively impact pollination, seed development,and seed quality, resulting in fewer, smaller and less viable seeds that lower both the economic value and regeneration potential of the crop (11). Similarly, in regions with a Mediterranean climate, like parts of the Middle East, where cool, wet winters with mean monthly temperatures between −3 °C and 18 °C are common (33), prolonged exposure to low temperatures can further impair safflower growth and productivity.

Safflower's capacity to accumulate proline and maintain low electrolyte leakage under various stresses enhances its survival in challenging climates, making it a promising crop for regions prone to climatic extremes. This adaptability is valuable for sustainable agriculture as it reduces dependency on intensive water and soil management and improves crop stability in unpredictable environments. With ongoing climate change, temperatures are predicted to rise, increasing evapotranspiration rates (34). In arid and semi-arid regions like Botswana, this suggests that water availability for crop production will decline, and soil salinity may increase. Higher temperatures also imply milder winters, reducing the risk of chilling injury. However, in regions with colder climates, this effect may be less pronounced leading to cold susceptibility in plants during the bolling stage. Given safflower's adaptive mechanisms, which confer resilience to various abiotic stresses, including cold, it holds potential as a climate-resilient crop that can help mitigate some effects of climate change.

The genomic data (shown in Table 3) demonstrated that most of the homologs selected to identify COR safflower genes came from *Cynara cardunculus var. scolymus*, commonly known as Artichoke/globe artichoke. Artichoke is a flowering perennial plant that belongs to the family Asteraceae (35), the same family as of safflower (4). Some research reported that during evolution, plants of the same family form genes with similar functions or structures through the process called whole genome duplication (36). This information suggested that the two plants could have evolved from a common ancestor hence high structural similarity, sequence homology and similar response to cold stress.

To associate safflower genes with cold responsiveness, the biological processes, functions, conserved domains and structure of *Cynara cardunculus var. scolymus* obtained from nucleotides blasting were studied. Genome annotation data, RPS-BLAST and CDD analyses revealed that E3 ubiquitin protein ligases CIP8-like and RING finger transmembrane domain-containing protein 2-like are RING finger proteins of RING/U-box superfamily (37–39). Under cold stress, the RING fingers act as E3 ubiquitin protein ligases and interact with E2 ubiquitin conjugating enzymes, thereby promoting ubiquitination of specific target transcription factors (37).

In rice, the ubiquitination activity and expression pattern of the RING-H2 finger protein COLDINDUCIBLE (OsCOIN) was positively induced by low temperature (40). Relating to the current study, CtAH10T0001700.1 with a similar structure as E3 ubiquitin ligase (RING-H2) was highly expressed at bolling than rosette stages of safflower. However, following the outcome of the current study (32), safflower plants in the rosette stage were cold tolerant while those in the bolling stage were susceptible, contradicting to the expression pattern of RING-H2 finger protein. Overexpression of CtAH10T0001700.1 in the bolling stage could be attributed to its CIP8-like character. CIP8 RING finger proteins are known to be repressor of light-responsive genes critical at plants flowering stage (41). These RING finger proteins also possess the ubiquitin ligase activity and directly interact with bZIP transcription factor hypocotyl 5 (HY5), a positive regulator of photomorphogenesis, for degradation in darkness to reduce or inhibit flowering. Under cold stress and darkness, the dual role of E3 ligase gene prompts its production in large quantities, hence overexpression of CtAH10T0001700.1 at bolling than rosette stage (41).

The CtAH10T0001500.1 which was like RING finger transmembrane domain-containing protein 2-like (HC) was downregulated at the rosette stage and upregulated at bolling stage. Based on RING-HC finger protein High Expression of Osmotically Responsive Genes 1 (HOS1), these genes are downregulated under cold stress. In Arabidopsis and rice, the RING-HC finger protein interacted with Inducer of CBF Expression 1 (ICE1) to mediate ICE1 ubiquitination and degradation through the 26S proteasome pathway, hence inhibiting expression of CBF transcription factors which are essential for expression of Dehydration responsive element (DRE) required to increase osmoregulation substances in order to attain cold tolerance (37, 42, 43). The results implied that CtAH10T0001500.1 at rosette stage followed the expression pattern of RING-HC proteins to attain cold tolerance. While cold susceptibility at bolling stage could be attributed to overexpression of HOS1 which totally inhibits the expression of CBF transcription factors, thus resulting in unstable cell membranes prone to cold damage.

The gene expression results further showed that CtAH10T0000500.1 safflower gene like *Cynara cardunculus* var. *scolymus* Scarecrow was significantly upregulated at the rosette stage while down regulated at the bolling stage. The gene expression level increased with decreasing temperature and increasing duration of exposure and was at peak at cold treatment of 0 °C for 12 h. The expression pattern of CtAH10T0000500.1 followed a similar trend as of GRAS genes. GRAS genes are a family of plant-specific transcriptional factors formed by three functional proteins named: GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA), and SCARECROW (SCR) (44, 45). In most plant species, cold stress significantly induces upregulation of the GRAS genes relative to controls (46–48). Slightly differing in banana transplants, GRAS gene expression exhibited multidirectional regulation. Out of 7 genes studies, four showed positive expression while three were negatively regulated (45). It was thus observed that the increase in GRAS gene expression was positively correlated with plant resistance to cold stress (47, 48).

Furthermore, the correlation analysis demonstrated that E3 ligase genes associated with REL differently. CtAH10T0001700.1 was positively correlated to REL while the opposite was noted for CtAH10T0001500.1 in both phenological stages. These two genes conferred cold tolerance by upregulation of CtAH10T0001700.1 and downregulation of CtAH10T0001500.1. Overexpression of the genes in the bolling stage rendered safflower plants more sensitive to cold stress, hence high solutes leakage and membrane damage. High electrolyte leakage has been associated with overexpression of E3 ligase genes such as AtCHIP and OsHOS1 in Arabidopsis and rice (49, 50). On the other hand, the suppression of RING finger gene OsSRFP1 was reported to increase free proline content of cold stressed rice leaves (50). Therefore, it could be concluded that both CtAH10T0001700.1 and CtAH10T0001500.1 with lower expression at the rosette stage of safflower plants had higher proline content as evidenced by the results of the present study (Fig. 2, 5 & 6). Meanwhile, the GRAS gene CtAH10T0000500.1 positively correlated with REL and proline content in the rosette stage and inversely associated with the two biochemical variables at the bolling stage of safflower plants. Experiments have reported a significant positive correlation between electrolyte leakage and proline content (51). Thus, as cold stress triggers electrolyte leakage, upregulation of the GRAS gene triggers proline synthesis and accumulation hence conferring cold tolerance to safflower plants at the rosette stage. While suppression of the gene decreases proline synthesis and increases electrolyte leakage, leading to greater susceptibility of safflower plants to cold stress at the bolling stage.

Conclusion

Out of 33 safflower pseudo-maker snap genes accessed from The Safflower Database Genome, 3 genes exhibited cold responsiveness and were involved in cold tolerance at the rosette stage of safflower. The gene CtAH10T0001700.1 and CtAH10T0001500.1 exhibited similar character as the E3 ligase genes while CtAH10T0000500 successfully matched the GRAS genes and followed the genes expression pattern. Overexpression of some E3 genes like CIP8 and HOS 1 could be associated with the cold susceptibility of safflower plants at the bolling stage. To enhance a better understanding of the mechanisms behind cold tolerance and susceptibility of safflower, in-depth studies on the roles of these E3 ligase genes and GRAS genes in cold stress response at different growth stages, particularly focusing on gene expression patterns and their effects on plant physiology during the bolling stage could be conducted. These studies could also incorporate multiple genotypes to observe the genotypic response of safflower to cold stress.

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Authors' contributions

DK conceived the study, conducted all laboratory experiments and drafted the manuscript. GM validated the methodologies used, assisted in statistical analysis and interpretation, reviewed and edited the draft. VEE conceived the study, reviewed and edited the draft. UB participated in validation of methods, reviewed and edited the draft. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: All authors declare no conflict of interests.

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References

1. Kopecká R, Kameniarová M, Cerný M, Brzobohatý B, Novák J. Abiotic stress in crop production. Int J Mol Sci. 2023;24(7):6603. https://doi.org/10.3390/ijms24076603
2. United Nations. Population: The world in 2100 [Internet] [Accessed on the 19Mar 2024]. https://www.un.org>global-issues
3. Iizumi T, Shen Z, Furuya J, Koizumi T, Furuhashi G, Kim W, et al. Climate change adaptation cost and residual damage to global crop production. Clim Res. 2020;80:203-18. https://doi.org/10.3354/cr01605
4. Li D, Hu B, Wang Q, Liu H, Pan F, Wu W. Identification and evaluation of reference genes for accurate transcription normalization in Safflower under different experimental conditions. PLoS One. 2015;10(10). https://doi:10.1371/journal.pone.0140218
5. Emongor VE, Emongor RA. Safflower (*Carthamus tinctorious* L.). Chapter 24. Elsevier Inc; 2023. p. 683-731.
6. Johnson RC, Dajue L, Bradley V. Autumn growth and its relationship to winter survival in diverse Safflower germplasm. Can J Plant Sci. 2006;86:701-09. https://doi.org/10.4141/P05-104
7. Landry EJ, Fuchs SJ, Bradley VL, Johnson RC. The effect of cold acclimation on the low molecular weight carbohydrate composition of Safflower. Heliyon. 2017;3:e00402. https://doi:10.1016/j.heliyon.2017.e00402
8. Emongor V. Safflower (*Carthamus tinctorius* L.) the underutilized and neglected crop: A Review. Asian J Plant Sci. 2010;9(6):299-306. https://doi:10.3923/ajps.2010.299.306
9. Vaclavik L, Mishra A, Mishra KB, Hajslova J. Mass spectrometry-based metabolomic fingerprinting for screening cold tolerance in *Arabidopsis thaliana* accessions. Anal Bioanal Chem. 2013;405 (8): 2671-83.https://doi.org/10.1007/s00216-012-6692-1
10. Johnson RC, Petrie SE, Franchini MC, Evans M. Yield and yield components of winter-type Safflower. Crop Sci. 2012;52:2358-64. https://doi.org/10.2135/cropsci2011.12.0659
11. Kolenyane MO. The influence of nitrogen and phosphorus on growth and yield components of Safflower (*Carthamus tinctorious* L.). Botswana University of Agriculture and Natural Resources Press; 2022. p. 46. https://moodle.buan.ac.bw/handle/13049/534
12. Lee BH, Henderson DA, Zhu JK. The *Arabidopsis* cold responsive transcriptome and its regulation by ICE1. Plant Cell. 2005;17:3155-75. https://doi.org/10.1105/tpc.105.035568
13. Ren C, Li H, Wang Z, Dai Z, Lecourieux F, Kuang Y, et al. Characterization of chromatin accessibility and gene expression upon cold stress reveals that the RAV1 transcription factor functions in cold response in *Vitis amurensis*. Plant Cell Physiol. 2021;62 (10):1615-29. https://doi.org/10.1093/pcp/pcab115
14. Ritonga FN, Chen S. Physiological and molecular mechanism involved in cold stress tolerance in plants. Review Plants. 2020;9 (560):1-13. https://doi.org/10.3390/plants9050560
15. Chai F, Liu W, Xiang Y, Meng X, Sun X, Cheng C, et al. Comparative metabolic profiling of *Vitis amurensis* and *Vitis vini*fera during cold acclimation. Hortic Res. 2019;6:8. https://doi.org/10.1038/s41438-018-0083-5
16. Hasanuzzaman M, Nahar K, Alam MM, Roychowdhary R. Physiological, biochemical and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci. 2013;14:9643-84. https://doi.org/10.3390/ijms14059643
17. Leuendorf JE, Frank M, Schmülling T. Acclimation, priming and memory in the response of *Arabidopsis thaliana* seedlings to cold stress. Sci Rep. 2020;10(1):689. https://doi.org/10.1038/s41598-019-56797-x
18. Aazami MA, Asghari-Aruq M, Hassanpouraghdam MB, Ercisli S, Baron M, Sochor J. Low temperature stress mediates the antioxidants pool and chlorophyll fluorescence in *Vitis vinifera* L. cultivars. Plants. 2021;10:1877. https://doi.org/10.3390/plants10091877
19. Kovaleski PA, Gossman JJ. Standardization of electrolyte leakage data and novel liquid nitrogen control improve measurement of cold hardiness in woody tissues. Plant Methods. 2021;17:53. https://doi.org/10.1186/s13007-021-00755-0
20. Liu W, Yu K, He T, Li F, Zhang D, Lui J. The low temperature induced physiological responses of *Avena nuda* L, a cold-tolerant plant species. Sci World J**.** 2013;658793:1-7. https://doi.org/10.1155/2013/658793
21. Carillo P, Gibon Y. Protocol: extraction and determination of proline. [Internet]. Prometheuswiki. Researchgate Net; 2011 [cited 2024 June 23]. https://www.researchgate.net
22. Zhang Z, Huang R. Analysis of malondialdehyde, chlorophyll proline, soluble sugar and glutathione content in *Arabidopsis* seedling. Bio Protoc. 2013;3(14):e817. https://doi.org/10.21769/BioProtoc.817
23. The genome Database of *Carthamus tinctorious* repository [Internet] [Cited 2024 March 3]. https://www.safflower.scuec.edu
24. Lu S, Wang J, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, et al. CDD/SPARCLE: The Conserved Domain Database in 2020. Nucleic Acids Res. 2020;48:D265-68. https://doi.org/10.1093/nar/gkz991
25. Wang J, Chitsaz F, Derbyshire MK, Noreen R, Gonzales NR, Marc-Gwadz Lu S, et al. The Conserved Domain Database in 2023. Nucleic Acids Res. 2023;51:D384-88. https://doi.org/10.1093/nar/gkac1096
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-}\Delta\Delta CT$ Method. Methods. 2001;25:402-08. https://doi.org/10.1006.meth.2001.1262
27. Hatsugai N, Katagiri F. Quantification of plant cell death by electrolyte leakage assay. Bio Protoc. 20188(5):e2758. https://doi.org/10.21769/BioProc.2758
28. Amala E, Singh KP, Panwar S, Namita B, Neelu J, Kumar S, et al. Physiological and morphological responses of marigold (*Tagetes erecta*) genotypes under cold stress. Indian J of Agric Sci. 2022;92(7):923-27. https://doi.org/10.56093/ijas.v92i7.119495
29. Rani A, Kiran A, Sharma KD, Prasad PVV, Jha UC, Siddique KHM, et al. Cold tolerance during the reproductive phase in chickpea (*Cicer arietinum* L.) is associated with superior cold acclimation ability involving antioxidants and cryoprotective solutes in anthers and ovules. Antioxidants. 2021;10(11):1693. https://doi.org/10.3390/antiox10111693
30. Verbruggen N, Hermans C. Proline accumulation in plants; A review. Amino Acids. 2008;35(4):753-59. https://doi.org/10.1007/s00726-0080061-6
31. Tatar O, Ozalkan C, Atasoy GD. Partitioning of dry matter, proline accumulation, chlorophyll content and antioxidant activity of chickpea (*Cicer arietinum* L.) plants under chilling stress. Bulg J Agric Sci. 2013;19(2):260-65. https://www.researchgate.net/publication/269987702
32. Kereilwe D, Emongor VE, Malambane G. Temperature and duration of exposure on chilling injury of safflower. EJFOOD. 2023;5 (2):23-26. http://dx.doi.org/10.24018/ejfood.2023.5.2.660
33. Urdiales-Flores D, Zittis G, Hadjinicolaou P, Cherchi A, Alessandri A, Peleg N, et al. A global analysis of historical and future changes in mediterranean climate-type regions. Int J Climatol. 2024;0:1-14. https://doi.org/10.1002/joc.8655

34. Mosupiemang M, Emongor VE, Malambane G. A review of drought tolerance in safflower. IJPSS. 2022;34(10):140-49. https://doi.org/10.9734/IJPSS/2022/v34i1030930

35. López-Anido F, Martin E. Globe artichoke (*Cynara cardunculus* var. *scolymus* L.) breeding: In: Al-Khayri JM, Mohan-Jain S, Johnson DV, editors. Advances in plant breeding strategies: Vegetable crops, Volume 10; Leaves, Flowerheads, Green pods, Mushrooms and truffles; 2021. p. 303-04. https://doi.org/10.1007/978-3-030-66969-08

36. Cannon SB, Mitra A, Baumgarten A, Young ND, May GJB. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol. 2004;4(10):1-21. https://doi.org/10.1186/1471-2229-4-10

37. Han G, Qiao Z, Li Y, Yang Z, Wang C, Zhang Y, et al. RING zinc finger proteins in plant abiotic stress tolerance. Front Plant Sci. 2022;13:877011. https://doi.org/10.3389/fpls.2022.877011

38. Wang S, Lv X, Zhang J, Chen D, Chen S, Fan G, et al. Roles of E3 ubiquitin ligases in plant responses to abiotic stresses. Int J Mol Sci. 2022;23:230. https://doi.org/10.3390/ijms23042308

39. Wang P, Zhu L, Li Z, Cheng M, Chen X, Wang A, et al. Genome-wide identification of the U-Box E3 ubiquitin ligase gene family in cabbage (*Brassica oleracea* var. *capitata*) and its expression analysis in response to cold stress and pathogen infection. Plants. 2023;12(7):1437.https://doi.org/10.3390/plants12071437

40. Liu K, Wang L, Xu Y, Chen N, Ma Q, Li F, et al. Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. Planta. 2007;226(4):1007-16. https://doi.org/10.1007/s00425-007-0548-5

41. Sun J, Sun Y, Imtiaz-Ahmed R, Ren A, Xie M. Research progress on plant RING finger proteins. Genes. 2019;10(12):973. https://doi.org/10.3390/gene10120973

42. Lourenço T, Sapeta H, Figueiredo DD, Rodrigues M, Cordeiro A, Abreu IA, et al. Isolation and characterization of rice (*Oryza sativa* L.) E3-ubiquitin ligase OsHOS1 gene in the modulation of cold stress response. Plant Mol Biol. 2013;83,351-63. https://doi.org/10.1007/s11103-013-0092-6

43. Al-Saharin R, Hellmann H, Mooney S. Plant E3 ligases and their role in abiotic stress response. Cells. 2022;11:890. https://doi.org/10.3390/cells11050890

44. Lu X, Liu W, Xiang C, Li X, Wang Q, Wang T, et al. Genome-wide characterization of GRAS family and their potential roles in cold tolerance of cucumber (*Cucumis sativus* L.). Int J Mol Sci. 2020;21(11):3857. https://doi.org/10.3390/ijms21113857

45. Tong N, Li D, Zhang S, Tang M, Chen Y, Zhang Z, et al. Genome-wide identification and expression analysis of the GRAS family under low-temperature stress in bananas. Front Plant Sci. 2023;14:1216070. https://doi.org/10.3389/fpls.2023.1216070

46. Peng G, Xin X, Hua J, Yan D. Cloning and functional studies of maize ZmSCL7. Chin Agric Sci. 2013;46(12):2584-91. https://doi.org/10.3864/i.issn.0578-1752.2013.12.020

47. Li LL, Zeng WJ, Li YH, Ge FW, Lu H, Lei W, et al. Analysis of GRAS transcription factors and Lascl18 gene cloning and cold correlation. Mol Plant Breed. 2017;15(9):3428-37. https://doi.org/10.13271/j.mpb.015.003428

48. Ye JJ. Cold temperature stress response and promoter sequence analysis of ERF 6 and GRAS genes in citrus plants. Hang Zhou: Zhejiang Normal University; 2013.

49. Yan JQ, Wang J, Li QT, Hwang JR, Patterson C, Zhang H, et al. AtCHIP, A U-box-containing E3 ubiquitin ligase, plays a critical role in temperature stress tolerance in *Arabidopsis*. Plant Physiol. 2003;132(2):861-69. https://doi.org/10.1104/pp.103.020800

50. Fang H, Meng Q, Zhang H, Huang J. Knockdown of a RING finger gene confers cold tolerance. Bioengineered. 2016;7(1):39-45. https://doi.org/10.1007/s11103-015-0294-1

51. Li J, Yang Y, Iqbal A, Qadri R, Shi P, Wang Y, et al. Correlation analysis of cold related gene expression with physiological and biochemical indicators under cold stress in oil palm. PLoS One. 2019;14(11):e0225768. https://doi.org/10.1371/journal.pone.0225768 .