INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN THE RESPONSE TO COLD IN *Arabidopsis thaliana*

Tesis que presenta

Natalia Beatriz Torres-Serrallonga

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Juan Francisco Jiménez Bremont

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Constancia de aprobación de la tesis

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Dr. Juan Francisco Jiménez Bremont
Director de la tesis

Dr. Ángel Gabriel Alpuche Solís
Miembro del Comité Tutoral

Dr. Braulio Gutiérrez Medina
Miembro del Comité Tutoral

Dra. Margarita Rodríguez y Domínguez Kessler
Miembro del Comité Tutoral

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MAESTRA EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Natalia Beatriz Torres Serrallonga

sobre la Tesis intitulada:

Identificación y caracterización de genes involucrados en la respuesta a frío en Arabidopsis thaliana

que se desarrolló bajo la dirección de

Dr. Juan Francisco Jiménez Bremont

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Dedicatoria

A las personas más importantes en mi vida

y que más amo en el mundo,

Mi mamá y mi abuelita

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Resumen

Identificación y caracterización de genes involucrados en la respuesta a frío en *Arabidopsis thaliana*

Las plantas han desarrollado mecanismos bioquímicos y moleculares para hacer frente al estrés por frío. Si bien, estudios recientes de secuenciación del genoma de plantas han proporcionado información acerca de los complejos mecanismos transcripcionales que operan durante el estrés por frío, los mecanismos de regulación y adaptación involucrados no son del todo conocidos. El presente trabajo se divide en dos capítulos. En el primero, se realizó una secuenciación masiva del transcriptoma de *Arabidopsis thaliana* bajo tres temperaturas de frío 0ºC, 4ºC y 10ºC durante 24h**.** Considerando los 50 genes más inducidos en cada condición, 7 genes se encuentran compartidos entre las tres temperaturas. Con base en estos resultados, nosotros analizamos la expresión de 5 genes, en plántulas de Arabidopsis sometidas a 0ºC, 4ºC y 10ºC mediante qRT-PCR, en donde observamos una respuesta diferencial a los 3 tiempos analizados (24, 48 y 72h). En el segundo capítulo, se evaluaron los niveles de expresión de los cuatro genes que codifican para dehidrinas ácidas que posee *A. thaliana,* bajo las mismas temperaturas de frío, durante 24, 48 y 72h. Observamos que estas dehidrinas presentan diferente expresión con relación al tiempo y la temperatura, principalmente en 0ºC y 4ºC. Con los resultados obtenidos en este estudio, se aporta información relevante para profundizar en los mecanismos mediante los cuales las plantas sobreviven a bajas temperaturas.

Palabras clave: Estrés por frío, mecanismos de regulación transcripcional, dehidrinas

Abstract

Identification and characterization of genes involved in the response to cold in *Arabidopsis thaliana*

Plants have developed biochemical and molecular mechanisms to cope with cold stress. Recent genome sequencing studies of plants have provided information about the complex transcriptional mechanisms that operate during this stress. However, the mechanisms of regulation and adaptation to cold stress in plants are not completely known. The present study is divided into two chapters. In the first one, a massive sequencing of the *Arabidopsis thaliana* transcriptome under three cold temperatures 0ºC, 4ºC y 10ºC during 24h was made. Taking into consideration the fifty most highly induced genes on each condition, 7 genes are shared between the three temperatures. Based on these results, we analyzed the expression of five genes on Arabidopsis plants, under 0ºC, 4ºC y 10ºC through qRT-PCR, where we observed a differential response on the three analyzed periods of time (24, 48 and 72h). On the second chapter, the expression levels of the four acidic dehydrin genes of *A. thaliana* were evaluated under the same cold temperatures during 24, 48 and 72h. We observed that these dehydrins display different expression patterns in relation to time and temperature, mainly under 0ºC and 4ºC. With the results obtained in this study, relevant information is contributed to delve into the mechanisms by which plants survive under low temperatures.

Keywords: Cold stress, transcriptional mechanisms, dehydrins

CAPÍTULO I

Identificación de genes expresados diferencialmente durante la respuesta a frío en *Arabidopsis thaliana*

1.1 Resumen

Las plantas son altamente sensibles al estrés térmico y responden a éste en diferentes escalas de tiempo. Posterior a la exposición de las plantas al frío, se induce una serie de alteraciones bioquímicas y fisiológicas a nivel molecular y celular para auxiliar al crecimiento de la planta y su supervivencia bajo condiciones de estrés por frío. Algunos de los genes que se inducen en esta condición se encuentran directamente involucrados en la defensa a frío a través de la biosíntesis de compuestos osmóticos, la generación de antioxidantes y el incremento de la fluidez de la membrana; otros pueden funcionar en la regulación de la expresión génica y la transducción de señales. Sin embargo, cómo las plantas pueden continuar con su crecimiento y desarrollo bajo estas condiciones, no está completamente comprendido. En el presente estudio, con el objetivo de analizar los cambios en el perfil transcriptómico de *Arabidopsis thaliana* bajo temperaturas de frío, plántulas de 14 días de edad fueron sometidas a 0ºC, 4ºC y 10ºC durante 24h. Utilizando el análisis transcriptómico RNAseq, encontramos que entre las tres temperaturas siete genes se encuentran compartidos (UGT78D3, ELIP2, LTI78, HSP17.6, GPT2, At1g80130 y GOLS3). Además, entre los 50 genes más inducidos en las tres diferentes condiciones, se encuentran algunos previamente reportados en la respuesta a frío, tales como proteínas anticongelantes, dehidrinas ácidas y genes regulados por frío (KIN1, LTI65, LTI78, COR15, ERD10 y COR47 entre otros). Validamos estos cambios transcripcionales mediante qRT-PCR de cinco genes

seleccionados: At1g80130, At1g71000, At5g05220, At1g59530 (AtBZIP4) y At1g09350 (GOLS3). Los resultados aquí presentados, nos brindan un punto de partida para continuar investigando aquellos genes que nos puedan ayudar a comprender el proceso mediante el cual las plantas sobreviven bajo temperaturas de frío.

Palabras clave: Bajas temperaturas, *Arabidopsis thaliana,* RNA-seq, expresión génica diferencial

CHAPTER I

Identification of genes differentially expressed during the response to cold in *Arabidopsis thaliana*

1.2 Abstract

The plants are highly sensitive to temperature stress and respond over different time scales. After the plants exposure to cold, a series of biochemical and physiological alterations at the molecular and cellular level are induced to aid plant growth and survival under cold stress conditions. Some of the genes induced in this condition are directly involved in defense to cold through biosynthesis of osmotic compounds, generation of antioxidants and increase in membrane fluidity, and other may function in regulation of gene expression and signal transduction. However, how the plants can continue their growth and development under these conditions, is not fully understood. Here, in order to analyze the transcriptome profiling changes in response to cold temperatures in *Arabidopsis thaliana*, 14 days old plantlets were subjected to 0ºC, 4ºC and 10ºC during 24h. Using the RNAseq transcriptome analysis, we found that between the three temperatures seven genes are shared (UGT78D3, ELIP2, LTI78, HSP17.6, GPT2, At1g80130 y GOLS3). Moreover, among the fifty most highly induced genes in the three different conditions, are some that have been previously reported for being involved in the response to cold such as anti-freeze proteins, acidic dehydrins and cold regulated genes (KIN1, LTI65, LTI78, COR15, ERD10, COR47, among others). We validated these transcriptional changes through qRT-PCR of five selected genes: At1g80130, At1g71000, At5g05220, At1g59530 (ATBZIP4) and At1g09350 (GOLS3).

The results here presented gives us a start point to investigate the genes that can help us understand the plant's process to survive under cold temperatures.

Kewywords: Low temperatures, *Arabidopsis thaliana,* RNA-seq, differential gene expression

1.3 Introduction

In plant physiology, the stress is defined as any environmental factor, biotic or abiotic that diminishes the rate of any physiological process (growth, photosynthesis, etc) below the optimal or maximal rate that it could reach. The abiotic stress can be divided into physical or chemical depending on the causal agent. Among the physical factors are: the excess or deficit of water, extreme temperatures, salinity and ultraviolet radiation [1]. As for the chemical factors, the environmental contamination by heavy metals, toxins, salinity and deficit of minerals can be mentioned [2, 3].

Abiotic stress results in a series of morphological, physiological, biochemical and molecular changes that affect the plant's productivity and growth. Factors such as drought, salinity, extreme temperatures and oxidative stress are frequently interconnected and can induce similar cellular damages [1, 2, 3]. As a consequence, these types of environmental stress activate similar signaling pathways and cellular responses, such as: antistress protein production, antioxidant enzymes regulation and compatible solute accumulation [3, 4, 5, 6]. In particular, temperature is determinant in diverse cellular and physiological processes such as cellular division, photosynthesis, respiration, sugar accumulation, germination and nutrient absorption, among others [7, 8]. Just as temperature is fundamental for these metabolic processes, it can also be a barrier for the adequate growth and development of the plant. Although various physiological processes normally work under a temperature range between 0ºC to 40ºC (depending on the plant species), temperatures below 13ºC are associated with damage by cold [9]. Therefore, low temperatures are one of the main abiotic factors that

limit the growth and geographical distribution of crops, as well as their quality and production [4, 7].

At the physiological level, the most common effects of low temperatures in plants are: the decrease in the growth rate, chlorosis and injuries on leaves like withering, (which occurs when the root is directly affected). In order to cope with the stress by cold, different plant species have developed physiological and molecular adaptations to increase their tolerance to low temperatures; including the regulation of gene expression [10, 11, 12].

Such stress-responsive signal transduction pathways have been previously studied [3, 9], but there aren't many studies using a next- generation sequencing technology like RNA-Seq, which provides information on gene expression independent of genomic sequence knowledge. Likewise, RNA-Seq has also the advantage of higher sensitivity and greater dynamic range of gene expression than array- based technologies [13, 14]. Considering the above information and knowing there are few studies evaluating the response to different cold temperatures using this technology, in this study we used the model plant *Arabidopsis thaliana*, which was subjected to three different low temperatures (0ºC, 4ºC and 10ºC) during 24h. Using RNA-seq, we analyzed changes in the transcriptome of *A. thaliana* induced by low temperatures. This strategy leads to the understanding of the mechanisms by which the plant can survive in adverse temperature conditions, such as cold.

1.4 Material and Methods

1.4.1 Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) were surface-sterilized for 5 min with 20% chlorine solution and rinsed five times in sterile distilled water. Aseptic stratified and vernalized seeds (2 days at 4ºC) were germinated and grown *in vitro* for two weeks on plates containing Murashige and Skoog medium (0.5X) on a growth chamber with controlled temperature conditions (22ºC±1ºC) under 16h light (13,000lux)/ 8h. Fourteen days old plantlets were subjected to low temperatures of 0ºC, 4ºC and 10ºC during 24h, 48h and 72h, the control plants were kept on the growth chamber at 22ºC. After the stress treatment, plants were collected, immediately frozen in liquid nitrogen and stored at -80ºC, until used. For each temperature condition, three biological replicates were made, each consisting in groups of 12 plants.

1.4.2 RNA extraction

Total RNA was extracted from plant material using the ConcertTM Plant RNA Reagent protocol (Invitrogen Life Technologies. Catalogue no. 12322-012). The concentration of RNAs was measured using a NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc., Wilmington,DE, USA). The structural integrity of the RNAs was checked in a 1% agarose and TBE 0.5X gel. Only the samples with 24h of treatment were prepared to be shipped according to the RNAstable® protocol for room temperature preservation of RNA to be analyzed through RNA-seq.

1.4.3 Illumina RNA-Seq sample preparation

Total RNA of samples from the control and cold treated plants underwent a successive procedure consisting of mRNA enrichment by oligo(dT)- attached magnetic beads, mRNA fragmentation, cDNA synthesis with random hexamer-primer, addition of the tailing A and ligation of the adapters, the suitable fragments were purified and selected for sequencing templates by the Illumina Hiseq^{TM} 2500 RNA-seq system.

1.4.4 Mapping sequence reads to the reference genome and gene hit counts extraction

Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The reads were then mapped to the *Arabidopsis thaliana* TAIR10 reference genome available on ESEMBL using the STAR aligner. The RNA-seq aligner was executed using a splice aligner which detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique exon hit counts were calculated using Feature counts from the Subread package.

1.4.5 Comparison of Gene Expression

After mapping and total gene hit counts calculation, the total gene hit counts was used for downstream differential expression analysis using DESeq2. Each temperature condition was compared with the control condition at 22ºC. Genes with adjusted p-value <0.05 and absolute Log2Fold Change >1 were called as significant differentially expressed genes for each comparison.

1.4.6 Validation of differential expression using qRT-PCR

Real- time quantitative RT-PCR was carried out using the StepOne™ Real-Time PCR System (Applied Biosystems. Catalogue no. 4376374). The elongation factor alpha (*EF1α*) gene was used as internal control and amplified in parallel with the target gene allowing gene expression normalization and providing quantification. The list of the genes and primers used are listed in Table 1. Detection of real-time RT-PCR products was done using SYBR® Green Supermix protocol (Applied Biosystems). Dilutions of 100ng of the cDNAs were used as a template for PCR. The PCR cycling conditions comprised 40 PCR cycles of 10s at 95ºC (denature) and 30s at 60ºC (anneal/extend). At the end of the PCR runs, melting curves were performed starting at 65ºC and gradually increasing the temperature every 0.5ºC up to 95ºC. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results.

Table 5 Primers used for qRT-PCR

GENE/LOCUS	DESCRIPTION	PRIMERS
EF1α. At5g60390	GTP binding elongation factor Tu family protein. GTPase activity, translation elongation factor activity.	FW- TCCAGC TAAGGGTGCCGC RV- AACGCCTGTCAATCTTGGTCA
GOLS3 At1g09350	Predicted to encode a galactinol synthase. Involved in response to cold and response to oxidative stress	FW- GTGCCAAAGCTCCATCCGC RV-ACAATTTCCTAAGTAAACATCACCAG
At1g80130	Encodes a tetratricopeptide repeat (TPR)-like superfamily protein which is known to respond to oxidative stress	FW- GGCCATGTGCCTCCAACTA RV-TTTACCTAAATACGAACATTCACGA
ATBZIP4 At1g59530	Basic leucine-zipper 4. Involved in regulation of transcription, has DNA binding transcription factor activity	FW- GGCCATGCAATCGCCAATCT RV- CGTTTGACCTTTACAAAAATGCTCA
At1g71000	Encodes a chaperone DNAJ-domain superfamily protein. Involved in protein folding.	FW- CACAGATGTCTCTACCGCTTTC RV- CACAGTCCGAGTCTCCTAGAG
At5g05220	Hypothetical protein	FW- CATCAAAGAACAGAGAGAGCAGTC RV- GAGGTACAACGGTACAAGGAATC

1.4.7 Data Analysis

The cycle number at threshold (Ct value) was used for calculations of relative mRNA expression levels. The Ct value from each target gene was normalized by subtraction of the Ct value from the *EF1α* gene. The fold change in gene expression relative to control samples (plants kept at 22ºC for each period of time) was calculated using the $2^{-\Delta\Delta Ct}$ method [15]. In the case of ratios being lower than 1, the inverse ratio was estimated and graphed as negative.

Statistical significance was estimated by One-way ANOVA and t test at $p < 0.05$ through GraphPad Prism version 6.0 (GraphPad Software, California, USA).

1.5 Results

In order to analyze the negative effect of cold stress, 14 days old plantlets of *Arabidopsis thaliana* were subjected to 0ºC, 4ºC and 10ºC during 24h (Figure 1).

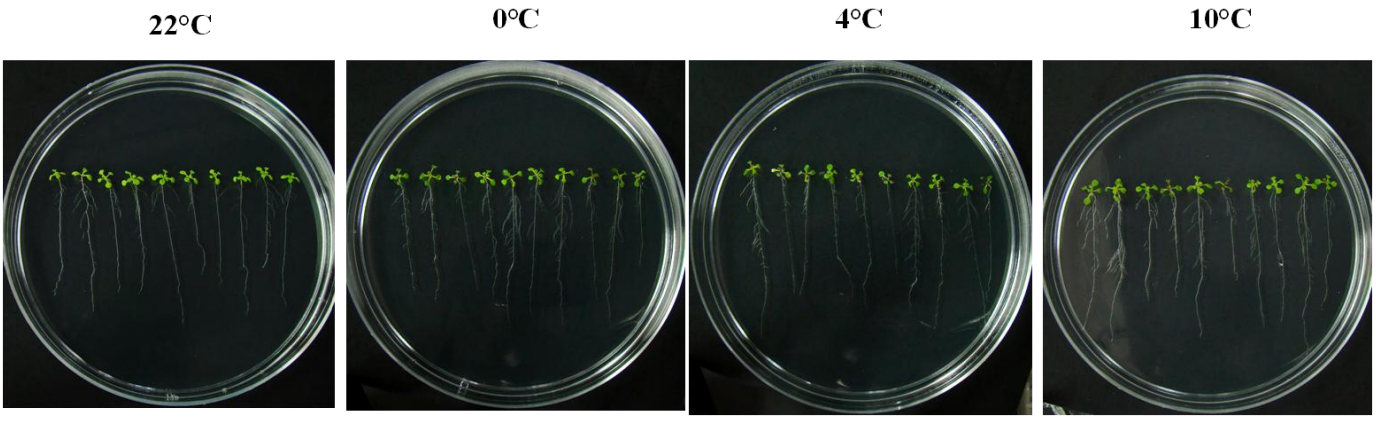

Figure 8 Plantlets of *Arabidopsis thaliana* exposed to cold. Representative plate from each temperature treatment (0ºC, 4ºC and 10ºC) with its control plants at 22ºC. The 14 days old plantlets were subjected to low temperatures for 24h.

1.5.1 RNA sequencing results. Three replicates of each temperature used for cold treatment (0ºC, 4ºC and 10ºC) during 24h, as well as the control at 22ºC, were subjected to RNA sequencing using Illumina Hiseq 2500 sequencing platform. High quality base reads were obtained. The raw reads were trimmed by removing low quality reads and the adapters, after which the mapped ratio reached at least 95% (Table 2).

Table 6 RNAseq mapping statistics. Samples of each temperature condition and their three replicas are shownned

SAMPLE ID	TOTAL READS	TOTAL MAPPED READS	%TOTAL MAPPED READS	UNIQUE MAPPED READS	%UNIQUE MAPPED READS
22°C-R1	32,056,705	31.486,416	98.22	30,973,138	96.62
22°C -R2	38,984,602	38,247,047	98.11	37,639,752	96.55
22°C -R3	33,296,040	32,673,432	98.13	32,133,476	96.51
0°C -R1	31,702,257	31,097,234	98.09	30,663,876	96.72
0°C -R2	33,838,903	33,172,828	98.03	32,618,617	96.39
0°C -R3	33,770,077	33,107,231	98.04	32,631,557	96.63
4°C -R1	40,469,591	39,690,258	98.07	39,033,516	96.45
4°C -R2	32,324,382	31,689,656	98.04	31,170,645	96.43
4°C -R3	34,580,145	33,867,285	97.94	33,280,836	96.24
10°C -R1	36,694,539	35,888,243	97.8	35,318564	96.25
10°C -R2	34,371,063	33,661,531	97.94	32,935,245	95.82
10°C -R3	32,319,648	31,717,542	98.14	31,232,005	96.63

We organized and selected the fifty most highly induced genes in each condition (0ºC, 4ºC and 10ºC) based on their Log^2 fold change with a range of 10.9 to 3.4. Tables 3, 4 and 5 show the 50 most highly induced genes of each condition respectively, with their expression value and a brief description of them.

Table 7 Genes differentially expressed at 0ºC in Arabidopsis. The fifty most highly expressed genes under the 0ºC treatment respecting the control condition at 22ºC. They are organized according to their Log2 fold change and a description of each of them is presented. **.*Two columns of 25 genes are shown.**

GENE	LOG2FOLD CHANGE	DESCRIPTION	GENE	LOG2FOLD CHANGE	DESCRIPTION
AT2G33090	10.9129	Transcription elongation factor (TFIIS) family protein	**SAMDC2**	7.3949618	Adenosylmethionine decarboxylase 2
AT1G71000	10.13952	Encodes a chaperone DNAJ-domain superfamily protein	**AT2G42150**	7.3747765	DNA-binding bromodomain-containing protein
AT5G48270	10.025916	DUF868 family protein (DUF868)	**AT3G17130**	7.2173385	Plant invertase/pectin methylesterase inhibitor superfamily protein
AT5G05220	9.9831736	Hypothetical protein	**ATBZIP4**	7.2041449	Basic leucine-zipper 4
UGT78D3	9.8228193	UDP-glucosyl transferase 78D3	**AT2G40925**	7.190817	F-box and associated interaction domains-containing protein
GA2OX3	9.4638171	Gibberellin 2-oxidase 3	**HSP22.0**	7.1699754	HSP20-like chaperones superfamily protein
GOLS3	9.1697358	Predicted to encode a galactinol synthase	**AT1G19410**	7.1072775	FBD / Leucine Rich Repeat domains containing protein
AT1G21940	9.0546497	Transmembrane protein	**HSP17.6**	7.0847447	Heat shock protein 17.6
DOF1.3	8.7736729	Dof-type zinc finger DNA-binding family protein	**AT2G17660**	7.0230436	RPM1-interacting protein 4 (RIN4) family protein
AT3G14820	8.7116409	GDSL-like Lipase/Acylhydrolase superfamily protein	**AT4G12210**	7.0065589	RING/U-box superfamily protein
LEA4-5	8.5095599	Late Embryogenesis Abundant 4-5	**AT5G50361**	7.0021861	Hypothetical protein
AT4G08145	8.4690487	Transposable element gene	**AT2G05580**	6.9888265	Glycine-rich protein family
AT1G04570	8.1741089	Transposable element gene	**PUB19**	6.9568079	ARM repeat superfamily protein
AT2G24560	8.0898769	GDSL-like Lipase/Acylhydrolase family protein	**AT1G67856**	6.867526	RING/U-box superfamily protein
HSP17.7	7.9250445	Heat shock transcription factor A9	**GPT2**	6.8490891	Glucose-6-phosphate/phosphate translocator 2
AT1G16850	7.8677689	Transmembrane protein	**LTI65**	6.8276093	Low temperature responsive protein 65
AT1G70640	7.8677185	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	**AT1G80130**	6.7816499	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1G21529	7.7196249	DRIR. Long non coding RNA	**AT2G05441**	6.7643063	Pseudogene. Unknown protein
ADH1	7.6880703	Alcohol dehydrogenase1	**DEP**	6.7319222	RING finger protein involved in ABA sensitivity during seed development. Regulates the expression of ABI3
ELIP2	7.651798	Early light inducible protein 2	**PP2B8**	6.7256226	Putative F-box protein
ENODL5	7.6219376	Early nodulin-like protein 5	**AT1G69140**	6.720498	Pseudogene. Pseudogene of hypothetical protein
CML37	7.5585191	Calmodulin like 37	**AT5G45630**	6.6962137	Senescence regulator (Protein of unknown function, DUF584)
AT1G43895	7.4504343	Pseudogene of Protein kinase superfamily protein	**AT3G55580**	6.6826216	Regulator of chromosome condensation (RCC1) family protein
AT1G05330	7.430968	Hypothetical protein	**AT5G03920**	6.6641199	F-box protein
LTI78	7.4084199	Low-temperature-responsive protein 78	**DREB2H**	6.6395883	Dehydration-responsive element binding protein.

Table 8 Genes differentially expressed at 4ºC in Arabidopsis. The fifty most highly expressed genes under the 4ºC treatment respecting the control condition at 22ºC. They are organized according to their Log2 fold change and a description of each of them is presented.

GENE	Log2 FOLD CHANGE	DESCRIPTION	GENE	Log2 FOLD CHANGE	DESCRIPTION
UGT78D3	9.879588017	UDP-glucosyl transferase 78D3	**AtRLP33**	6.266244819	Receptor like protein 33
GOLS3	9.064345253	Predicted to encode a galactinol synthase	**AT1G64340**	6.082859888	Serine/Threonine-kinase
BGLU27	8.698683723	Beta glucosidase 27	**KIN1**	6.075860126	Stress responsive protein
AT1G09360	8.683023131	Plant invertase/pectin methulesterase inhibitor superfamily	**AT2G05610**	5.936358397	Transposable element gene
AT2G24560	8.294406657	GDSL-like lipase/Acylhydrolase family protein	**AT2G23910**	5.911444148	NAD(P)-binding Rossmann-fold superfamily protein
ELIP2	8.010645315	Early light- induced protein	**AT5G05220**	5.870417147	Hypothetical protein
AT5G13655	7.968835816	Transposable element gene	**HSP17.6**	5.843744023	Heat shock protein 17.6
AT5G35965	7.963579848	Transposable element gene	**DOF1.3**	5.812416068	Dof-type zinc finger DNA-binding family protein
AT1G70640	7.550448928	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	**AT3G17130**	5.811828977	Plant invertase/pectin methylesterase inhibitor superfamily
AT5G44990	7.477690314	Glutathione S-transferase family protein	**ATBZIP4**	5.804246153	Basic leucine-zipper 4
AT1G16850	7.344269957	Transmembrane protein	**ERD10**	5.798966705	Early responsive to dehydration 10
LTI78	7.27990811	Low temperature responding protein 78	**ERF115**	5.77432596	Integrase-type DNA-binding superfamily
AT1G04570	7.235721814	Major facilitator superfamily protein	**ENODL5**	5.71160867	Early nodulin-like protein 5
PXMT1	7.089128841	S-adenosyl-L-methionine dependent methyltransferases superfamily protein	**GPT2**	5.696855492	Glucose-6-phosphate/phosphate translocator 2
AT4G08145	7.068986636	Transposable element gene	**AT5G50360**	5.60827112	Von willebrand factor A domain protein
AT5G66780	7.022611213	Late embryogenesis abundant protein	**AO**	5.580098397	L-aspartate oxidase involved in the early steps of NAD biosynthesis
COR15A	6.936258277	Cold regulated gene	**COR15B**	5.481676586	Cold regulated gene
ADH1	6.893844175	Alcohol deshydrogenase 1	**AT1G06540**	5.480853264	Hipothetical protein
LEA4-5	6.831654347	Late embryogenesis abundant protein	**HSP17.7**	5.447784242	Heat shock transcription factor A9
GSTU12	6.791246141	Glutathione S-transferase TAU12	**KIN2**	5.430969023	Stress responsive protein
AT3G04150	6.695851399	RmlC-like cupins superfamily protein	**AT4G01985**	5.369394209	Hypothetical protein
UGT91A1	6.486644596	UDP-glucosyl transferase 91A1	**UGT78D4**	5.35864783	UDP-glucosyl transferase 78D4
AT1G80130	6.458284003	Tetratricopeptide repeat (TPR)-like superfamily protein	**AT2G44800**	5.320886799	2-oxoglutarate (2OG) and Fe (II)-dependet oxygenase superfamily protein
MIR858A	6.452993007	MicroRNA ath-MIR858a precursor	**AT5G62210**	5.281767512	Embryo-specific protein 3
AT1G69140	6.402959976	Pseudogene of hypothetical protein	**AT5G17460**	5.268862727	Glutamyl-tRNa (Gin) amidotransferase subunit C

.*Two columns of 25 genes are shown.

Table 9 Genes differentially expressed at 10ºC in Arabidopsis. The fifty most highly expressed genes under the10ºC treatment respecting the control condition at 22ºC. They are organized according to their Log2 fold change and a description of each of them is presented.

GENE	Log2 FOLD CHANGE	DESCRIPTION	GENE	Log2 FOLD CHANGE	DESCRIPTION
ELIP2	7.49435231	Early light- induced protein	**AT1G04570**	3.98938634	Major facilitator superfamily protein
AT5G13655	7.31164448	Transposable element gene	**AT1G67856**	3.98875898	RING/U-box superfamily protein
SYP124	6.55425064	Syntataxin of plants 124	**AT5G16960**	3.97553053	Zinc-binding dehydrogenase family protein
MYB90	6.10617869	Myb domain protein 90	**DOF1.3**	3.96466282	Dof-type zinc finger DNA-binding family protein
GPT2	5.98435291	Glucose-6-phosphate/phosphate translocator 2	**TBL26**	3.95332925	Trichome birefringence-like 26
UGT78D3	5.62438685	UDP-glucosyl transferase 78D3	**COR15A**	3.90489141	Cold regulated gene
AT4G12735	5.22551335	Hypothetical protein	**AT2G04460**	3.88836525	Transposable element gene
DTXL2	5.12887124	Detoxifying efflux carrier for plant-derived antibiotics and other toxic compounds	**AT5G54450**	3.80369128	Hypothetical protein (DUF295)
AT4G03292	4.91394147	Polynucleotidyl transferase, ribonuclease H-like superfamily protein	**UGT73C1**	3.7604034	UDP-glucosyl transferase 73C1
CRC	4.87074305	Plant specific transcription factor YABBY family protein	**SAUR41**	3.74001075	SAUR-like auxin responsive protein family
SNOR30	4.72779749	snoRNA	**CYP710A3**	3.67319748	Cytochrome P450, family 710, subfamily A, polypeptide 3
AT1G70640	4.64156669	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	**GSTF12**	3.66252689	Glutathione S-transferase phi 12
AtcwINV5	4.35184972	Cell wall invertase 5	**FH12**	3.65481356	SEC14-like 12
AT1G32880	4.29778906	ARM repeat superfamily protein	**A3G2XYLT**	3.64870001	Anthocyanidin-3-O-glucoside 2”-O-xylosultransferase
GOLS3	4.22082971	Predicted to encode a galactinol synthase	**DTXL1**	3.61541155	Detoxification protein
LTI78	4.18554839	Low temperature responding protein 78	**ERF071**	3.61437553	Ethylene-responsive transcription factor
LHY	4.17443114	Homeodomain-like superfamily protein	**AT2G41730**	3.57189944	Calcium-binding site protein
DTX1	4.17005612	Protein detoxification	**COR15B**	3.56425904	Cold regulated gene
DFRA	4.12111535	Dihydroflavonol 4-reductase	**AtRLP33**	3.56299136	Receptor like protein 33
AT1G06540	4.0970328	Hypothetical protein	**SULTR1;3**	3.51259986	Sulfate transporter 1.3
AT1G16635	4.05920309	Other RNA	**GBSS1**	3.49314782	UDP-Glycosyltransferase superfamily protein
KIN1	4.05053885	Stress responsive protein	**AT1G80130**	3.49007253	Tetratricopeptide repeat (TPR)-like superfamily protein
AT5G62210	4.02954394	Embryo-specific protein 3	**FLS1**	3.47934443	Flavonol synthase 1
AKR4C9	4.00526682	NAD(P)-linked oxidoreductase superfamily protein	**CHS**	3.46922293	Disease resistance protein
HSP17.6	3.99335997	Heat shock protein 17.6	**AT3G15650**	3.4381724	Alpha/beta- hydrolases superfamily protein

.*Two columns of 25 genes are shown.

Taking into consideration these fifty genes on each condition, we found that between the three conditions (0ºC, 4ºC and 10ºC) seven genes were shared, being 4ºC and 10ºC the two temperatures that shared more genes with 17 of them, followed by 0ºC and 4ºC with six genes. Meanwhile, between 0ºC and 10ºC only two genes were shared (Figure 2).

Among these fifty genes, they were included some that had been previously reported to respond under low temperatures such as KIN1, LTI65, LTI78, COR15a, COR15b, ERD10, COR47 and GOLS3 [3, 16, 17, 18, 19, 20].

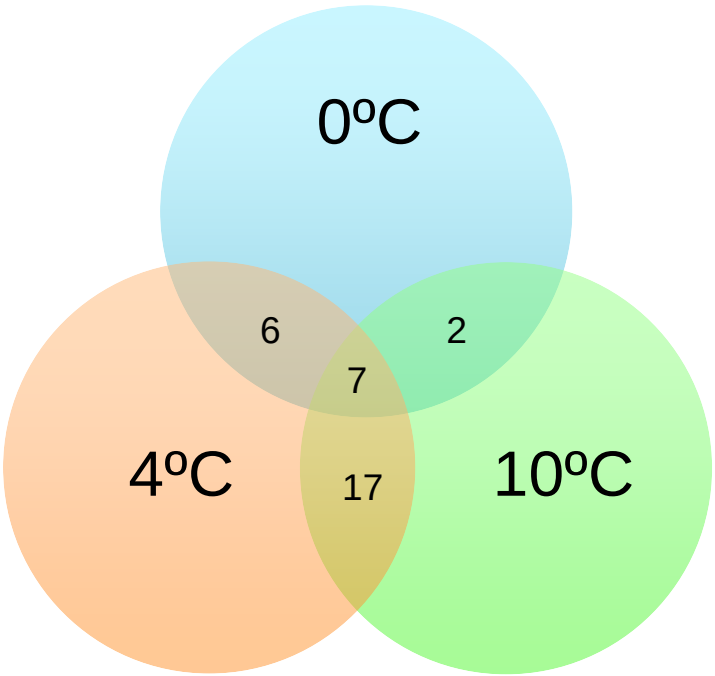

Figure 9 Number of differentially expressed genes in the three conditions. The Venn diagram shows the common genes induced under the three low temperature conditions. The significantly different genes were defined as transcripts with fold changes ≥3.

1.5.2 Validation of differential expression using qRT-PCR.

To confirm the DEGs induced by the three low temperatures, we performed qRT-PCR analysis of the five selected genes that were differentially expressed under the low temperatures treatments at 24h; we also examined these genes in cDNA samples of plants exposed to 48h and 72h at 0ºC, 4ºC and 10ºC, in order to analyze their behavior through time.

Figure 3 shows the differential expression of GOLS3, a gene which has been previously reported under cold temperature conditions. Here, we observed that its most higher expression was at 4ºC treatment during 24h of exposure, with levels of transcript reaching near 1400-fold respecting control at 22ºC, which decreased at 48h (almost 300-fold) but still represents a statistically significant difference, and by 72h of exposure the transcript levels of GOLS3 were similar to control. A later response was observed for the GOLS3 gene under 0ºC, being its maximum expression levels at 48h with 600 times above control, which remained high through the last 72h of exposure (500-fold). In the 10ºC condition, we observed an induction of the GOLS3 gene at 24 and 72h, but they did not represented statistically significant differences.

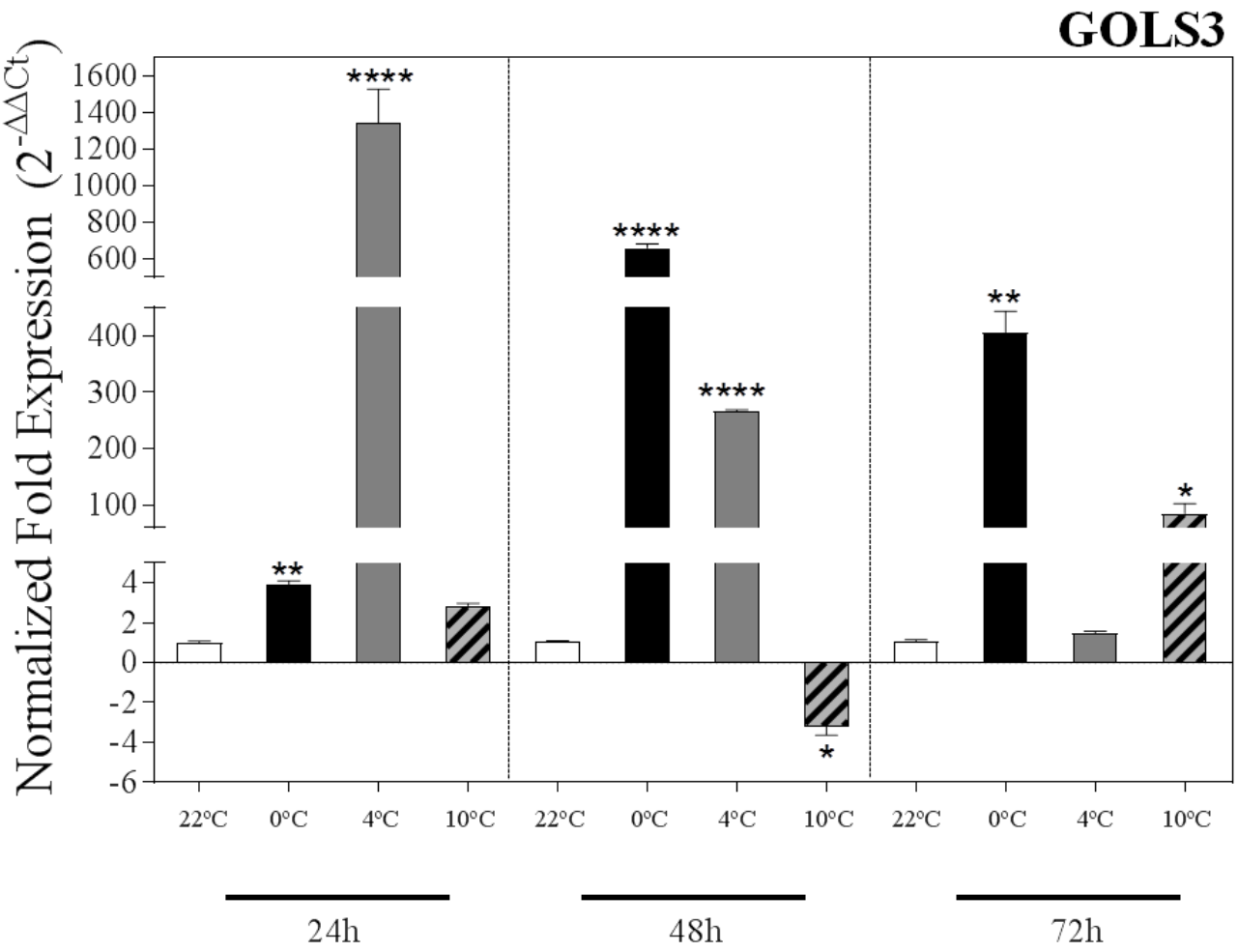

Figure 10 qRT-PCR validation of GOLS3. mRNA expression levels of GOLS3 under low temperature conditions of 0ºC, 4ºC and 10ºC, during 24, 48 and 72h of exposure . The qPCR reactions were performed using cDNA of three replicates for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC.

We also selected the *At1g80130* gene, which was present among the three conditions, and we observed that at 0ºC, the tendency was to increase the transcript levels through time (Figure 4), from similar levels to control at 22ºC at 24h, to 32.7-fold at 72h. Whereas the behavior under the 4ºC and 10ºC condition, shared the opposite tendency decreasing the expression of *At1g80130* through time, reaching its highest levels under the 4ºC condition at the first 24h of exposure with above 103-fold respecting control. Overall, higher levels of transcript were observed in the qRT-PCR than those collected from the sequencing data where, very similar levels were observed for 0ºC and 4ºC on 24h of exposure (6.78 and 6.45 respectively).

Figure 5 shows the expression of *At1g71000* gene, which encodes a chaperone DNAJ-domain superfamily protein. Interestingly, on the data from the RNAseq at 24h this gene only appears induced under the 0ºC condition, with transcript levels very similar with those showed here (10-fold respecting control), but we also observed a higher expression at 4ºC of almost 50 times above control, and on the collected data it doesn't appear under this condition. On the following hours of exposure, we observed that at 0ºC, the transcript levels increased significantly up to 1518-fold and, although they diminished a little bit on the next 72h, it remained high (554-fold). On the other hand, the expression under the 4ºC condition decreased through time so that, by 72h of exposure the gene was repressed regarding control. Finally, at 10ºC no statistically significant differences were observed in the expression levels of *At1g71000* gene.

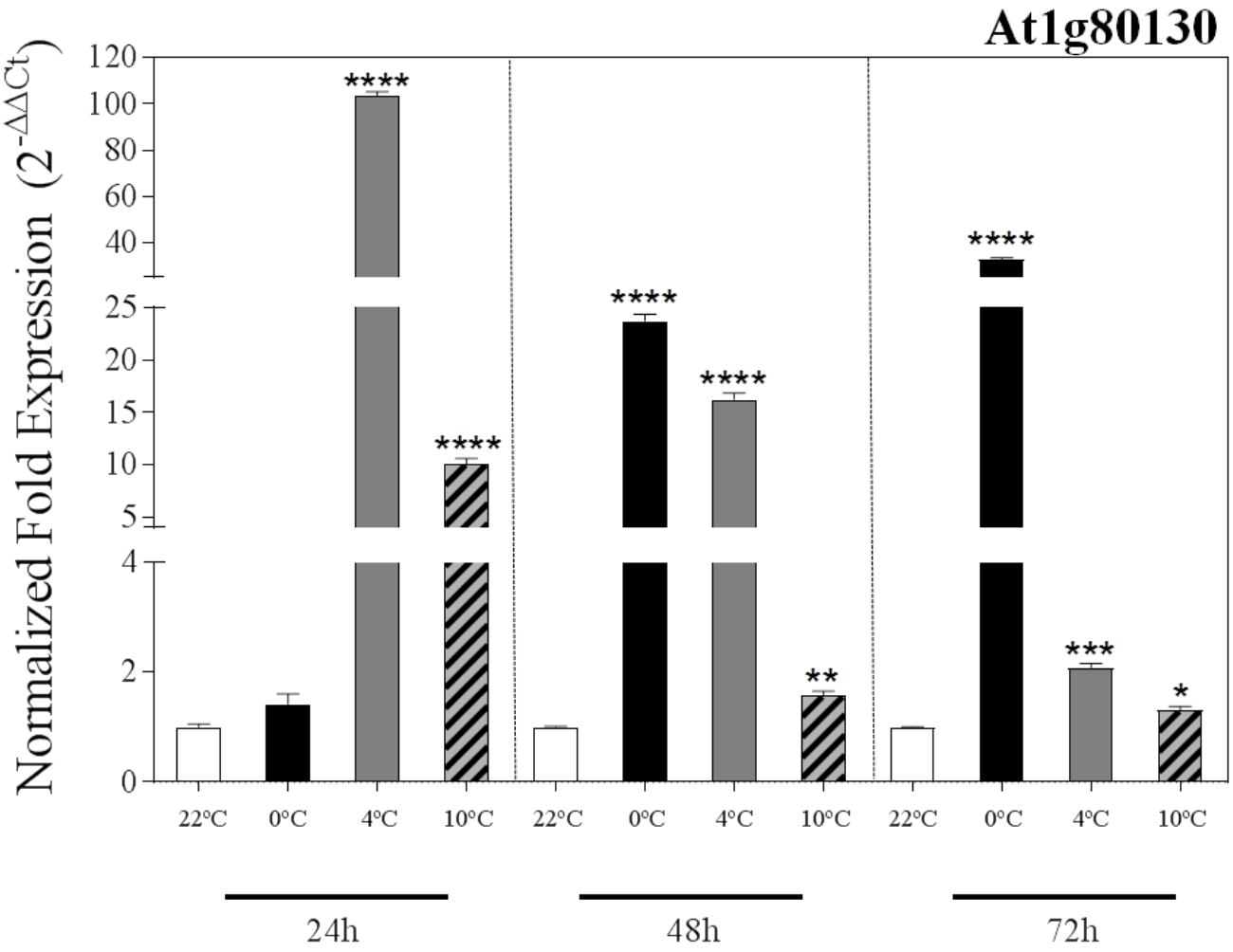

Figure 11 qRT-PCR validation of At1g80130. mRNA expression levels of At1g80130 under low temperature conditions of 0ºC, 4ºC and 10ºC, during 24, 48 and 72h of exposure . The qPCR reactions were performed using cDNA of three replicates for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC

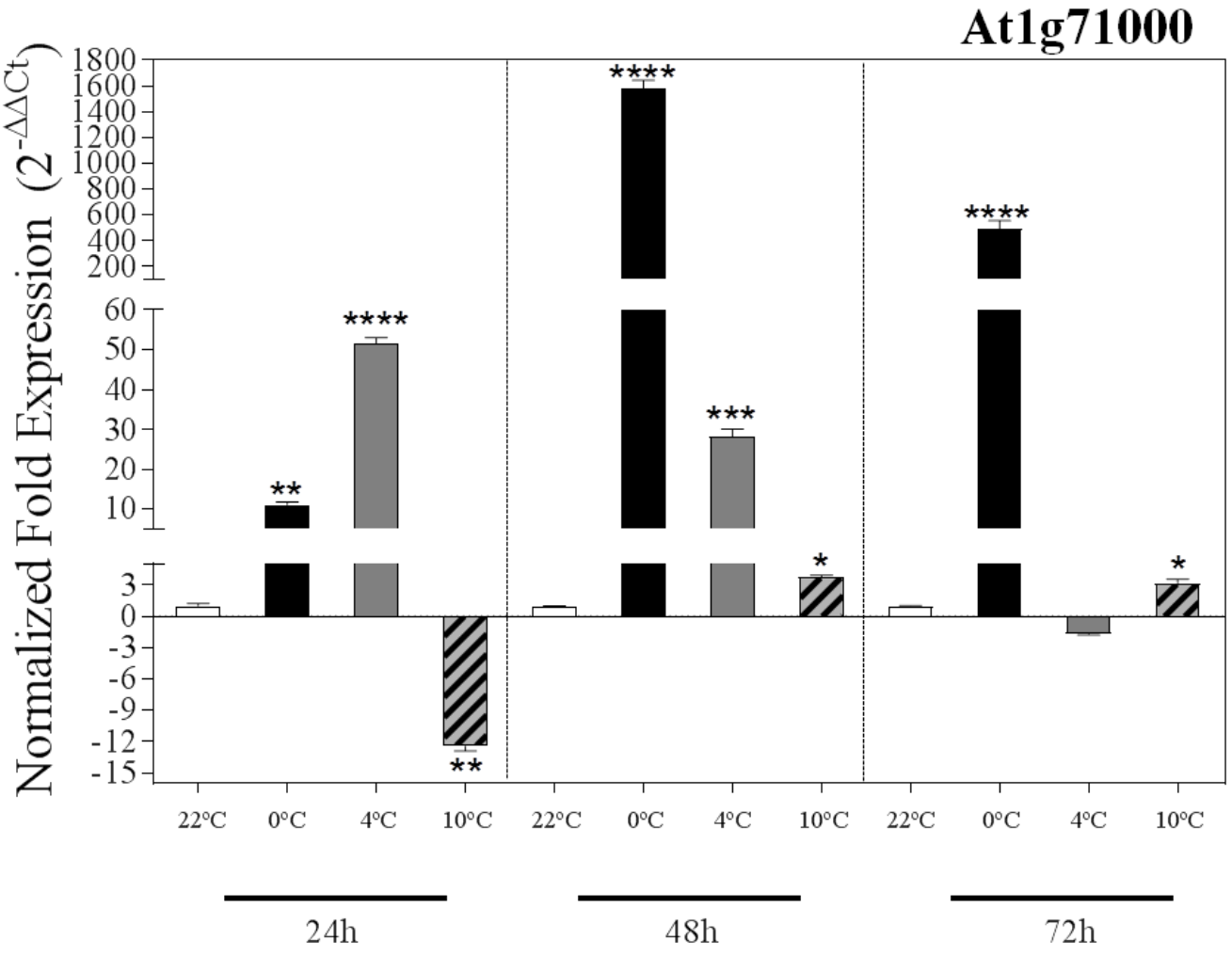

Figure 12 qRT-PCR validation of At1g71000. mRNA expression levels of At1g71000 under low temperature conditions of 0ºC, 4ºC and 10ºC, during 24, 48 and 72h of exposure. The qPCR reactions were performed using cDNA of three replicates for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One-way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC

Another gene that we selected for validation was *At5g05220* that on the results from the RNAseq, at 24h of exposure, its highest expression was obtained at 0ºC. In Figure 6 we can observe that at 24h, the transcript levels are higher under the 4ºC condition with 16-fold respecting control at 22ºC, nevertheless the expression of *At5g05220* diminished through time under this condition down to 2.3-fold. Notwithstanding that at 0ºC during the first 24h, the expression remained on control levels, it incremented through time reaching 211 times above control, by the last 72h of exposure (Figure 6). On the other hand, under the 10ºC condition, the gene remained repressed under the first 24h and 48h and reestablished its expression by the last 72h, but not on levels that represented a statistically significant difference respecting control.

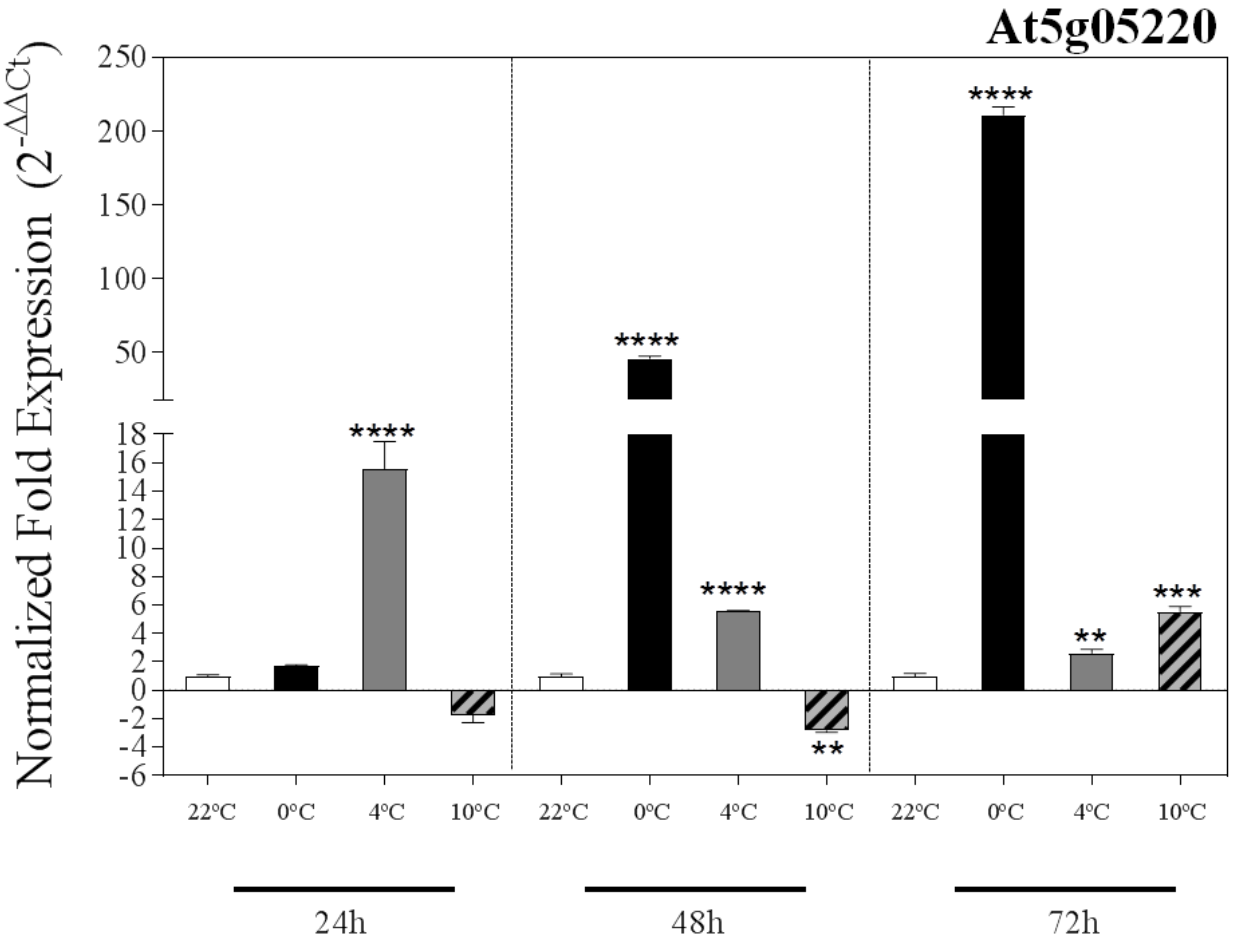

Figure 13 qRT-PCR validation of At5g05220. mRNA expression levels of At5g05220 under low temperature treatments of 0ºC, 4ºC and 10ºC, during 24, 48 and 72h of exposure . The qPCR reactions were performed using cDNA of three replicates for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC

Finally, the fifth gene that we selected was ATBZIP4 (*At1g59530*), which on the RNAseq results, the expression levels were detected under the 0ºC and 4ºC condition at 24h; whereas, on the qRT-PCR (Figure 7), we observed that ATBZIP4 was repressed under at 0ºC and it was until the last 72h of exposure to low temperatures, that its transcript levels went up to 403 times above control at 22ºC. Under the 4ºC condition during the first 24h, the expression of ATBZIP4 showed 37-fold respecting control, but at 72h it reached almost 65 times. Also, at 10ºC an increase in transcript levels was observed until the last 72h (17-fold respecting control), despite of being repressed during the first two periods of time.

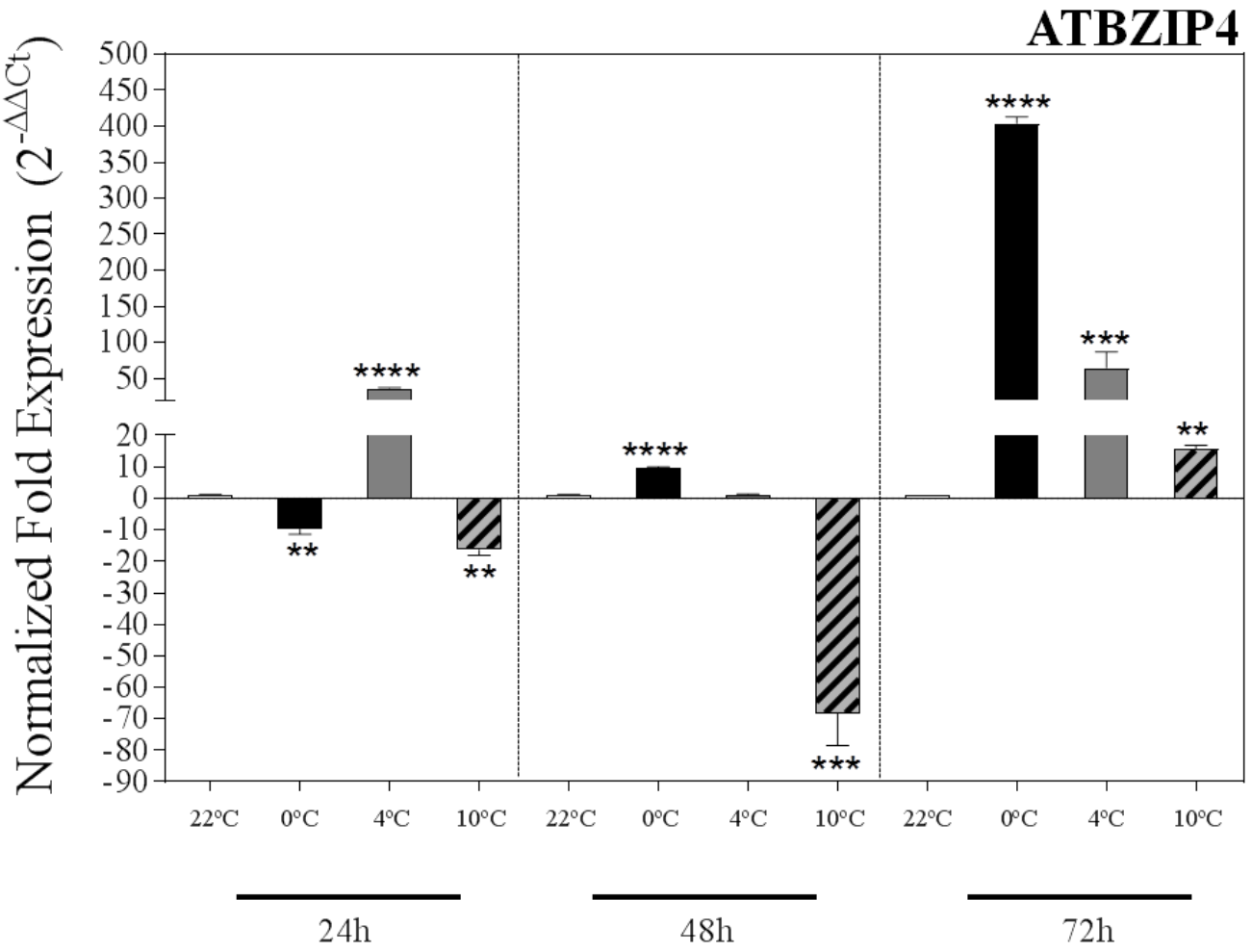

Figure 14 qRT-PCR validation of ATBZIP4. mRNA expression levels of ATBZIP4 under low temperature treatments of 0ºC, 4ºC and 10ºC, during 24, 48 and 72h of exposure . The qPCR reactions were performed using cDNA of three replicates for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC

1.6 Discussion

Plants have an elaborated response system to external stimuli. Such stress-responsive signal transduction pathways have been widely studied, although they are not fully understood, many of the key components of these pathways are protein-coding genes [14]. During the past decades, microarrays have been the most important and widely used approach for such analyses, but recently high-throughput sequencing of cDNA (RNA-seq) has emerged as a powerful alternative [21].

Transcriptome analysis is an important tool for characterization and understanding of the molecular basis of various physiological processes. The most common use of transcriptome profiling is in the search of differentially expressed genes; meaning, genes that show differences in their expression levels between conditions or somehow, are associated with given predictors or responses [21, 22, 23]. In *Arabidopsis thaliana*, tolerance to cold temperatures involves the accumulation of sugars (like raffinose) and aminoacids, the increase in the concentration of compatible solutes that function to stabilize enzymes, membranes and other cellular components, or the modification of the membrane's lipid composition [24].

The above information is in accordance with the present study where, through the RNA-seq technology, we obtained the transcriptome profiling of *A. thaliana* plantlets exposed to three different low temperatures: 0ºC, 4ºC and 10ºC, during 24 h. With the obtained information, and considering the first 50 upregulated genes in each of the

conditions, we observed that most of these genes are involved in the processes previously mentioned.

In order to validate the differential expression of our results, we selected five genes to analyze through qRT-PCR. Additionally, we subjected to these analysis samples of plantlets that were also exposed during 48h and 72h to the same temperatures, to observe their expression through time.

GOLS3 gene is predicted to encode a galactinol synthase that catalyzes the synthesis of galactinol from UDP-galactose and myo-inositol. Galactinol, is an exclusively galactosyl donor for raffinose family oligosaccharides biosynthetic pathway. In this regard, GOLS is potentially a metabolic control point, a key enzyme of a primary metabolite, and its products play an important role in abiotic stress tolerance [16, 25]. In the present study we found, through the qRT-PCR analysis, a major increase on the transcript levels of this gene under the 4ºC condition the first 24h, which diminished through time.

The opposite behavior was observed at 0ºC where the maximum transcript levels were obtained after 48h of exposure. This is in agreement with the reported by [16], where through western and northern blot assays, they observed a high expression of GOLS3 under 4ºC condition on early exposure times from 3h to 24h. The authors also found that this gene is the only from its family to be a target of DREB1A, having DRE and DRE-like core motifs on its promoter, which make it very responsive to cold stress

conditions, and with its expression more raffinose is synthesized, helping to the membrane stabilization during cold, and therefore during dehydration conditions.

The next gene we analyzed was At1g80130, which encodes a tetratricopeptide repeat (TPR)-like superfamily protein and it is known to have CTR/DRE elements within its promoter [26]. In the RNAseq results, we found that under the 4ºC condition, the At1g80130 gene was induced, this result concurs with the reported on [27] where, while trying to understand the extent to which freezing tolerance is dependent on a functional CBF pathway, they exposed plantlets of *A. thaliana* to low temperature conditions (4ºC during 24h) and through real time PCR, also found this gene being induced. These results are also in agreement with our qRT-PCR analysis where, at 4ºC, the At1g80130 gene was highly expressed, but its transcript levels diminished through time, a similar pattern to what happened under the 10ºC condition. On the other hand, at 0ºC, the opposite was observed where its induction was higher on the 48 and 72h, in comparison with the transcript levels on the 24h.

The remaining three genes that we selected had not been described under cold stress conditions. The At1g71000 gene, which encodes a chaperone DNAJ-domain superfamily protein, was found highly induced at 0ºC during 24h and its transcript levels remained very high through time. Once again, the opposite behavior was observed under the 4ºC condition where the transcript levels decreased through time until the At1g71000 gene was repressed. The DnaJ containing proteins(like At1g71000) are not restricted to a chaperone function, but are very diverse and may be involved in

ribosome biogenesis, posttranscriptional processes, protein import, translocation involvement and signal transduction [28]. Many of these processes are key to the low temperature response on the plant that may explain the high levels of expression of this gene.

The At5g05220 gene has been previously reported on [29] in a microarray showing the response profiles of 85 *Arabidopsis thaliana* highly induced genes (HIGs), to various abiotic stress conditions such as heat, drought, salt and cold, among other stimuli. Under the cold condition, At5g05220, as well as At1g71000, were showed up-regulated. Additionally, in a study to identify the genes of *A. thaliana* that are induced by abscisic acid (ABA) and abiotic stress [30], they reported At5g05220 gene to be one of the top 40 predicted ABA- inducible and stress-inducible genes, due to the presence of ABRE and CE's within its promoter, which explains its response to low temperature conditions. This can also be proven with the results here presented where the response of this gene reaches high levels of expression under the 0ºC condition in a late response manner, and at 4ºC in an early response, different to the 10ºC condition where, only at the last 72h of exposure an increase of the transcript levels was observed.

Finally, At1g59530 (AtbZIP4) gene is a member of the S-group of basic leucine zipper transcription factors, that are known to be involved in the endoplasmic reticulum stress response. Different to the A-group members, which are involved in dehydration tolerance through ABA signaling and abiotic stress responses [31]. Nevertheless, the

herein study, we observed expression of this gene under the lower temperatures such as 4ºC on the 24 and 72h of exposure and the highest levels of AtbZIP4 were obtained at 0ºC in the last 72h, indicating that AtbZIP4 gene is also probably involved in the dehydration response as well as the abiotic stress response, particularly, to cold temperatures.

1.7 Conclusions

1. Both GOLS3 (At1g09350) gene and At1g80130 gene showed an early response under the 4ºC and a late response at 0ºC.

2. At1g71000 was the gene that increased its expression in higher levels than the other four genes, in a late response manner during 48 and 72h at 0ºC.

3. At5g05220 and ATBZIP4 (At1g59530) showed an early response at 4ºC, but reached their highest transcript levels under the 0ºC condition in a late response manner at 72h.

4. In the present study we show data about some genes that are induced during the response to cold in *A. thaliana*, which can be further functionally characterized in order to understand their role in the response to cold.

1.8 References

[1] Zhu, J. K. (2016). Abiotic stress signaling and responses in plants. Cell, 167(2), 313-324.

[2] Montoliu, V. A. (2010). Respuestas fisiológicas de los cítricos sometidas a condiciones de estrés biótico y abiótico aspectos comunes y específicos (Doctoral dissertation, Tesis Doctoral. Universidad de Jaume, España).

[3] Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, 218(1), 1-14.

[4] Hetherington, S. E, He, J. & Smilie, R. M. (1989). Photoinhibition at low temperature in chilling-sensitive and -resistant plants. *Plant Physiology* 90: 1609-1615.

[5] Song, J. B., Gao, S., Wang, Y., Li, B. W., Zhang, Y. L., & Yang, Z. M. (2016). miR394 and its target gene LCR are involved in cold stress response in Arabidopsis. Plant Gene, 5, 56-64.

[6] Muzi, C., Camoni, L., Visconti, S., & Aducci, P. (2016). Cold stress affects H+-ATPase and phospholipase D activity in Arabidopsis. Plant Physiology and Biochemistry, 108, 328-336.

[7] Adbelrahman, M., Burritt, D. J., & Tran, L. S. P. (2017). The use of metabolomic quantitative trait locus mapping and osmotic adjustment traits for the improvement of crop yields under environmental stresses. In: *Seminars in Cell & Developmental Biology*. Academic Press.

[8] Körner, C., & Larcher, W. (1988). Plant life in cold climates. In Symposia of the Society for Experimental Biology, 42, 25-57.

[9] Zhu, J., Dong, C. H., & Zhu, J. K. (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current opinion in plant biology, 10(3), 290-295.

[10] Taiz, L., & Zeiger, E. (2002). Photosynthesis: physiological and ecological considerations. *Plant Physiology*. Massachusetts: Sinauer Associates, 171-192.

[11] Navarro, G. M. (2014). Manejo del Estrés por Temperatura en Cultivo Hortícolas. Curso de capacitación Intagri. Instituto para la Innovación Tecnológica en la Agricultura Diciembre 2014.

[12] Barrios, A., Caminero, C., García, P., Krezdorn, N., Hoffmeier, K., Winter, P., & de la Vega, M. P. (2017). Deep Super-SAGE transcriptomic analysis of cold acclimation in lentil (*Lens culinaris*). Biomed Central Plant Biology, 17(1), 111.

[13] Severin, A. J., Woody, J. L., Bolon, Y. T., Joseph, B., Diers, B. W., Farmer, A. D., & Graham, M. A. (2010). RNA-Seq Atlas of Glycine max: a guide to the soybean transcriptome. BMC plant biology, *10*(1), 160.

[14] Di, C., Yuan, J., Wu, Y., Li, J., Lin, H., Hu, L. & Lu, Z. J. (2014). Characterization of stress-responsive lncRNAs in Arabidopsis thaliana by integrating expression, epigenetic and structural features. The Plant Journal, *80*(5), 848-861.

[15] K.J.Livak, T.D.Schmittgen (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods, 25(4), 402-408.

[16] Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M & Shinozaki, K. (2002). Important roles of drought-and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. The Plant Journal, 29(4), 417-426.

[17] Chinnusamy, V., Zhu, J., & Zhu, J. K. (2007). Cold stress regulation of gene expression in plants. Trends in Plant Science, 12(10), 444-451.

[18] Yadav, S. K. (2010). Cold stress tolerance mechanisms in plants. A review. Agronomy for Sustainable Development, 30(3), 515-527.

[19] Huang, G. T., Ma, S. L., Bai, L. P., Zhang, L., Ma, H., Jia, P. & Guo, Z. F. (2012). Signal transduction during cold, salt, and drought stresses in plants. Molecular Biology Reports, 39(2), 969-987.

[20] Cedeño, C., Żerko, S., Tompa, P., & Koźmiński, W. (2017). 1H, 15N, 13C resonance assignment of plant dehydrin early response to dehydration 10 (ERD10). Biomolecular NMR assignments, 11(2), 127-131.

[21] Soneson, C., & Delorenzi, M. (2013). A comparison of methods for differential expression analysis of RNA-seq data. BMC bioinformatics, 14(1), 91.

[22] Haas, B. J., & Zody, M. C. (2010). Advancing RNA-seq analysis. Nature biotechnology, 28(5), 421

[23] Zenoni, S., Ferrarini, A., Giacomelli, E., Xumerle, L., Fasoli, M., Malerba, G. & Delledonne, M. (2010). Characterization of transcriptional complexity during berry development in Vitis vinifera using RNA-Seq. Plant physiology, 152(4), 1787-1795

[24] Cabello, J. V., Arce, A. L., & Chan, R. L. (2012). The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. The Plant Journal, 69(1), 141-153.

[25] Wang, D., Yao, W., Song, Y., Liu, W., & Wang, Z. (2012). Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in Salvia miltiorrhiza. Journal of plant physiology, 169(18), 1838-1848.

[26] Boyce, J. M., Knight, H., Deyholos, M., Openshaw, M. R., Galbraith, D. W., Warren, G., & Knight, M. R. (2003). The sfr6 mutant of Arabidopsis is defective in transcriptional activation via CBF/DREB1 and DREB2 and shows sensitivity to osmotic stress. The Plant Journal, 34(4), 395-406.

[27] Park, S., Lee, C. M., Doherty, C. J., Gilmour, S. J., Kim, Y., & Thomashow, M. F. (2015). Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. The Plant Journal, 82(2), 193-207.

[28] So, H. A., Chung, E., & Lee, J. H. (2014). Arabidopsis atDjC53 encoding a type III J-protein plays a negative role in heat shock tolerance. Genes & genomics, 36(6), 733-744.

[29] Inzé, A., Vanderauwera, S., Hoeberichts, F. A., Vandorpe, M., Van Gaever, T. I. M., & Van Breusegem, F. (2012). A subcellular localization compendium of hydrogen peroxide-induced proteins. Plant, cell & environment, 35(2), 308-320.

[30] Zhang, W., Ruan, J., Ho, T. H. D., You, Y., Yu, T., & Quatrano, R. S. (2005). Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic

acid-and abiotic stress-responsive genes in Arabidopsis thaliana. Bioinformatics, 21(14), 3074-3081.

[31] Yang, O., Popova, O. V., Süthoff, U., Lüking, I., Dietz, K. J., & Golldack, D. (2009). The Arabidopsis basic leucine zipper transcription factor AtbZIP24 regulates complex transcriptional networks involved in abiotic stress resistance. Gene, 436(1), 45-55.

CAPÍTULO II

Expresión diferencial de las dehidrinas ácidas de *Arabidopsis thaliana*, COR47, ERD10, ERD14 y At4g38410 en bajas temperaturas

2.1 Resumen

Las proteínas inducidas por deshidratación (dehidrinas) son miembros del grupo II de la familia de proteínas abundantes de la embriogénesis tardía (LEA, por sus siglas en inglés). El estrés abiótico tal como sequía, frío y salinidad, causa la regulación de los niveles de mRNA y de proteína de las dehidrinas. La planta modelo *Arabidopsis thaliana,* posee 10 genes que codifican dehidrinas los cuales, con base en su punto isoeléctrico se encuentran divididos en básicos y ácidos. De manera específica, las dehidrinas ácidas (COR47, ERD10, ERD14, At4g38410) son conocidas por trabajar como estabilizadores de la membrana durante la deshidratación inducida por bajas temperaturas y congelamiento. En el presente estudio, con el objetivo de analizar los niveles de expresión de los cuatro genes de dehidrinas ácidas a bajas temperaturas, plántulas de 14 días de edad de *Arabidopsis thaliana* fueron sometidas a 0ºC, 4ºC y 10ºC durante periodos de 24, 48 y 72h. Nosotros observamos que COR47, ERD10 y ERD14 presentan niveles de expresión más altos bajo el tratamiento de congelamiento en tiempos de exposición tardíos; mientras que At4g38410, presenta un incremento en los niveles de transcrito bajo el tratamiento por frío tanto en respuesta temprana como tardía. Nuestros resultados muestran la particularidad de la transcripción de los genes de estas dehidrinas durante temperaturas y tiempos de exposición específicos bajo condiciones de estrés por frío.

Palabras clave: *Arabidopsis thaliana,* dehidrinas ácidas, estrés por frío, regulación transcripcional.

CHAPTER II

Differential expression of Arabidopsis acidic dehydrins COR47, ERD10, ERD14 and At4g38410 at low temperatures

2.2 Abstract

Dehydration proteins (dehydrins) are group II members of the Late Embryogenesis Abundant (LEA) protein family. Abiotic stresses such as drought, cold and salinity cause the upregulation of dehydrin mRNA and protein levels. *Arabidopsis thaliana* has 10 genes that encode dehydrins, which based on their isoelectric point are divided into basic and acidic. Specifically, acidic dehydrins (COR47, ERD10, ERD14, At4g38410) are known to work as membrane stabilizers during low temperatures and freezing induced dehydration. In this study, in order to analyze the expression levels of the four acidic dehydrin genes, 14 days old plants of *Arabidopsis thaliana* were exposed to freezing and cold temperatures during 24, 48 and 72h. We observed that COR47, ERD10 and ERD14 had higher expression levels under the freezing treatment on late periods of exposure, whereas At4g38410 showed increased transcript levels under the cold treatment both, at early and late response. Our results show the particularity of this dehydrin genes transcription during specific temperatures and periods of exposure to cold stress conditions.

Keywords: *Arabidopsis thaliana*, acidic dehydrins, cold stress, transcriptional regulation.

2.3 Introduction

Dehydrins (DHNs) are proteins that are classified as members of the LEA (Late Embryogenesis Abundant) protein family D-11 or group II. The LEAs constitute a complex plant protein family that plays an important role on protection against dehydration, and during abiotic stress conditions, such as drought, salinity and low temperatures [1, 2, 3]. DHNs are characterized for being hydrophilic and thermostables due to their property of disordered proteins [4]. Another characteristic of the DHNs is the presence of the K-segment, which is a region of 15 conserved aminoacids (EKKGIMDKIKEKLPG) that has been identified as multicopy on the reported DHNs [5,6]. This and other domains, including a phosphorylable segment constituted of serine residues (S-segment), and a consensus sequence at the N-terminal end (Y-segment), can be also identified on DHNs [5,7,8]. Based on the conserved motifs number of K-, S- and Y- segments, the DHNs are divided into five classes: $YnSK_2$, Kn, KnS, SKn and Y_2Kn [6,9,10].

On the model plant *Arabidopsis thaliana*, ten DHNs have been reported, and based on their isoelectric point, they are classified as: acidic dehydrins AtCOR47 (pI 4.68, At1g20440), AtERD10 (pI 4.98, At1g20450), AtERD14 (pI 5.28, At1g76180) and At4g38410 (pI 6.09); neutral dehydrins: At4g39130 (pI 6.38), At2g21490 (pI 6.38), HIRD11 (pI 7.23, At1g54410) and AtRAB18 (Pi7.57, At5g66400); and basic dehydrins: AtXERO1 (pI 9.66, At3g50980) and AtXERO2 (pI 9.84, At3g50970) [11]. It has been proposed that acidic DHNs work as membrane stabilizers during dehydration caused by

freezing and low temperatures, that they have cryoprotectant activity, and work as possible osmoregulators [2, 12, 13].

DHNs gene expression is a key factor for plant tolerance to cold stress, because the accumulation of these proteins on high levels is an indicator of the plant's response capacity to low temperatures and its cold stress resistance [11]. Despite numerous studies, mostly with individual *Arabidopsis* DHNs, such as ERD10, COR47 and ERD14, inducing their expression under cold stress conditions, there is not a detailed study carried out under several low temperatures and stress periods of time. That is why, the aim of this study was to evaluate the differential expression of the four *Arabidopsis thaliana* acidic dehydrins, COR47, ERD10, ERD14 and At4g38410, in response to freezing stress (0ºC) and cold stress (4ºC and 10ºC) during three periods of time (24h, 48h and 72h) in order to observe their behavior both in early and late response.

2.4 Materials and Methods

2.4.1 Plant material, growth conditions and stress treatments

Seeds of *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) were surface-sterilized for 5 min with 20% chlorine solution and rinsed five times in sterile distilled water. Aseptic stratified and vernalized seeds (2 days at 4ºC) were germinated and grown *in vitro* for two weeks on plates containing Murashige and Skoog medium (0.5X) on a growth chamber with controlled temperature conditions (22ºC±1ºC) under 16h light (13,000lux)/ 8h dark. For cold treatment, 14 days old plantlets were incubated in growth chambers at 0ºC, 4ºC and 10ºC. The plants were subject to stress treatments for various periods of 24h, 48h and 72h. The control plants were kept in the growth chamber at 22ºC. In addition to this, another experiment was carried out, in which 14 days old plants were subjected to an extra acclimation of 10ºC during 24h prior to cold treatment and then, incubated in growth chambers at 0ºC and 4ºC during 48h. After the stress treatments, plants were immediately frozen in liquid nitrogen and stored at -80ºC, until used. For each temperature condition and period of time, three biological replicates were done, each consisting in groups of 12 plants.

2.4.2 RNA extraction and Reverse Transcription

Total RNA was extracted from plant material using the Concert^{TM} Plant RNA Reagent protocol (Invitrogen Life Technologies. Catalogue no. 12322-012). The concentration of RNAs was measured using an Epoch 2 microplate reader (Biotek Instruments,

Winnoski, USA). The structural integrity of the RNAs was checked in a 1% agarose and TBE 0.5X gel. To remove contaminating genomic DNA, RNAs (2.5 µg) were treated with Ambion™ DNase I (RNAse-free) (Thermo Fisher Scientific, Carslbad,USA. Catalog no. AM2222) according to the manufacturer's procedure. The absence of DNA was proved by a PCR reaction. DNase- treated RNA samples were reverse- transcribed using the SuperScript™ II Reverse Transcriptase (Invitrogen. Carslbad,USA. Catalogue no.1806404) and quantified on an Epoch 2 microplate reader (Biotek Instruments, Winnoski, USA). The cDNAs were then stored at -20ºC until used in real-time PCR amplification.

2.4.3 Real-time quantitative RT-PCR

Real- time quantitative RT-PCR was carried out using the StepOne™ Real-Time PCR System (Applied Biosystems. Catalogue no.4376374). The Elongation factor alpha (*EF1α*) gene was used as internal control and amplified in parallel with the target gene allowing gene expression normalization and providing quantification. Detection of real-time RT-PCR products was done using SYBR® Green Supermix protocol (Applied Biosystems). Dilutions of 100ng of the cDNAs were used as a template for PCR. Primers used for the amplification of target genes, *COR47*, *ERD10*, *At4g38410* and *ERD14* (as well as the internal control *EF1α*) are listed in Supplementary Table 1. PCR cycling conditions comprised 40 PCR cycles of 10s at 95ºC (denature) and 30s at 60ºC (anneal/extend). At the end of the PCR runs, melting curves were performed starting at 65ºC and gradually increasing the temperature every 0.5ºC up to 95ºC. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results.

Table 1 Primer sequences and technical information.

Gene	Accession Number	Primer Name	Primer Sequences (5'-3')	Amplicon size (bp)
EF1α	At5g60390	FWEF1α	TCCAGCTAAGGGTGCCGC	152 bp
		RVEF1α	AACGCCTGTCAATCTTGGTCAA	
COR47	At1g20440	FwRTCOR47	GTCTGATGATTAAGCAAAATGG	184bp
		RvRTCOR47	GGATCAAATGCAATCAACGAAA	
ERD10	At1g20450	FwRT20450	CTAGCTGTTCTCCAAGTTTGT	105bp
		RvRT20450	GAGAATTTATCCATTGAAGCTC	
ERD14	At1g76180	FwRT76180	GTAGAGGAGGAGAAGAAAGAT	149bp
		RvRT76180	GCCAACAAACTTACACATCAC	
At4g38410	At4g38410	FwRT38410	GATGAGAAGAAGAAGGAAACC	124bp
		RvRT38410	GTTGGCATTTCACTGACACAT	

2.4.4 Data analysis

The cycle number at threshold (Ct value) was used for calculations of relative mRNA expression levels. The Ct value from each target gene (*COR47*, *ERD10*, *ERD14* and *At4g38410*) was normalized by subtraction of the Ct value from the *EF1α* gene. The fold change in gene expression relative to control samples (plants kept at 22ºC for each period of time) was calculated using the $2^{-\Delta\Delta Ct}$ method [7]. In the case of ratios being lower than 1, the inverse ratio was estimated and graphed as negative.

Statistical significance was estimated by One-way ANOVA and t-test at $p < 0.05$ through GraphPad Prism version 5.0 (GraphPad Software, California, USA).

2.5 Results

In order to analyze the expression levels of the COR47, ERD10, ERD14 and At4g38410 genes under cold stress, 14 days old *Arabidopsis thaliana* plantlets were incubated at 0ºC, 4ºC and 10ºC during 24, 48 and 72h. Expression analysis of these acidic dehydrins was carried out by qRT-PCR.

In the case of the COR47 gene (Figure 1), real time-PCR analysis showed a significant increase through time at 0ºC, where the transcript levels of COR47 during 48 and 72h increased in almost 59 and 26 times more than control plantlets (22ºC), respectively. We observed a strong response during the first 24h under the 4ºC condition of 58 times respecting the control plants at 22ºC, while at 48h diminished to 10-fold. No statistically significant differences on COR47 expression levels were observed under the 10ºC condition.

When we analyzed ERD10 gene, a similar behavior to COR47 was observed (Figure 2) where, at 0ºC the ERD10 expression levels increased through time, whereas at 4ºC the opposite happened. Under the 0ºC condition, we observed that after 48h of treatment, the mRNA expression levels of ERD10 increased 120 times respecting control at 22ºC, that remained high until the last period at 72h with 118 times above the control plants (22ºC). An early response was obtained during the first 24h under the 4ºC condition of 74 times above the control that diminished through time until it reached 2.6-

fold respecting the control on the last 72h of treatment. Once again, no statistically significant differences in the ERD10 expression levels were observed at 10ºC.

In the case of ERD14 (Figure 3), lower levels of transcript were observed but it presented a similar behavior tendency than the previous two genes. Under the 0ºC condition during the first 24h the expression levels of ERD14 were repressed in comparison with the control at 22ºC. However, after 48h of treatment we observed an increase of 5.2 times of transcript levels of ERD14 which remained until the last 72h of exposure. Again, an early response was observed during the first 24h, specifically under the 4ºC condition with a 2.5 fold increase of the transcript levels that decreased to 1.4 times during the next 48h and remained stable until the last 72h of exposure to low temperatures. The ERD14 expression response was diminished under the 10ºC condition at 24h, but it was re-established at 72h.

Different to these three genes, At4g38410 showed a diminished expression at 0ºC being repressed on the first 24h and the last 72h of exposure to low temperatures (Figure 4). Meanwhile, we observed an increased response under the 10ºC condition but at much lower transcript levels where the highest expression was obtained on the first 24h with 2 times more with respect to the control at 22ºC. Also, during the last 72h at 10ºC similar levels of expression were observed; however they remained almost the same as the obtained on the control conditions to 22ºC.

Regarding the acclimation experiment (Figure 5), where the Arabidopsis plantlets were subjected to a previous treatment for 24h at 10ºC, and then to a 48h exposure to 0ºC and 4ºC. We observed that the transcript levels on the four genes were diminished in a proportional manner. Under the 0ºC condition, COR47 remained as the most expressed gene with almost 80-fold with respect to the control plants that were returned to 22ºC. In the case of ERD10 a 70- fold increase was observed, followed by At4g38410 with an increase of more than 7 times and ERD14 showed a 3-fold increase above control. On the other hand, at 4ºC only in the case of ERD14 the transcript levels showed a statistically significant difference in comparison to the control at 22ºC.

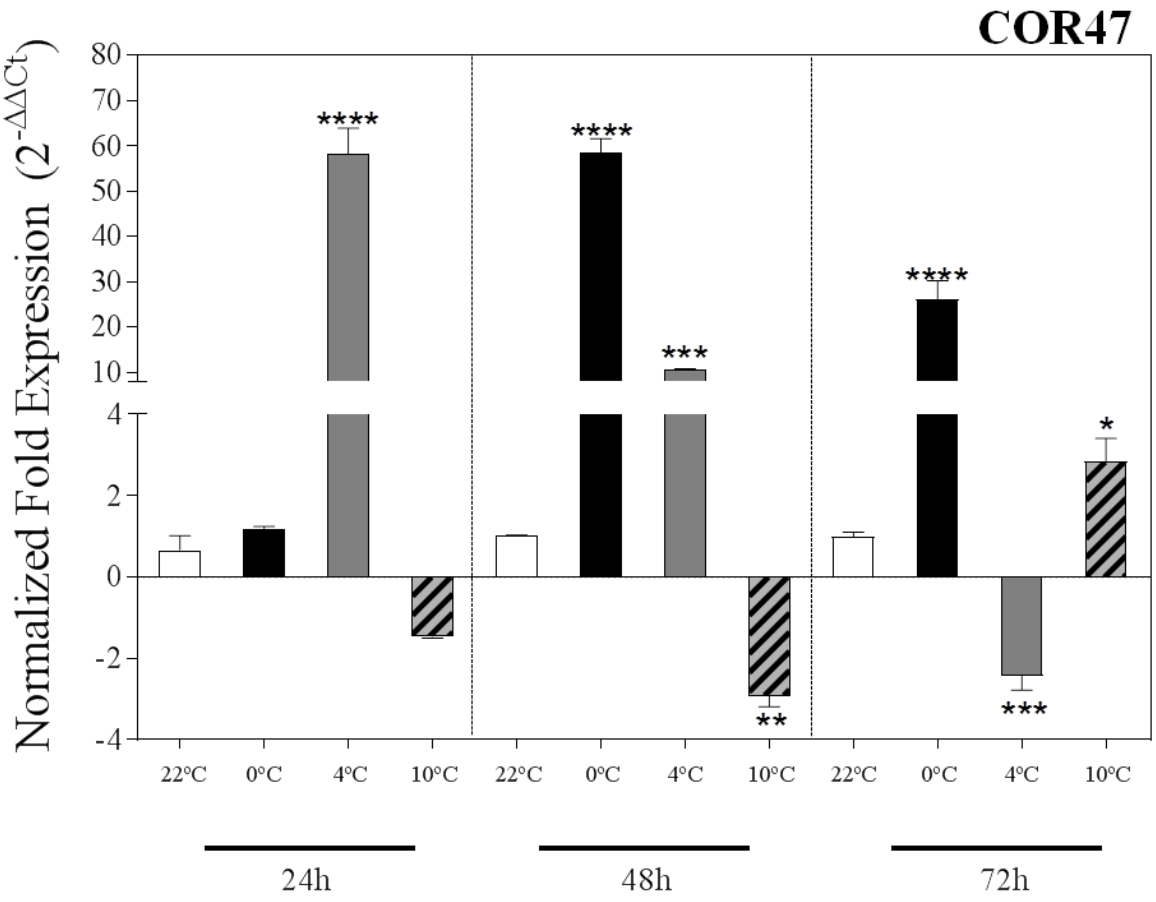

Figure 1 COR47 mRNA expression levels under low temperatures. Effect of freezing (0ºC) and cold stress temperatures (4ºC and 10ºC) on 14 days old plantlets of Arabidopsis thaliana during 24, 48 and 72h of treatment. The qPCR reactions were performed using cDNA of three replicas for each sample using the fluorescent dye SYBR Green® and the elongation factor alpha (EF1α) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent $P<0.05$ respecting control plants at 22ºC

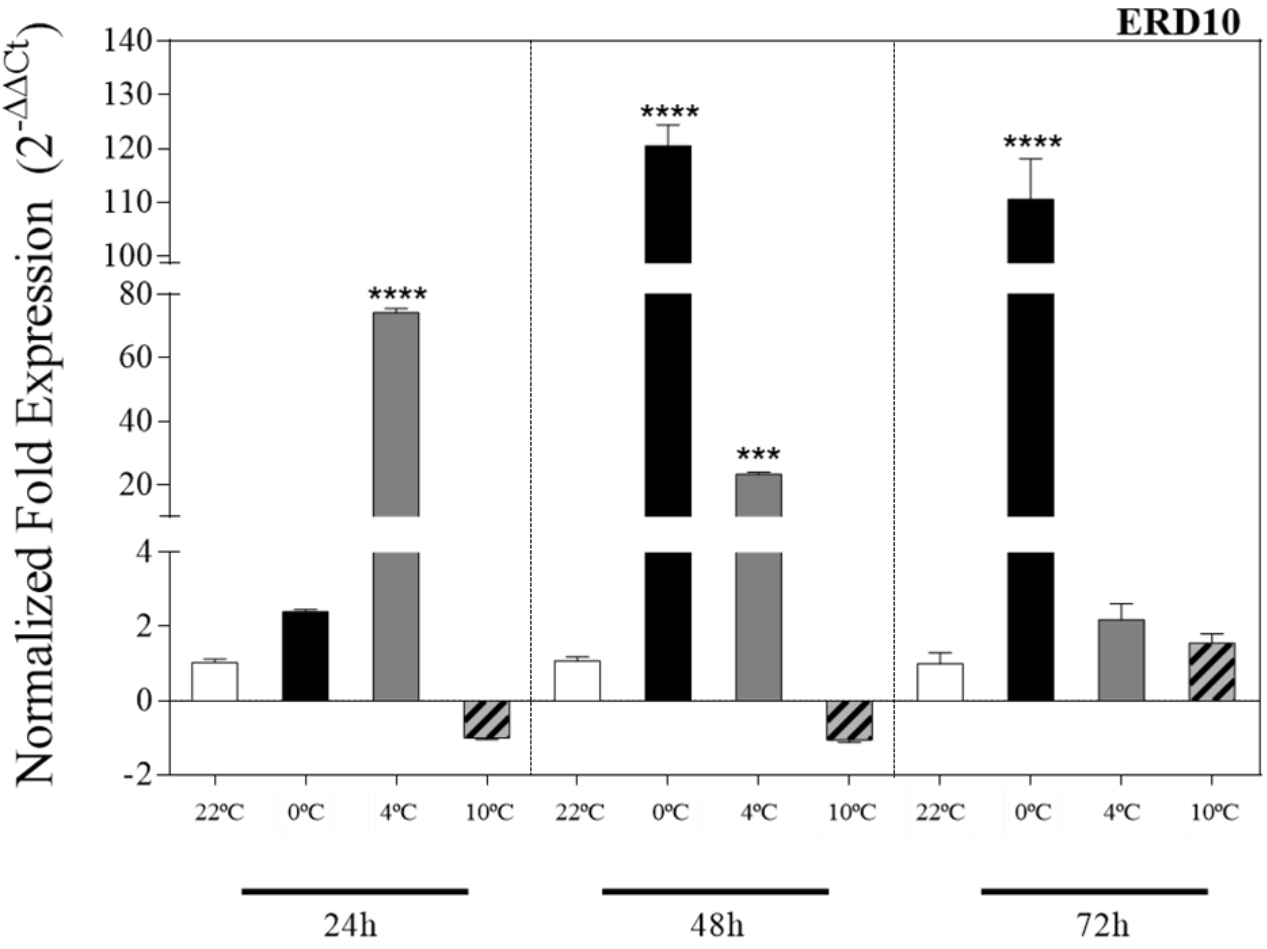

Figure 2 ERD10 mRNA expression levels under low temperatures. Effect of freezing (0ºC) and cold stress temperatures (4ºC and 10ºC) on 14 days old plantlets of *Arabidopsis thaliana* during 24, 48 and 72h of treatment. The qPCR reactions were performed using cDNA of three replicas for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent $P<0.05$ respecting control plants at 22ºC.

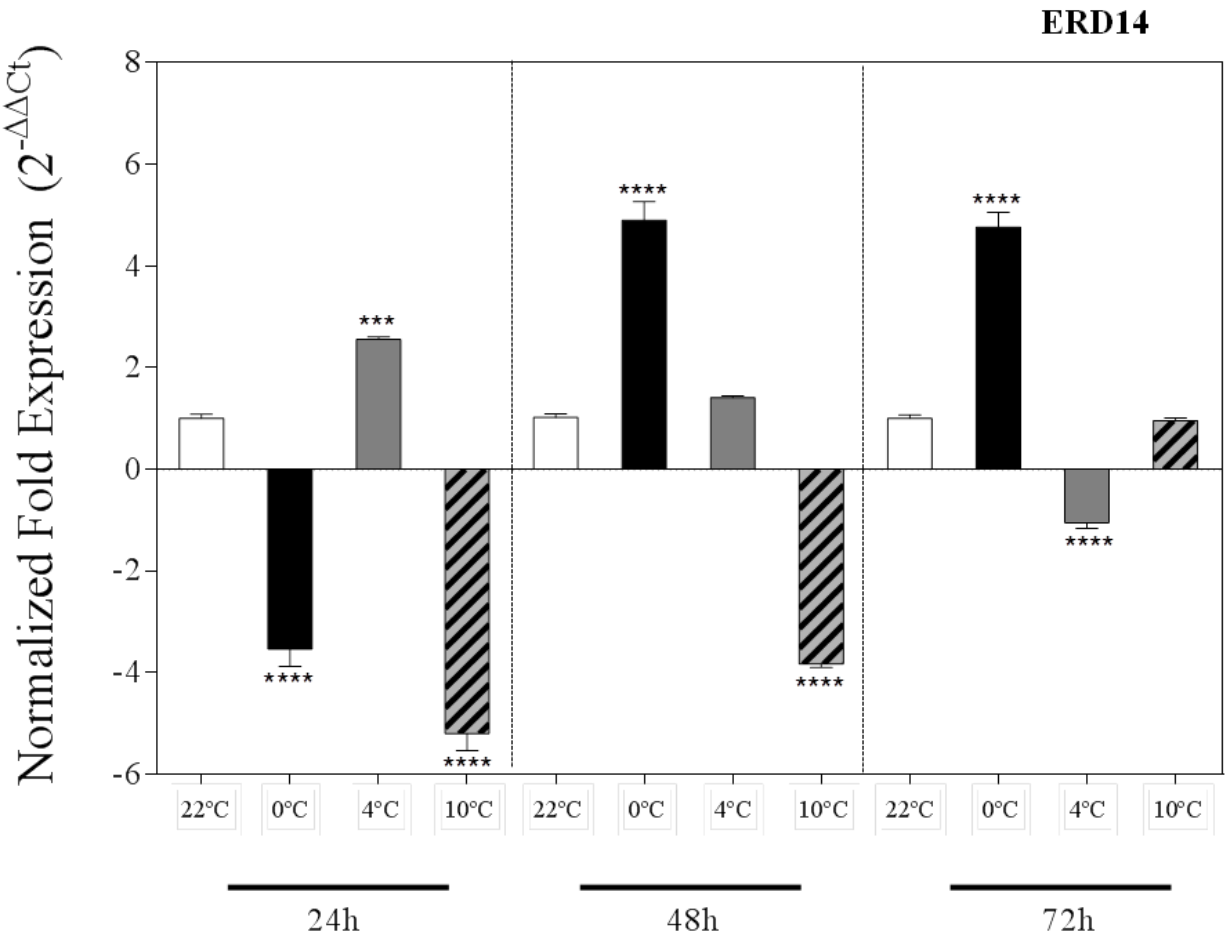

Figure 3 ERD14 mRNA expression levels under low temperatures. Effect of freezing (0ºC) and cold stress temperatures (4ºC and 10ºC) on 14 days old plantlets of *Arabidopsis thaliana* during 24, 48 and 72h of treatment. The qPCR reactions were performed using cDNA of three replicas for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-test. Asterisks represent P<0.05 respecting control plants at 22ºC.

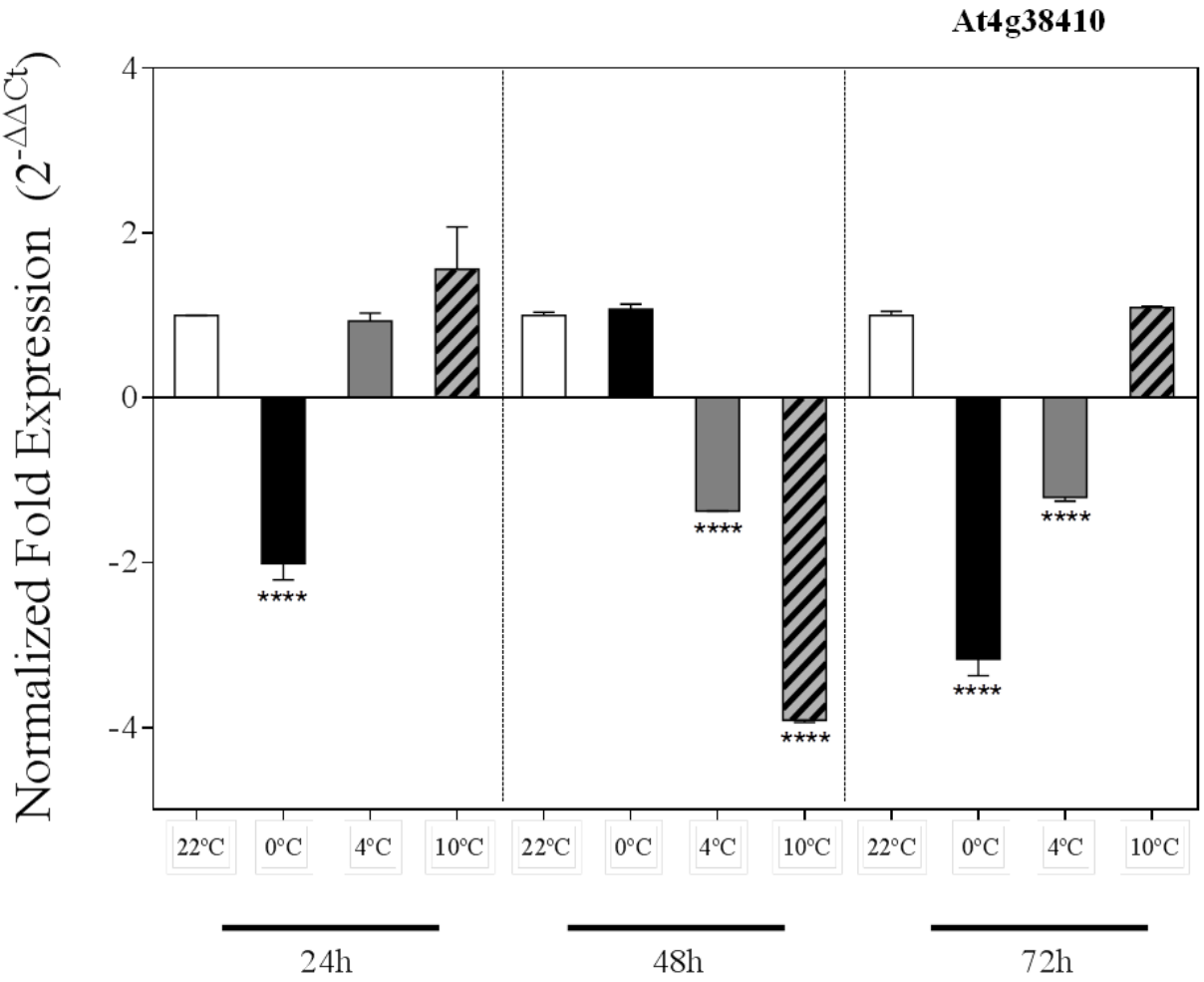

Figure 4 At4g38410 mRNA expression levels under low temperatures. Effect of freezing (0ºC) and cold stress temperatures (4ºC and 10ºC) on 14 days old plantlets of *Arabidopsis thaliana* during 24, 48 and 72h of treatment. The qPCR reactions were performed using cDNA of three replicas for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and t-tes. Asterisks represent $P<0.05$ respecting control plants at 22ºC

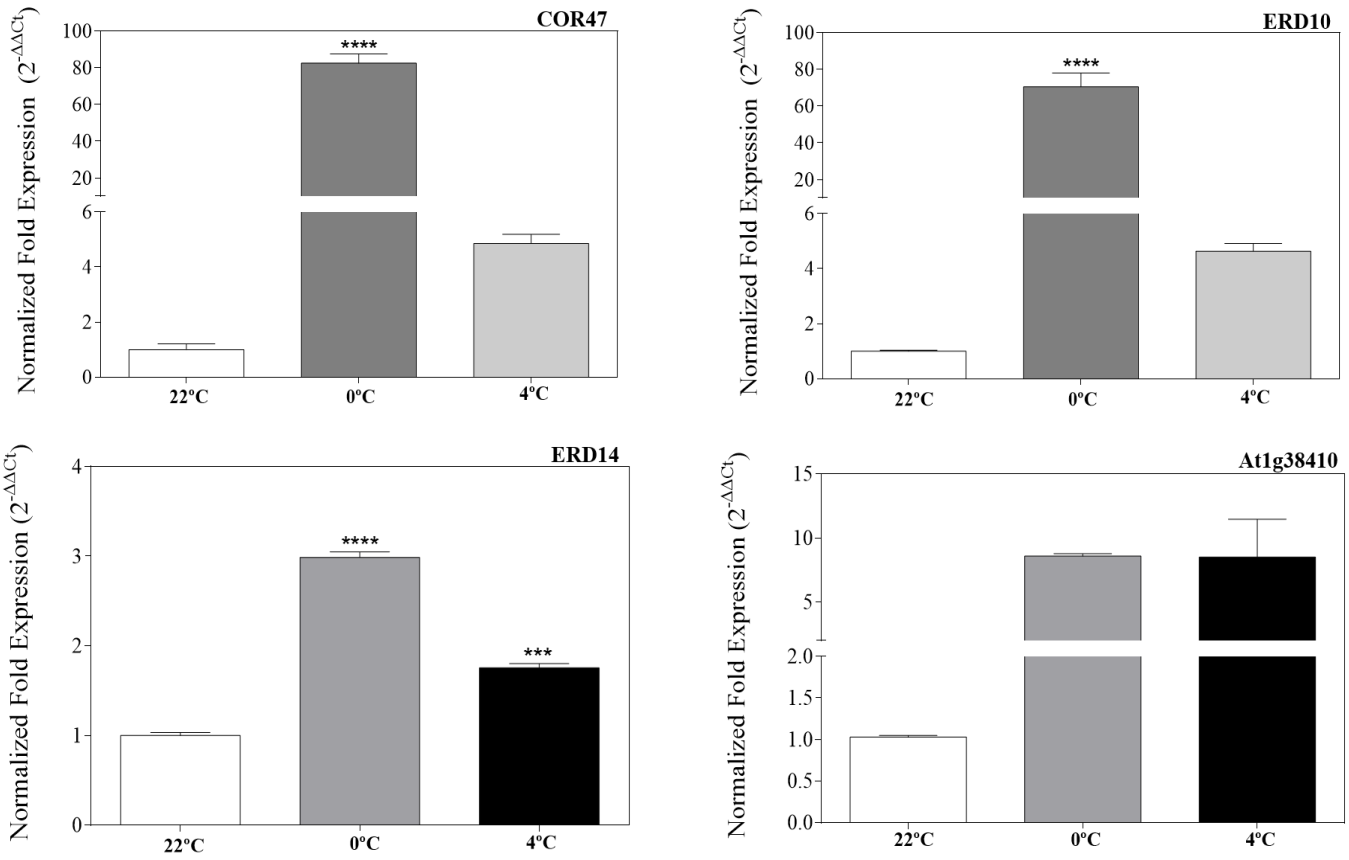

Figure 5 COR47, ERD10, ERD14 and At4g38410 differential expression under the acclimation experiment. 14 days old plantlets of *Arabidopsis thaliana* were submitted to a 10ºC acclimation during 24h prior to a 48h cold (4ºC) and freezing (0ºC) treatment. Control plants were also submitted to acclimation at 10ºC for 24h and then incubated to 22ºC. Real time PCR reactions were performed using cDNA of three replicas for each sample using the fluorescent dye SYBR Green® and the *elongation factor alpha* (*EF1α*) gene was used as load control. Statistical analysis was carried out for each period of time through One- way ANOVA and Bonferroni's multiple comparison. Asterisks represent P<0.05 respecting control plants at 22ºC

2.6 Discussion

It is well known that abiotic stress caused by drought, high salinity, and cold conditions result in dehydration, meaning, a reduction of the amount of free water available to the cell. One family of proteins that is expressed during dehydration stress is the dehydrins (DHNs) [6, 10, 13]. Despite all 10 *Arabidopsis* DHNs do not present a high identity percentage on their sequences, when a phylogenetic analysis is performed, two major groups are evident, one of them (the acidic DHNs group) includes COR47, ERD10, ERD14 and At4g38410 (Q9T), and they present protein sequences closely related [15]. Specifically, the acidic nature DHNs are known to respond to cold temperatures conditions [2, 4, 10, 12, 13, 20], that is why they were evaluated in this study.

Previous studies analyzing the DHNs expression were carried out using techniques such as Northern blot and semiquantitative RT-PCR. In [16], using plants of *A. thaliana* ecotype Landsberg erecta, under a 4ºC treatment for periods of time between a half hour and 48h, they observed through Northern blot, a strong accumulation of COR47 RNA at the first 4h and 8h. Nevertheless, also at 24h an increase in the amount of RNA was obtained which seems to diminish through time until the last 48h. In this study, a similar tendency was observed for COR47, where there was a high expression at 24h, followed by a decrease at 48h, these results substantiate the early response manner in which COR47 works. Also in agreement with our results, in [17] authors evaluated the expression levels of various cold responsive genes on 4

week old plantlets of *A. thaliana* exposed to a 4ºC treatment during 1 to 24h (early response time) and they reported the highest expression of COR47 gene also at 24h.

Although most of studies evaluating COR47 gene expression applied 4ºC treatment [16, 17, 18], studies performed by [19], where they exposed plants to 0ºC for 3, 6, 12, 24 and 36h, report the higher mRNA levels of COR47 at 24h. This differs with the obtained in the present study where, although an expression was observed on the first 24h under the 0ºC treatment, this increased highly within the next 48h to almost 60 times above control at 22ºC. In general, we found that a more sustained expression of COR47 gene is obtained under 0ºC which remained high until the last 72h of treatment, meaning a late response preference.

The case of the ERD10 (Early Response to Dehydration 10) gene results to be very interesting, because in this study we observed that ERD10 was the gene with the highest expression over the other three, both at freezing temperatures (0ºC) and chilling temperatures such as 4ºC and at late and early response, respectively. These results concur with those mentioned on [20], where they evaluated the expression of ERD10 in wild-type Col-0 *A. thaliana*, exposing the plants to cold treatment of 4ºC during 24h through Northern blot and RT-PCR and they reported that the expression of ERD10 increased in wild-type plants; they also generated an *erd10* knock-out mutant and observed that it was less tolerant to cold treatment.

Furthermore, the authors found that in the *erd10* mutant, the transcription factors CBF/DREB1 (C-repeat Binding Factor/Dehydration-Responsive Element Binding 1), which are well-known transcription factors with major roles on the activation of the COR genes expression [22, 23. 24, 25, 26]**,** were not activated in response to cold, suggesting that ERD10 is necessary for the accumulation of cold-inducible transcription factors like these, and their downstream targets including COR47. This could explain the tendency that we observed with ERD10 and COR47, which accumulated during the same periods of time and temperatures, but with higher transcript levels of ERD10 that would be necessary for the expression of COR47.

Another Early Response to Dehydration gene that we evaluated was ERD14, which has the particularity of being strongly phosphorylated and this event results in a significant increase in calcium binding activities, compared to the previously mentioned DHNs ERD10 and COR7 [27]. Our results show a similar behavior of ERD14 to its related gene ERD10, where under 4ºC at earlier times there is transcript accumulation which decreases through time, and under 0ºC regardless it is repressed at 24h, at later periods of time the expression of ERD14 increased but at lower levels than the last one. These results are also in accordance with [20] where a similar inducible pattern is described. Also, on [28] an evaluation of the mRNA levels of both ERD10 and ERD14 was reported by exposing 5 weeks old plants of *A. thaliana* to 4ºC cold treatment for 1, 2, 5, 10 and 24 h. They observed that the induction of both genes occurred in two phases, an early induction at 1h that decreased on the next lapses and a second peak at 5h, reaching maximum levels that remained through the final 24h. Although we did

not evaluate earlier hours than 24h, we observed a similar response on this lapse of time, also similar to the expression pattern of an homologue of ERD14 in soybean, named GmERD14, which under a 4ºC treatment for 48h appeared to be highly induced, both at mRNA levels, seen through RT-PCR; and at protein levels, by Western blot. Data mentioned above suggest that the response of ERD14 gene at 4ºC happens earlier, but at 0ºC it remains more stable through longer periods of time.

Finally, we evaluated the expression of At4g38410 under low temperature conditions, although in its nature this DHN is also acidic, we observed that the pattern of its expression was very different from the other three DHNs. Regarding on the lowest temperatures of 0ºC and 4ºC this gene remained repressed and it was only under the 10ºC treatment that seems to increase its transcript levels at an early response on 24h and later at 72h of exposure. The At4g38410 gene, often referred as Q9T [15, 27], has been described as an acidic dehydrin [7, 15, 27], but also as neutral [21], this nature can explain why it doesn't present a similar expression pattern to the other three acidic DHNs analyzed herein. Additionally, another factor that could play a role on the differential expression of At4g38410 is that, while COR47, ERD10 and ERD14 have high correlations on their expression patterns, At4g38410 does not mirror these patterns even though they share close phylogenetic relationships. This can be explained by its restricted expression in the roots and seed [15].It is worth mentioning that, while it did not showed to be as responsive to abiotic stress (particularly to cold temperatures) as the other acidic DHNs, in the root it has been found that it tends to mimic the other acidic DHNs response to abiotic stress [15].

Cold shock induces an immediate rise in the levels of cytosolic free calcium [23, 24, 28, 30]. The initial perception of this calcium signal is via the binding of calcium to various calcium sensors (including calmodulin, calmodulin domain protein kinase and calcerulin B-like protein), which transmit the stress signal down-stream in the pathway [31] and activates the transcription of cold induced genes, acidic DHNs among them.

Arabidopsis activates cold acclimation in response to persisting or oscillating cold stress and it is rapidly lost once the temperatures increase. On this regard, it has been shown that *Arabidopsis* is capable of memorize an earlier (priming) cold stress for several days [32]. This priming can improve the plant's performance upon a future stress having an effect of its cold response mechanisms, like the one we presented in this study where *Arabidopsis* plants underwent an acclimation of 10ºC for 24h and then a cold stress of 0ºC and 4ºC for 48h in order to analyze the transcript levels of the four acidic DHNs. We observed that the levels of these DHNs were much lower than the plants that did not underwent this "priming", since these DHNs genes were already transcribed with the 10ºC treatment, improving the plant's tolerance response, not having to rise the levels of transcript of these cold-responsive genes.

2.7 Conclusions

These results indicate that these four DHNs genes are specialized on certain temperatures as well as different periods of time.

1. Significant expression levels of DHNs were observed under the lower temperatures such as 0ºC in periods of late response, and 4ºC in an early response.

2. In accordance to the control conditions, ERD10 was the gene that increased more times its transcript levels, followed by COR47, ERD14, and last At4g38410 gene.

3. The acclimation results suggest that the previous exposure to 10ºC has an effect on the plant's tolerance response to lower temperatures, where the plant activates response mechanisms that would make the expression of the DHNs genes decrease once the plants are transferred to 0ºC and 4ºC.

2.8 References

[1] Hara,M., Terashima, S., Kuboi, T. (2001). Characterization and cryoprotective activity of cold- responsive dehydrin from *Citrus unshiu*, J. Plant Physiol. 158, 1333-1339.

[2] Ochoa- Alfaro, A.E., Rodríguez-Kessler, M., Pérez-Morales,M.B., Delgado-Sánchez,P., Cuevas- Velázquez, C.L., Gómez- Anduro, G., Jiménez-Bremont, J.F. (2012). Functional characterization of an acidic SK3 dehydrin isolated from an *Opuntia streptacantha* cDNA library, Planta. 235(3) 565-578.

[3] Hussain, S., Niu, Q., Qian, M., Bai, S., Teng, Y. (2015). Genome-wide identification, characterization, and expression analysis of the dehydrin gene family in Asian pear (*Pyrus pyrifolia*), Tree genetics & genomes. 11(5), 110.

[4] Hernández- Sánchez,I.E., Martynowicz, D.M., Rodríguez-Hernández, A.A., Pérez-Morales, M.B., Graether, S.P., Jiménez-Bremont, J.F. (2014). A dehydrin-dehydrin interaction: the case of SK_3 from *Opuntia streptacantha*, Frontiers in plant science. 5, 520.

[5] Close, T.J. (1994). Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins, Physiologia plantarum. 97(4), 795-803.

[6] Close, T.J. (1997). A commonality in the response of plants to dehydration and low temperature, Physiologia plantarum. 100(2), 291-296.

[7] Alsheikh, M.K., Svensson, J.T., Randall, D.K. (2005). Phosphorylation regulated ion-binding is a property shared by the acidic subclass dehydrins, Plant, cell and environment, 28, 1114-1122.

[8] Cao, Y., Xiang, X., Geng, M., You, Q., Huang, X. (2017). Effect of HbDHN1 and HbDHN2 genes on abiotic stress responses in Arabidopsis, Frontiers in plant science. 8, 470.

[9] Zhu, B., Choi, D.W., Fenton, R., Close, T.J. (2000). Expression of the barley dehydrin multigene family and the development of freezing tolerance, Mol. Gen. Genet. 264, 145-153.

[10] Allagulova, Ch. R., Gimalov, F.R., Shakirova, F.M., Vakhitov, V.A. (2003). The plant dehydrins: Structure and putative functions, Biochemistry, 68(9), 945-951.

[11] Yamasaki, Y., Koehler, G., Blacklock, B.J., Randall, S.K. (2013). Dehydrin expression in soybean. Plant physiology and biochemistry, 70, 213-220.

[12] Puhakainen, T., Hess, M.W., Mäkelä, P., Svensson, J., Heino, P., Palva, E.T. (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis, Plant molecular biology, 54(5), 743-753.

[13] Graether, S.P., Boddington, K.F. (2014). Disorder and function: a review of the dehydrin protein family. Frontiers in plant science. 5, 576.

[14] Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods, 25(4), 402-408.

[15] Koehler, G., Weisel, T. J., & Randall, S. (2007). Transcript expression analysis indicates distinct roles for dehydrin subclasses. Current Topics in Phytochemistry, 8, 73-83.

[16] Welin, B. V., Olson, Å., Nylander, M., & Palva, E. T. (1994). Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in Arabidopsis thaliana. Plant molecular biology, 26(1), 131-144.

[17] Chen, Q. F., Xu, L., Tan, W. J., Chen, L., Qi, H., Xie, L. J. & Zhang, J. H. (2015). Disruption of the Arabidopsis defense regulator Genes SAG101, EDS1, and PAD4 confers enhanced freezing tolerance. Molecular plant. 8(10), 1536-1549.

[18] Gilmour, S. J., Artus, N. N., & Thomashow, M. F. (1992). cDNA sequence analysis and expression of two cold-regulated genes ofArabidopsis thaliana. Plant molecular biology, 18(1), 13-21.

[19] Lee, H., Xiong, L., Ishitani, M., Stevenson, B., & Zhu, J. K. (1999). Cold-regulated gene expression and freezing tolerance in an Arabidopsis thaliana mutant. The Plant Journal, 17(3), 301-308.

[20] Kim, S. Y., & Nam, K. H. (2010). Physiological roles of ERD10 in abiotic stresses and seed germination of Arabidopsis. Plant cell reports, 29(2), 203-209.

[21] Yamasaki, Y. (2013). Responses of soybean to cold temperature/Dehydrins in Arabidopsis and soybean (Doctoral dissertation, Purdue University).

[22] Chinnusamy, V., Zhu, J., & Zhu, J. K. (2007). Cold stress regulation of gene expression in plants. Trends in Plant Science, 12(10), 444-451.

[23] Yadav, S. K. (2010). Cold stress tolerance mechanisms in plants. A review. Agronomy for Sustainable Development, 30(3), 515-527.

[24] Huang, G. T., Ma, S. L., Bai, L. P., Zhang, L., Ma, H., Jia, P. & Guo, Z. F. (2012). Signal transduction during cold, salt, and drought stresses in plants. Molecular Biology Reports, 39(2), 969-987.

[25] Nakashima, K., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2014). The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. Frontiers in plant science, 5, 170.

[26] Qin, T., Zhao, H., Cui, P., Albesher, N., & Xiong, L. (2017). A Nucleus-localized Long Non-Coding RNA Enhances Drought and Salt Stress Tolerance. Plant physiology, pp-00574.

[27] Chacha, A. R. (2014). Functional dissection of ERD14 phosphorylation-dependent calcium binding activity (Doctoral dissertation).

[28] Kiyosue, T., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). Characterization of two cDNAs (ERD10 and ERD14) corresponding to genes that respond rapidly to dehydration stress in Arabidopsis thaliana. Plant and Cell Physiology, 35(2), 225-231.

[29] Theocharis, A., Clément, C., & Barka, E. A. (2012). Physiological and molecular changes in plants grown at low temperatures. Planta, 235(6), 1091-1105.

[30] Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, 218(1), 1-14.

[31] Huang, C., Ding, S., Zhang, H., Du, H., & An, L. (2011). CIPK7 is involved in cold response by interacting with CBL1 in Arabidopsis thaliana. Plant science, 181(1), 57-64.

[32] Cvetkovic, J., Müller, K., & Baier, M. (2017). The effect of cold priming on the fitness of Arabidopsis thaliana accessions under natural and controlled conditions. Scientific Reports, 7, 44055.