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Ciencias Naturales e Ingenierías

Expresión de genes involucrados en la respuesta a factores abióticos en *Capsicum annuum*

Expression of gene involved in the response to abiotic factors in *Capsicum annuum*

Dafne Alejandra Ríos-Molina[1a]
Edgar Querubín Aquino-Medina[1a]
Yeny L. Couoh-Uicab[1,2]
Lorenzo Guevara-Olvera[1]
Guillermo A. Silva-Martínez[1]
Gerardo Acosta-García[1]
Humberto R. Medina[1]

[1] Tecnológico Nacional de México en Celaya, Laboratorio de Biología Molecular, Departamento de Ingeniería Bioquímica
[2] Instituto Tecnológico Superior de Purísima del Rincón, División de Ingeniería Bioquímica, Purísima del Rincón
[a] Those authors contributed equally to this work

Autor para correspondencia: Humberto R. Medina, E-mail: hramirez@conacyt.mx

Resumen

Introducción: Estrés abiótico ocasionado por frío o déficit de agua altera muchos procesos celulares que modifican la fisiología y bioquímica de plantas; esto resulta en reducción del rendimiento de cultivos agrícolas. Las giberelinas son ácidos carboxílicos diterpenoides tetracíclicos que inducen crecimiento y desarrollo de plantas. La transcripción de muchos genes se modifica durante estrés abiótico o por aplicación de giberelinas exógenas; algunos de ellos codifican para proteínas como LEA que confieren protección contra temperatura baja y deshidratación, WRKY y FT participan en la respuesta a estrés abiótico, FT que regula el tiempo

de floración y GA20ox1 que sintetiza giberelinas. La generación de conocimiento sobre los mecanismos moleculares que regulan la respuesta de las plantas a estrés abiótico es esencial para el mejoramiento del cultivo de *Capsicum annuum*. Para ello, en este estudio nos enfocamos en analizar el efecto de estrés abiótico y la aplicación de fitohormonas exógenas sobre el desarrollo *C. annuum*, principalmente en producción de fruto (chile) y la expresión de genes involucrados en la respuesta a dichas condiciones.

Método: El genoma de *C. annuum* contiene homólogos de las proteínas LEA, WRKY, FT y GA20ox1, por lo que estimamos mediante Real-Time PCR (qPCR) y análisis de fenotipo, la expresión de genes y producción de frutos en plantas expuestas a temperatura baja (4 ºC), déficit de agua o tratadas con giberelinas exógenas (GA_3).

Resultados: Los transcritos de los genes *CaLEA73* y *WRKY40* se incrementaron durante estrés por frío en hojas. La expresión del gen *CaGA20ox1* disminuyó durante estrés por frío, aplicación de GA_3 y estrés hídrico-GA_3 en hojas. Este efecto se observó también en botones florales de plantas crecidas bajo déficit de agua, tratadas con giberelinas exógenas, o crecidas bajo estrés hídrico-GA_3; curiosamente, los transcritos de este gen fueron ligeramente abundantes en plantas crecidas bajo déficit de agua.

La transcripción de *CaFT* se indujo por estrés por frío y GA_3 en hojas y botones florales, respectivamente; sin embargo, la transcripción de *FT* fue reprimida por estrés hídrico y GA_3-estrés hídrico en ambos tejidos. Estrés por frío y aplicación de fitohormonas exógenas incrementaron la producción de frutos.

Conclusión: De acuerdo con estos resultados, suponemos que el tratamiento con frío induce los mecanismos de defensa de la planta mediante activación de factores de transcripción como WRKYs y proteínas LEA e incrementa el desarrollo de la planta mediante inducción de la ruta de señalización de FT. Nuestro estudio contribuye al entendimiento de los mecanismos moleculares que controlan las respuestas a estrés abiótico y la participación de las giberelinas en el desarrollo de *C. annuum* para mejorar el rendimiento del cultivo de chile.

Palabras clave: *Capsicum annuum*; factores de transcripción WRKY; giberelinas; proteínas FT; proteínas LEA; estrés abiótico

Abstract

Introduction: Abiotic stress caused by cold or water-deficit alters many cellular processes that modificate the physiology and biochemistry of plants, which reduces yield of agricultural crops. Gibberellins are phytohormones that can induce growth and development of the plants. There are many genes whose transcription is modified during abiotic stress or by exogenous-gibberellins application; some of them encode for proteins such as LEA that confer protection against low temperature and dehydration, WRKY and FT that take part in the response to abiotic stress, FT that regulates the flowering time, and GA20ox1 that synthesized gibberellins. The understanding of molecular mechanism that regulates the plant responses to abiotic stress or exogenous gibberellins application is essential for *Capsicum annuum* (pepper) agriculture improvement. To this aim, we have proceeded to study the effect of biotic stress and exogenous phytohormones on *C. annuum* development, mainly in fruit (chili) production and expression of genes involved in the response to these conditions.

Method: The genome of *Capsicum annuum* contains homologues to the proteins LEA, WRKY, FT and GA20oxy, so we estimate by Real-Time PCR (qPCR) and phenotype analysis, the gene expression and fruits production in plants grown under abiotic stress and after treatment with exogenous gibberellins.

Results: The transcripts of *CaLea73* and *CaWRKY40* increased by cold stress in leaves. While, *CaGA20ox1* expression was down-regulated by cold stress, GA_3, and hydric stress-GA_3 in leaves. This effect was also observed in flower buds of plants grown under water-deficit, treated with gibberellins or hydric stress-GA_3; curiously, the transcripts from this gene became slightly abundant in plants grown under water-deficit. *CaFT* transcription was induced by cold stress and GA_3 in leaves and flower buds, respectively; however, transcription of this gene was almost abolished by hydric stress and GA_3–hydric stress in both tissues. Cold stress and exogenous phytohormones raised the fruits production.

Conclusion: According with these results, we propose that cold treatment induces the plant defense mechanisms through activation of transcription factors like WRKYs and LEA proteins and increases the plant development through induction of signaling pathway of FT. Our study contributes to understanding on molecular mechanisms governing the responses to abiotic stress and the participation of the gibberellins in *C. annuum* development and to improve the yield of the chili crop.

Keywords: *Capsicum annuum*; WRKY transcription factors; gibberellins; FT proteins; LEA proteins; abiotic stress

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Introduction

Abiotic stress caused by cold, including low temperature and freezing, modifies the physiology and biochemistry of plants (Rihan *et al*., 2018), which affects growth and development and, therefore reducing yield of agricultural crops (Josine *et al*., 2011; Sanghera *et al*., 2011). Low temperature, chilling (0–10 °C), limits the crop sustainability, mainly those of plants from tropical regions because they are cold sensitive and do not have the capacity to acclimatize (Thomashow, 1999). Low temperature induces dehydration of cells and tissues which affects many processes as cellular division, photosynthesis, and water transport (Beck *et al*., 2007); all they are essentials for growth and development. These cellular alterations induce changes in the expression of genes whose proteins are essentials for chilling tolerance (Jeknić *et al*., 2014). Cold stress induced genes have been grouped in two classes (Seki *et al*., 2002): the first one includes late embryogenesis abundant proteins (LEA) (Mertens *et al*., 2018), heat shock proteins, antifreeze proteins, lipid transfer proteins and others proteins, and the second one contains various transcription factors, which are involved in regulation of signal transduction and expression of cold-inducible genes (Sanghera *et al*., 2011).

Another condition of abiotic stress that directly affects the growth and development of plants is water-deficit (Gupta *et al*., 2016). The primary response of plants under this stress condition is to avoid further loss of water and to protect the cellular structure. For this, the proton pumps associated with the plasma membrane facilitate stomatal closure (Komatsu *et al*., 2009). Furthermore, lignification of cell wall is a preventive measure, which aids in preserving the intracellular moisture content and ion balance. In addition to, plants alter growth rates and

redistribute resources for better survival. Water-deficit response also includes modifications in dehydration-induced proteins, including chaperones, LEA proteins and dehydrins, among others involved in protecting the cellular components, and changes in expression levels of dehydration responsive transcription factors such as WRKY, bZIP, and AP2-domain (Bonhomme *et al*., 2012).

The gibberellins are tetracyclic diterpenoid carboxylic acids, synthetized by fungi, plants and other organisms, whose basic structure usually contains either 20 (inactive forms) or 19 carbon atoms (Binenbaum *et al*., 2018). The geranylgeranyl diphosphate is precursor of ent-kaurene which gives up vast assortment of gibberellins.

However, only some of them, such as GA_1, GA_3 and GA_4, are bioactive forms that induce the plant development, including cellular expansion and flowering (Sandoval-Oliveros *et al*., 2017). The biosynthesis of these diterpenoids involves several 2-oxoglutarate-dependent dioxygenases that carry out the latter stages of the biosynthetic pathway. These enzymes had been grouped into three families: GA20-oxidase family whose members catalyze three successive steps late in the GA pathway; GA3-oxidase family whose members convert several GAs produced by GA20-oxidase to bioactive GAs forms; and GA2-oxidase family whose members carry out the conversion of some bioactive GAs such as GA_1 and GA_4 to inactive GAs forms for reduce the bioactive GAs levels (Pearce *et al*., 2013). The environmental cues induce changes in the expression of genes encoding these enzymes, mainly the genes for *GA20ox*, which are presumably responsible for rate-limiting steps in the GAs biosynthesis. Moreover, *FT* induction by photoperiod is related to up-regulation of genes for GAs biosynthesis in the apices, but not in the leaves. In *Solanum lycopersicum*, the application of exogenous gibberellins induces cellular expansion and development of parthenocarpic fruit (de Jong *et al*., 2009). This effect is associated to GA_1-fitohormone accumulation that is related to either induction of expression of gene for GA20-oxydase or reduction of GA2-oxydases level which carry out the inactivation of GAs. In *Arabidopsis thaliana*, GAs induces the transition of vegetative to reproductive phases by expression of *SOC1* and *LFY* gene that encode for transcription factors.

The LEA hydrophilic proteins confer protection against abiotic stress conditions, such as dehydration and freezing. It has been showed that those proteins act as chaperones to reduce denaturation and inactivation of other proteins and to protect membrane structures (Bravo *et al*., 2003). The mechanism of these proteins occurs either by direct binding to protein surfaces and water replacement or by water control around the associated macromolecule (Reyes *et al*., 2005).

The LEA proteins according to their glycine content and hydrophilicity belong to a wide family that includes hydrophilins, dehydrins, and Why domain proteins, that also confer protection against dehydration. All they are found in many organisms. In plants, LEA proteins are expressed at varied levels, through all developmental stages in almost all tissues (Hong-Bo *et al*., 2005), and their accumulation occurs in both embryonic and vegetative tissues lacking water (Sharma *et al.*, 2016). The expression of these proteins is also modified by pathogenesis like a part of the plant hypersensitive response.

In *Capsicum annuum*, expression of *CaLEA73* gene occurs during development and it is modified by cold stress and abscisic acid application (Cortez-Baheza *et al*., 2008). Moreover, ectopic expression of this gene improves tolerance to water-deficit and osmotic stress caused by mannitol in transgenic lines of *Arabidopsis thaliana* (Acosta-García *et al*., 2014).

The WRKY transcription factors (WRKY TFs) regulate many biological processes including response to different conditions such as biotic and abiotic stresses, physiological changes and hormone signaling (Phukan *et al*., 2016; Banerjee and Roychoudhury, 2015). The WRKY TFs are proteins carrying a DNA binding domain, that is a 60 amino acid region defined by conserved sequence WRKYGQK (WRKY domain) at the N-terminous, adjacent to a zinc-finger motif at the C-terminus. Moreover, they contain nuclear localization, kinase domains, and rich regions in polar amino acids such as serine, threonine, or glutamine (Chen *et al*., 2012). The WRKY domain recognizes to sequence motif (T)(T)TGAC(C/T), called the W-box which invariant TGAC core is essential for WRKY TF binding to the promoter of target gene and to induce its expression. The WRKY domain can be found in one or two copies and some of them also bind another sites (Cai *et al*., 2008). The WRKY TFs form a large family that is frequently found in many plants. In *A. thaliana*, AtWRKY33 interacts with multiple VQ proteins, a class of proteins carrying a conserved motif (FxxxVQxLTG), to regulate abiotic stress (Wang *et al*., 2015). In *Capsicum annuum*, CaWRKY6 actives the *CaWRKY40* transcription during plant-patogen interaction to regulate the resistance and to provide tolerance against high-temperature and high-humidity (Cai *et al*., 2015).

The *Flowering Locus T* (*FT*) gene encodes a protein essential for the regulation of flowering, a developmental process in plants that involves cellular differentiation of shoot apical meristem into a floral meristem. The *FT* gene transcription is regulated by many proteins and external factors such as long days. The FT protein structure contains a segment B (a surface-

exposed loop region) and a residue Y85 that are essentials for flowering inducing. The FT protein synthesis occurs in the phloem cells of the leaf and then it is transported to the apical meristem cells (Corbesier *et al*., 2007), where it forms a complex with 14-3-3 proteins and the bZIP transcription factor FD. This complex, known as the florigen activation complex, is thought to translocate to the nucleus where it activates the floral meristem identity genes, thereby inducing flowering; this mechanism for formation and move of florigen complex was proposed some years ago (Taoka *et al*., 2012).

The study of Arabidopsis mutants affected in flowering enabled the identification of several flowering time control genes (Koornneef *et al*., 1991), including *Ft* gene (Kardailsky *et al*., 1999). FT-like proteins are members of the small phosphatidylethanolamine-binding protein family and they are classified in two groups (Danilevskaya *et al*., 2008) that can be subdivided into four subgroups in agree with Lee *et al*. (2013). In addition to, FT-like proteins act as either mobile or cell autonomous proteins that mediate developmental processes, such as growth, plant architecture control, fruit set and tuber formation (Navarro *et al*., 2011).

Capsicum is a genus that groups 32 species of plants at least, members of Solanaceae family, natives from tropical regions of America; these flowering plants are monoicous and autogamous and grow like a semi-bush (Moscone *et al*., 2007; Kim *et al*., 2014). Mexico is an excellent region for species domestication, holds a wide genetic diversity, and the second producer in the world, with almost 200 thousand ha cultivation that produce more than 3 million ton from many chili pepper varieties (FAOSTAT, 2018). Many species of Capsicum produces capsaicinoids, such as capsaicin, an alkaloid that accumulates in the fruit and confers a pungent flavor (chili peppers), and anthocyanin and carotenoids, pigments that confer color. Chili peppers are used like flavoring (spicy taste) in the foods and their ingestion produces benefits for health. Moreover, they are a source of capsaicin that used like active compound in some medicines (Reyes-Escogido *et al*., 2011).

The growth and development of *C. annuum* are affected by abiotic stresses such as cold and water-deficit, which decrease the crop yield. However, application of exogenous gibberellins induces the growth and development of the plants thus these phytohormones are a good alternative for reduce the adverse effect of abiotic stress. The understanding of molecular mechanism that regulates the plant responses to abiotic stress or exogenous gibberellins application is essential for pepper agriculture improvement. To this aim, we studied the effect of abiotic stress and the

participation of gibberellins on *C. annuum* development, mainly in fruit production and the expression of genes involved in the response to these conditions.

Materials and Methods

Seed germination and plant growth

The seeds from *Capsicum annuum* var. huichol were cleaned using absolute ethanol (96°), incubated at 23 ºC, and exposed to photoperiod (16 h light/8 h darkness) in a growth chamber (Lab-Line Instruments, ILL, EE.UU). Once in the cotyledon stage, they were transplanted into a soil mix (peat most/perlite/vermiculite, 3/1/1) and grown under controlled conditions (24 °C during the day and 20 °C at night) in the greenhouse. The plants were irrigated each three days.

Treatments

For cold stress, the plants carrying 4-6 true leaves (30 days-aged around) were incubated at 4 ºC during either 4 h or 6 h; for phytohormones treatment, gibberelic acid (GA_3, 10 μM) was spread, each 15 days, on the plants (Pichardo-González *et al*., 2018), until formation of flower-buds was observed like described Ouzounidou *et al*. (2010); for GA_3-cold stress treatment, the plants were treated with the phytohormone and then exposed to 4 ºC; for hydric stress, the plants that had reached the anthesis (flowering) stage were not irrigated for 15 days; and GA_3-hydrid stress treatment, the plants were treated with the phytohormone and then they were subjected to water-deficit. The control plants were irrigated with water only. Thus, tissue samples (0.5-2.0 g) either

leaf or flower buds, which were cut using a scalpel, were frozen into liquid nitrogen, and stored at -70 ºC.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from frozen leaf tissue (0.5 g around) following the TRIzol method (Chomczynski & Sacchi, 1987) and the recommendations of manufacturer (Invitrogen, CA, EE. UU.). The total RNA yield was estimated from 260/280nm and 260/230nm absorbances that were measured in a spectrophotometer NanoDrop 2000 UV-Vis (Thermo Fisher Scientific, OH, EE. UU.). The integrity of isolated RNA was corroborated by agarose gel electrophoresis using an Owl-Easy B1A System (Thermo Fisher Scientific, CA, EE. UU.). The DNA genomic was removed with DNase (RNase-free DNase set, Zymo Research, CA, EE. UU.). The cDNA was synthesized from mRNA (2.0 μg) using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific, CA, EE. UU.) following instructions of the manufacturer.

Real-Time PCR

The SYBER Green mix (Radiant Green Lo-ROX qPCR) (Alkali Scientific, FL, EE. UU.), cDNA (200 ng), and oligonucleotides (5´*TCCATCAATGGGAATCCATC*3´) and (5´*AGGTGGTACTGGTGGTCGTC*3´) for *FT* gene, (5´*CGGGGGAGAGAATAAAGGAG*3´) and (5´*GAAAGCGCAAAGAACAAAGC*3´) for *CaLEA73* gene (DQ902577.1), (5´*GTGGCAGCCGGTTAAATGTG*3´) and (5´*TGCTAGTCCTATCGGTGGCA*3´) for *wrky40* gene, (5´*GTTGCCTGCATAGAGCAGTG*3´) and (5´*CATTCGAGGGTTGTTGGAGT*3´) for *GA20ox1* gene, and (5´*GGCCTTATGACTACAGRRCACTCC*3´) and (5´*GATCAACCAACAGAGACATCCACAG*3´) for *gapdh* gene, were used for Real-Time PCR. The OligoPerfect program (Thermo Fisher Scientific, CA, EE. UU.) was used for oligonucleotides

design. The amplification (95°C for 2 min followed by 30 cycles at 95°C for 2 s and 55-60°C for 30 s) was performed in a Thermal Cycler CFX96 (Bio-Rad, CA, EE. UU.). The transcript levels were normalized to those of *gapdh* gene, which expression is supposed to be constitutive (endogenous control), in the same sample. The transcripts abundance was relative to control sample (control plants) expression. For this, C_T value was analyzed according to comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The amplification of specific product was corroborated by melting curves analysis. Three different biological samples were analyzed by duplicate.

Results and Discussion

CaLEA73 gene over-expression in plants exposed to low temperature

In order to study the role of LEA proteins in cold response in *C. annuum*, the *CaLEA73* expression (Cortez-Baheza *et al*., 2007) was estimated in plants that were exposed to low temperature. For this, total RNA was isolated from a sample of plant tissue (leaves), which was used like template for cDNA synthesis, and transcript abundance from *CaLEA73* was measured by Real-Time PCR. *CaLEA73* expression was increased in leaves of plants that were incubated at 4 ºC during 4 h (**Fig. 1**, left panel). This corroborates that *CaLEA73* is part of the molecular mechanism involved in cold response, which starts by low temperature sensing followed by expression of cold-regulated genes. The induction by cold stress of LEA proteins has also been observed in *A. thaliana* and other plants (Thomashow, 1998).

CaLEA73 is also up-regulated in root tissue of 2 months-old plants of *C. annuum* cv. caballero, a variety affected in seed germination, that were exposure to low temperature (4 ºC) during 16 h (Cortez-Baheza *et al*., 2008). However, transcripts from *CaLEA73* were not detected neither leaves nor stem in those experiments. Presumably, CaLEA73 protein has a function in membrane stabilization and to prevent protein aggregation by dehydration caused for low

temperature (Hundertmark *et al.*, 2008) in *C. annuum* roots. In addition, low temperature should activate the transcription of other genes whose proteins participate in processes involved in plant tolerance to abiotic stress. Our results showed that exposure to low temperature during 6 h represses the expression of *CaLEA73* in leaves; however, this effect was also observed in control plant leaves. Presumably, prolonged exposure to low temperature induce the frozen of tissues, which causes damage to cellular structure, even cells death.

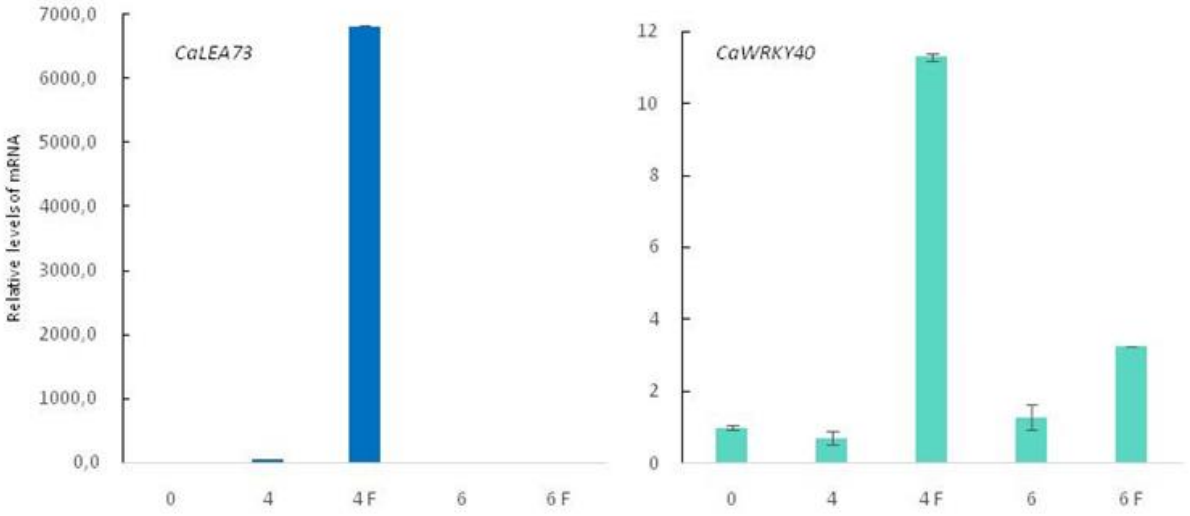

Fig. 1. Effect of cold stress on expression of two *C. annuum* genes: *CaLEA73* (late embryogenesis abundant protein), and *CaWRKY40* (transcription factor). 0, 4, and 6 untreated plants (Control); 4F and 6F, plants exposed to 4 ºC for 4 h and 6 h, respectively. The ordinates give the mean of at least two independent experiments of Real-Time PCR following reverse transcription of mRNA from two independent sets of plants. All measurements were calibrated with those of *gapdh* (for glyceraldehyde 3-phosphate dehydrogenase) in the same tissue and the mRNA amounts were calculated following the comparative method $2^{-\Delta\Delta Ct}$ (Livak & Schmitteng, 2001).

CaWRKY40 gene over-expression in plants exposed to low temperature

To investigate the role of *CaWRKY40* in cold response of *C. annuum*, the transcript abundance of *CaWRKY40* was estimated in chili pepper plants exposed to low temperature. For this, the tissue, nucleic acids, and Real-Time PCR was prepared and carried out like described previously (see above). *CaWRKY40* transcription was induced in plant leaves exposed at 4 ºC during 4 h, a 1.5-fold increase was observed (**Fig. 1**, right panel). This result was expected because WRKY TFs act as transcriptional regulators of many genes whose proteins participate in defense against abiotic stresses (Rushton *et al*., 2010). *CaWRKY40* is also up-regulated in pepper plant leaves exposed at 42 ºC (Dang *et al*., 2013). These results corroborate the participation of gene *CaWRKY40* in abiotic stress response of *C. annuum*, presumably, in the mechanisms regulating temperature sensing. Our results showed that exposure to low temperature during 6 h decreases the expression of *CaWRKY40*. However, the expression level was higher in treated plants than control plants. This suggests that prolonged exposure at low temperature induces the frozen of tissues, which causes damaged of cellular structures, even cellular death.

CaGA20ox1 expression in plants exposed to abiotic stress and treated with exogenous gibberellins

To study the involving of *CaGA20ox1* in *C. annuum* cold-stress response, the mRNA abundance of *CaGA20ox1* was estimated in plants that were exposed to low temperature (4 ºC). For this, the tissue and nucleic acids preparation and Real-Time PCR were carried out like described before (see above). *CaGA20ox1* expression was down-regulated by cold stress; thus, transcripts were lower in leaves that were exposed to cold during 4 h than control leaves (**Fig. 2**, left panel). Our results suggest that low-temperature activates the mechanism that regulates the transcription of gene *CaGA20ox1* that encodes for a dioxygenase that carry out the late steps in GAs biosynthesis which are related to growth and development. These cellular processes are almost stopped during cold-stress so that active phytohormone production can also be stopped as part of plants responses.

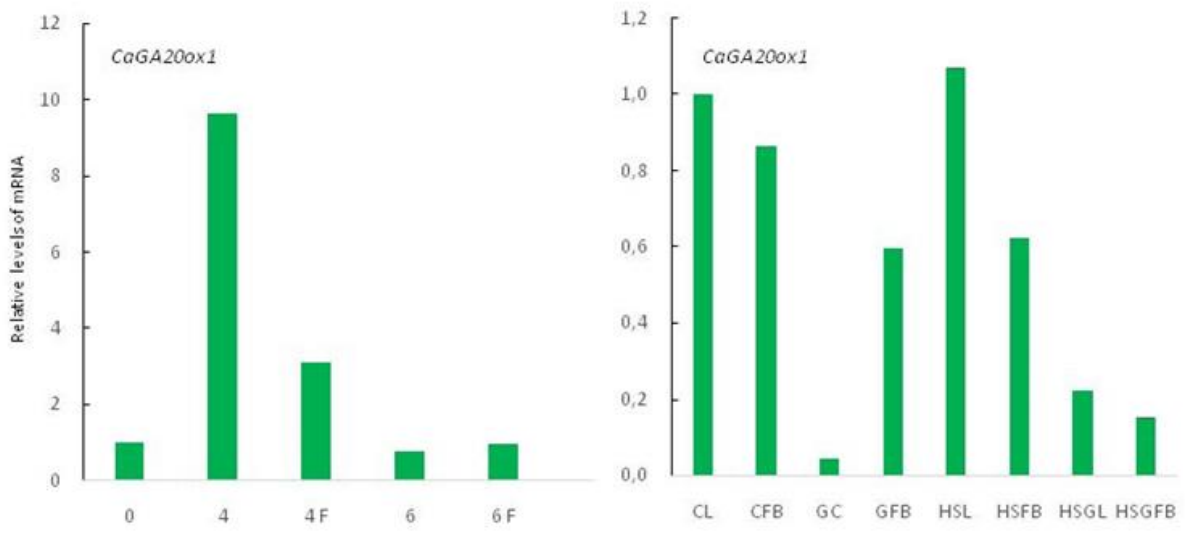

Fig. 2. Effect of abiotic stress and exogenous gibberellins on expression of *CaGA20ox1* (2-oxoglutarate-dependent dioxygenase) in *C. annuum*. Cold stress (left panel), 0, 4, and 6 untreated plants (Controls) and 4F and 6F, plants exposed to 4 ºC for 4 h or 6 h, respectively. Exogenous giberellins and hydric stress (right panel): C, untreated plants (Control); L, leaves; FB, flower buds; G, gibberellins and HS, hydric stress. The ordinates give the mean of at least two independent experiments of Real-Time PCR following reverse transcription of mRNA from two independent sets of plants. All measurements were calibrated with those of *GAPDH* (for glyceraldehyde 3-phosphate dehydrogenase) in the same tissue and the mRNA amounts were calculated following the comparative method $2^{-\Delta\Delta Ct}$ (Livak & Schmitteng, 2001).

We explored the participation of *CAGA20ox1* in *C. annuum* response to water-deficit and exogenous gibberellins application; a set of plants were grown under hydric stress and treated with GA_3 (10 μM). The transcription of this gene was decreased in leaves of plants treated with either gibberellins or both hydric stress and gibberellins; the transcript levels were lower in these plants than control plants (**Fig. 2**, right panel). However, this effect was not observed in leaves of plants grown under water-deficit; the transcripts level was higher in these plants than control plants, but this increase was not considerable. Moreover, hydric stress and gibberellins down-regulated the expression of the gene *CaGA20ox1* in flower buds; the transcription decreased almost at 50 % level, respectively.

In addition to, combination of these treatments (water-deficit and gibberellins) decreased even more the *CaGA20ox1* transcription, almost at 20 % in both tissues (leaves and flower buds). Our results demonstrate that hydric stress and exogenous gibberellins down-regulate the expression of *GA20ox1* in *C. annuum.* It is well known that application of exogenous phytohormones can modify the GAs biosynthesis, which is related to growth and development; thus the *GA20ox1*, that take part in the production of gibberellins, is not required because those cellular processes are almost stopped cause of abiotic stress. In addition to, a high level of gibberellins is reached which can carry out the feedback of the GAs pathway.

CaFT expression in plants exposed to abiotic stress and treated with exogenous gibberellins

To analyze the *CaFT* function in *C. annuum* response to cold stress, *CaFT* transcription was estimated in plants exposed at low temperature. For this, the tissue and nucleic acids preparation and Real-Time PCR were carried out like described before (see above). *CaFT* expression was induced in leaves that were exposed at 4 ºC during 4 h; the mRNAs level was increased, until 10 times more, in leaves treated than control leaves (**Fig. 3**, left panel). These results corroborated that *CaFT* expression is induced by abiotic stress in *C. annuum,* as it has also been observed in other plants. *CaFT* transcription is induced by CONSTANS protein, a transcription factor (Putterill *et al*., 1995; Suárez-López *et al*., 2001), and other factors such as temperature and stress by salicylic acid to promote flowering (Martínez *et al*., 2004). In *Populus trichocarpa* (poplar), two *FT* homologs controls the seasonal flowering cycle in response to temperature (Hsu *et al*., 2011). *PtFT2,* which supports vegetative growth and bud set inhibition during autumn, is up-regulated both by high temperatures and long photoperiods during the spring and summer. Conversely, *PtFT1*, which initiates reproductive growth, is repressed by high temperatures, but induced by low temperatures during winter. Moreover, low-temperatures also promote expression of *FT* genes in *Satsuma mandarin* (*Citrus unshiu*) (Nishikawa *et al*., 2007) and kiwifruit (Varkonyi-Gasic *et al*., 2013). Prolongated exposure at low-temperature does not modify the expression of *CaFT* as

demonstrated the low levels of detected transcripts in leaves from both treated and control plants. This effect cannot be attributed completely to neither cold stress nor time because gene *FT* messengers are able to move by plants, so that mRNAs low levels detected in leaves (6 h after) agree with *CaFT* movement.

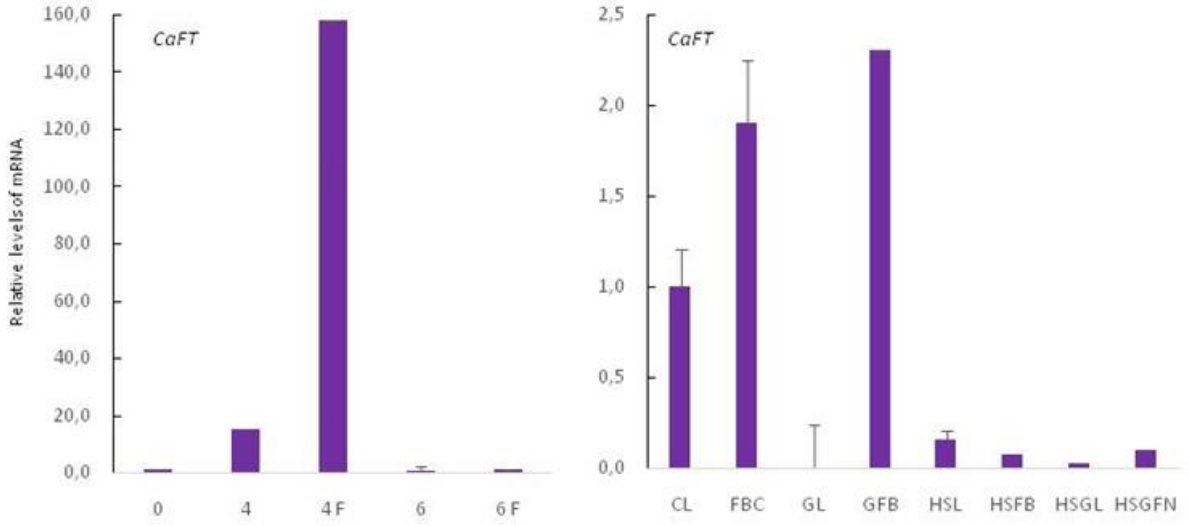

Fig. 3. Effect of abiotic stress and exogenous gibberellins on expression of *FT* (*Flowering Locus T*) in *C. annuum*.

Cold stress (left panel), 0, 4, and 6 untreated plants (Controls) and 4F and 6F, plants exposed to 4 ºC for 4 h or 6 h, respectively. Exogenous giberellins and hydric stress (right panel): C, untreated plants (Control); L, leaves; FB, flower buds; G, gibberellins and HS, hydric stress. The ordinates give the mean of at least two independent experiments of Real-Time PCR following reverse transcription of mRNA from two independent sets of plants. All measurements were calibrated with those of *GAPDH* (for glyceraldehyde 3-phosphate dehydrogenase) in the same tissue and the mRNA amounts were calculated following the comparative method $2^{-\Delta\Delta Ct}$ (Livak & Schmitteng, 2001).

We explored the function of *CaFT* in *C. annuum* response to water-deficit and gibberellins application. For this, we analyzed the *CaFT* transcription in tissue samples of leaves and flower buds from a set of plants grown under water-deficit or treated with GA_3 (10 μM). FT transcription was down-regulated by both gibberellins and water deficit in leaves; transcript levels were almost

abolished by exogenous phytohormones application (**Fig. 3**, right panel). However, *CaFT* expression was up-regulated by gibberellins in flower buds; the transcript level was increased, but this effect was not so considerable in comparison to control. Hydric stress and gibberellins-hydric stress down-regulated the *CaFT* expression.

These results demonstrated that exogenous gibberellins induce slightly the *CaFT* expression in flower buds, which will presumably modify the plant development, surely for flowering, because this gene has a main role in this cellular process. However, water-deficit did repress the expression of gene *CaFT*, which affect both growth and development. It is well known that abiotic stress modifies these cellular processes. In addition to, our results showed that exogenous gibberellins do not revert the water stress effect, as demonstrated the low level of *CaFT* mRNAs detected in both leaves and flower buds from plants treated with hydric stress-gibberellins.

Development and production of fruits in plants exposed to cold stress and treated with exogenous gibberellins

In order to analyze the effect of *CaFT* up-regulation by cold stress and exogenous gibberellins on crop yield of *C. annuum*, we evaluated the production and quality of fruits in plants exposed to these treatments. For this, several characteristics of the fruits were measured (**Fig. 4**). It was observed that low temperature for a short time (4 ºC, 4 h) and exogenous gibberellins (GA_3, 10 μM) increased the *C. annuum* yield; the plants treated produced more fruits than control plants. This effect was also observed in plants treated with cold stress only, but the increase was at 50 % around. In addition to, fruit weight was increased in plants treated with cold stress, exogenous gibberellins, or combination of these treatments. However, the fruit size was not modified by either treatment. All these observations suggest that cold stress and exogenous gibberellins induce the production and development of fruits in *C. annuum*.

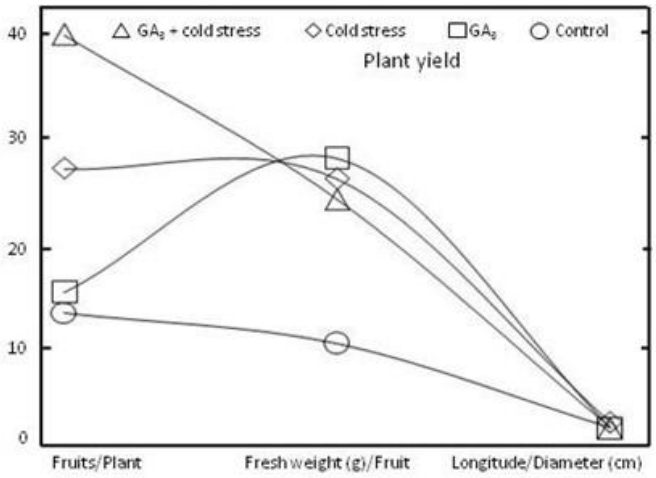

Fig. 4. Effect of cold stress and exogenous gibberellins on the yield of *C. annuum* plants.

Mean results of at least two independent sets of plants treated with GA_3 (*squares*), exposed to 4 ºC during 4 h (*diamonds*), combination of these treatments (*triangles*), and control (circles).

Conclusions

Low temperature increases the transcription of genes *CaLEA73* and *CaWRKY40*, whose proteins are involved in protection and defense to cold stress and regulation of genes involved in the response to this stress condition, respectively. *CaFT* transcription, which protein modulates differentiation processes that result in plant development, is increased by cold stress and exogenous gibberellins in leaves and flower buds, respectively. However, these phytohormones repress the transcription of *CaFT* in leaves, and during water deficit and exogenous gibberellins (in combination) down-regulated even more the *CaFT* transcription in the same tissues. The gene *CaGA20ox1* transcription is repressed in leaves and flower buds of plants grown under the abiotic stress and by exogenous gibberellins, its transcription is slightly increased in leaves, but this increase was not so considerable. The application of exogenous gibberellins and low temperature induces the transcription of *CaFT* in leaves and flower buds, respectively. We propose that cold

treatment induces the plant defense mechanisms through activation of transcription factors like WRKYs and LEA proteins and increases the plant development through induction of signaling pathway of *CaFT*. Our study contributes to understanding on molecular mechanisms governing the responses to abiotic stress and exogenous gibberellins in *C. annuum* and to improve the chili pepper agriculture.

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