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Full Length Article

Differential Gene Expression of Anthocyanin Biosynthetic Genes under Low Temperature and Ultraviolet-B Radiation in Bell Pepper (*Capsicum annuum*)

Rubén Gerardo León-Chan[1,2], Luis Alberto Lightbourn-Rojas[2], Melina López-Meyer[3], Luis Amarillas[1,2], J. Basilio Heredia[1], Talia Fernanda Martínez-Bastidas[1,2], Claudia Villicaña[4] and Josefina León-Félix[1*]

[1]*Centro de Investigación en Alimentación y Desarrollo A.C. Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110. Culiacán, Sinaloa*

[2]*Instituto de Investigación Lightbourn A.C. Carretera Las Pampas Km. 2.5 Col. Tierra y Libertad, C.P. 33980 Cd. Jiménez, Chihuahua*

[3]*Instituto Politécnico Nacional, CIIDIR-Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049 Guasave, Sinaloa, México*

[4]*CONACYT-Centro de Investigación en Alimentación y Desarrollo A.C. Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110. Culiacán, Sinaloa*

[*]For Correspondence: ljosefina@ciad.mx

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Abstract

Anthocyanins are colored water-soluble pigments found widespread in plants, which are strongly accumulated during development and under biotic and abiotic stress favoring plant adaptation. Bell pepper (*Capsicum annuum* L.) exhibits anthocyanin pigmentation during development and abiotic stress conditions, making it an interesting model to study anthocyanin biosynthesis. Therefore, the aim of this study was to investigate the temporal expression of the anthocyanin biosynthetic genes *MYB, F3H, F3´5´H, DFR* and *ANS*, in stems from *C. annuum* plants after exposure to LT, UV-B and combined LT+UV-B. In our study, we found a dramatic upregulation of *MYB* showing a peak at 16:00 h on day 31 in all treatments. *F3H* expression was upregulated by LT and LT+UV-B treatments showing a higher increase in the latter. Moreover, *F3´5´H* and *DFR* were strongly increased under LT treatments in bell pepper stems. Surprisingly, *F3H, F3´5´H* and *DFR* showed no changes in UV-B treatment, while *ANS* was only slightly upregulated several hours after UV-B radiation suggesting a late response. Based on our findings, anthocyanin biosynthetic genes were more influenced by LT than UV-B in *C. annuum* stems. The higher expression of *F3H* under LT+UV-B treatment may denote the biosynthesis of other flavonoids instead anthocyanins to protect plants from combined LT+UV-B stress. Furthermore, the higher increase on *DFR* expression in comparison to *ANS* by LT treatment may suggest an enhancement toward proanthocyanidin biosynthesis over anthocyanidin production by *ANS*. Collectively, our results provide new insights about the transcriptional regulation of anthocyanin biosynthetic genes in response to LT and UV-B alone or in combination in bell pepper stems.

Keywords: UV-B; Low temperature; Anthocyanin; Flavonoid; Flavonol; *Capsicum* stems

Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'5'H, flavonoid 3´5´hydroxylase; LT, low temperature; PAR, photosynthetically active radiation; RT-qPCR, real-time quantitative polymerase chain reaction; UV-B, ultraviolet-B radiation

Introduction

Anthocyanins are colored water-soluble pigments belonging to the flavonoid subclass of secondary metabolites that are ubiquitously found in all plants; these compounds are accumulated in vacuoles and responsible for the color of roots, leaves, stems, fruits, flowers, and vegetables (Xu *et al.* 2015). Anthocyanins possess potent antioxidant and free radical scavenging properties that appear to function as protective agents against oxidative damage on DNA and photosynthetic elements, chelator of metals and metalloids, and mediator of reactive oxygen species (ROS)-induced signal transduction pathways in plants (Landi *et al.* 2015; Rouholamin *et al.* 2015). In fact, anthocyanins are highly appreciated for human consumption given their antioxidant properties providing several health benefits related to the

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prevention and treatment of chronic and degenerative diseases such as cancer and metabolic syndrome (Lee *et al.* 2017; Lin *et al.* 2017). Moreover, anthocyanin pigments play other essential biological roles in plants participating in pollination and seed dispersal, as well as protection against various abiotic and biotic stresses providing better mechanisms for adaptation (Harborne and Williams 2000; Ahmed *et al.* 2015). Numerous studies have demonstrated that anthocyanins are strongly accumulated in response to ultraviolet (UV) radiation, low and high temperature, water stress, nutrient depletion, wounding, pathogen attack and high light intensity (Aza-González *et al.* 2012; Theocharis *et al.* 2012; Zlatev *et al.* 2012; Wiltshire 2017). Hence, the anthocyanin biosynthesis has raised special interest as target for genetic improvement based on the fact that anthocyanin accumulation may favor adaptation to stress conditions and climate change, as well as the development of anthocyanin-rich crops for consumers.

Anthocyanin content is dependent on genetic, developmental and environmental factors that collectively regulate anthocyanin metabolism. Anthocyanin biosynthetic pathway has been well established in many plant models, especially in *Solanaceous* vegetables, which is constituted by structural genes that encode enzymes participating in each reaction step, and regulatory genes encoding transcription factors to modulate the expression of structural genes (Liu *et al.* 2018). The anthocyanin biosynthetic pathway is a branch of the general flavonoid pathway, which is divided into genes involved early step of anthocyanin biosynthesis including chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*) and flavonoid 3´5´hydroxylase (*F3´5´H*); and the late biosynthetic genes, dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and flavonoid 3-O-glucosyltransferase (*UFGT*) (Dubos *et al.* 2010; Zhang *et al.* 2015). Flavonoid pathway initiates with the synthesis of naringenin chalcone by CHS, followed by isomerization into naringenin by CHI and conversion into dihydrokaempferol by F3H. Downstream steps involve the conversion of dihydrokaempferol into hydroflavonols, followed by colorless leucoanthocyanidins, then colored anthocyanidins and further some are glycosylated by *UFGT* (Liu *et al.* 2018). In addition, regulation of anthocyanin biosynthesis implies transcription factor types such as MYB, bHLH and WD40 repeats factors, which mostly regulates structural genes at different levels acting in a tissue-specific manner depending on the plant species (Quattroccio *et al.* 1993; Spelt *et al.* 2000). In *Petunia*, for example, expression of *DFR* and *ANS* genes were dependent of MYB-WD40 type regulatory factors, while *CHS*, *CHI* and *F3H* were independently expressed (Quattroccio *et al.* 1993). In other studies, overexpression of MYB transcription factors resulted in the upregulation of structural genes of anthocyanin biosynthesis in *Raphanus sativus*, *Nicotiana tabacum*¸ *Solanum lycopersicum*, *Malus domestica* and *Arabidopsis thaliana*, especially *DFR* and *ANS* genes (Espley *et al.* 2007; Lim *et al.* 2016; Jian *et al.* 2019), while MYB silencing abolished anthocyanin gene expression in *Capsicum annuum* (Zhang *et al.* 2015).

Bell pepper (*C. annuum* L.) is an economical important horticultural crop that exhibits anthocyanin pigmentation in several tissues, which make it a model for the study of anthocyanin biosynthesis (Dhar *et al.* 2015). Studies have demonstrated the expression of genes associated to anthocyanin biosynthesis in tissues such as fruits, flowers and leaves during development and some abiotic stress (Lightbourn *et al.* 2007; Stommel *et al.* 2009). Moreover, bell pepper has been reported as a very sensitive crop to low temperature (LT) and UV irradiation, but showed changes in phenolic compounds likely to cope against abiotic stresses (León-Chan *et al.* 2017; Perveen *et al.* 2018; Rodríguez-Calzada *et al.* 2019). Hence, the study of transcriptional regulation of anthocyanin biosynthetic genes in bell pepper may be useful to understanding the molecular mechanisms that alleviate the negative effects of LT and UV-B radiation, opening the possibility to use the transcription patterns to identify specific stress-induced responses in plants and propose candidate biomarkers for stress tolerance. In bell pepper, anthocyanin biosynthesis has been studied mostly in fruits and leaves, while little research has been done in stems. Therefore, the aim of this study was to investigate the temporal expression of the anthocyanin biosynthetic genes *MYB, F3H, F3´5´H, DFR* and *ANS*, in stems from *C. annuum* plants after exposure to LT, UV-B radiation and combined LT+UV-B.

Materials and Methods

Plant material and growth conditions

Bell pepper seeds Canon cv. (Zeraim Gedera; Israel) were germinated and maintained as previously described (León-Chan *et al.* 2017). Twenty-eight days after sowing (DAS) bell pepper plants were put into a plant growth chamber (GC-300TLH, JEIO TECH; South Korea) for three days at 25/20°C (day/night), a relative humidity of 65% and 12 h photoperiod (from 6:00 to 18:00 h) of photosynthetically active radiation (PAR) (972 μmol·m^{-2}·s^{-1}). Then, the UV-B radiation (UV-B), low temperature (LT) and low temperature with UV-B radiation (LT+UV-B) treatments were applied. For LT and LT+UV-B treatments, temperature was adjusted at 15/10°C the previous night (day 30 at 18:00 h) and maintained until sampling (day 31 and 32). For UV-B and LT+UV-B treatments, plants were irradiated with PAR for 6 h (from 06:00 to 10:00 and 16:00 to 18:00 h) and with UV-B irradiation (72 kJ·m^2) for 6 h (from 10:00 to 16:00 h) at day 31 (Fig. 1). The UV-B radiation was applied as described by León-Chan *et al.* (2017). The UV-B radiation treatment was started at day 31 and control samples were taken at 10:00 h just before starting the UV-B radiation. Then, samples were collected at 11:00 and 16:00 of day 31, and at 04:00 and 11:00 of day 32

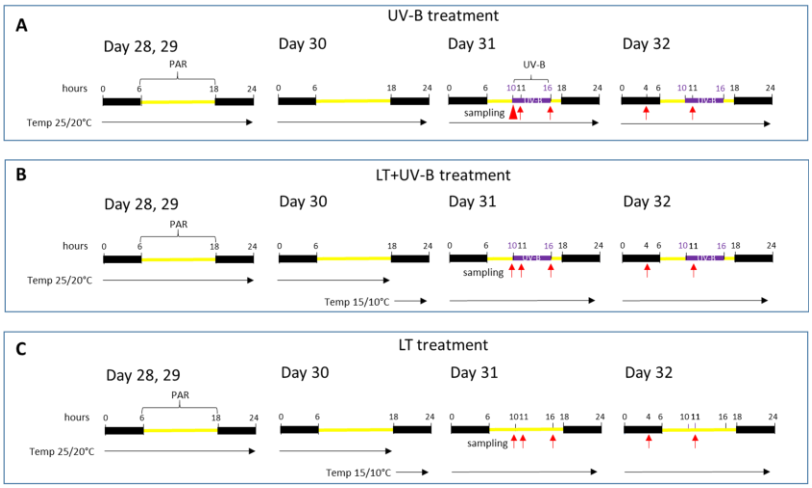

Fig. 1: Scheme that describes the application of UV-B light and low temperature to bell pepper plants from day 28 to 32, as well as the sampling times in UV-B (**A**), LT+UV-B (**B**), and LT (**C**) treatments. Samples were taken at the times indicated by a red triangle (calibrator sample) and red arrows on day 31 and 32. PAR, photosynthetically active radiation

(Fig. 1). Stems samples from 10 bell pepper plants were frozen in liquid nitrogen and stored at -80°C.

RNA isolation of bell pepper stems

Bell pepper stems were scraped using sterile scalpels, then immediately frozen in liquid nitrogen and stored at -80ºC until isolation of total RNA. Stems were pulverized with liquid nitrogen and total RNA was isolated from 50-100 mg of tissue with Trizol reagent (Ambion, Life Technologies, USA) according to the manufacturer´s instructions with the following modifications: two chloroform extractions; for precipitation step, we replaced 0.5 mL of isopropyl alcohol, with a mixture of 0.25 mL of isopropyl alcohol and 0.25 ml of saline solution (McDougall, 2018)); finally, RNA washes with 75% ethyl alcohol was carried out twice. Genomic DNA was removed with Turbo DNA free kit (Invitrogen, Life Technologies, USA). RNA concentration was determined using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and RNA integrity was analyzed by agarose gel electrophoresis.

Primer design and *In silico* analysis

Primers for the transcription factor (*MYB*) and anthocyanin biosynthesis structural genes (*F3H, F3´5´H, DFR*, and *ANS*) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) with the following features: amplicon size between 165 to 256 bp, primer size of 20 bp, melting temperature near to 60ºC and GC content between 50 and 60% (Table 1) (Raymaekers *et al.* 2009; Friedman *et al.* 2014). Primer sequences were analyzed for hairpin, homodimer and heterodimer formation *in silico* with OligoAnalyzer 3.1, and were also compared to NCBI database using BLAST (D´haene *et al.* 2010). As reference gene, we selected the primers for β-tubulin (*β-TUB*) gene (Wan *et al.* 2011). All primers were manufactured by Sigma Aldrich.

Real time quantitative PCR (RT-qPCR) analysis

The cDNA synthesis was carried out from 2 µg of total RNA using the Superscript III kit (Invitrogen, Life Technologies, USA), quantified with NanoDrop 2000c spectrophotometer and stored at -20ºC until its analysis. Primer efficiency was calculated using 2-fold dilution series from 500 to 7.81 ng of cDNA. RT-qPCR was performed in a final volume of 10 µL reaction mixture containing 1 µL of cDNA (100 ng/µL) plus 9 µL of master mix using a CFX96^{TM} Real-Time PCR detection system (Bio-Rad, USA). The master mix consisted of: 5 µL of SYBR SsoAdvanced^{TM} Universal SYBR^{TM} Green Supermix (Bio-Rad, USA), primer concentrations as indicated in Table 1, and finally, added water to complete volume. The amplification conditions were performed under the following conditions: initial denaturation, 95°C for 30 s; followed by 40 cycles of denaturation at 95°C for 10 s, and annealing temperature (T_a) for each primer set as described in Table 1 during 30 s. Melting curves were performed at the end of amplification program to determine primer specificity and PCR products were analyzed by agarose gel electrophoresis to verify amplicon size. Relative gene expression was calculated with the $2^{-\Delta\Delta Cq}$ method using the UV-B treatment at 10:00 h as calibrator and *β-TUB* as normalizer gene and the software CFX Manager 3.0 (Bio-Rad Laboratories, Inc.). For each sample, three biological replicates (n=3) were analyzed with two technical replicates.

Statistical analysis

The data were assessed by Two-way ANOVA and Fisher's

Table 1: Primer sequences used in this study. Fw, forward; Rv, reverse; Ta, annealing temperature

Gene	Primer sequence	Amplicon size (bp)	Ta (°C)	Primer concentration (nM)	Reference
MYB	Fw 5´-TACTAAGACCTCGCCCTCGG-3´ Rv 5´-ACTGCAGCCACATCTTCCTC-3´	238	63.3	280	This study
F3H	Fw 5´-ATGATGATGTGAAAGCAGCG-3´ Rv 5´-TTTCAACTGGTGGCTGCTAC-3´	256	58.3	317	This study
F3´5´H	Fw 5´-CATGCCACACGTGTCACTTG-3´ Rv 5´-GCACCTGCATTAGTTGGACG-3´	165	63	303	This study
DFR	Fw 5´-CGGCTGGATTTATCGGCTCT-3´ Rv 5´-CTTCCACGGTCAAGTCTGCT-3´	168	59.5	317	This study
ANS	Fw 5´-CAGACACCGATATCTCCGGC-3´ Rv 5´-CGCGGCCTCCAGGATTATAG-3´	207	63.6	233	This study
β-TUB	Fw 5´-GAGGGTGAGTGAGCAGTTC-3´ Rv 5´-CTTCATCGTCATCTGCTGTC-3´	167	56.5	317	Wan et al. (2011)

test to determine significant differences using Minitab® 17 statistical software (Minitab Inc.; Pennsylvania, USA). Relative gene expression is shown as the mean ± standard error. Differences at $P \leq 0.05$ were considered significant.

Results

To gain insights about anthocyanin regulation at transcriptional level by abiotic stress on stem bell pepper, we decided to investigate the temporal expression of MYB transcription factor under LT, UV-B and combined LT+UV-B treatments, because MYB has been reported as the major determinant for anthocyanin biosynthesis (Hichri *et al.* 2011). Interestingly, *MYB* was dramatically upregulated in bell pepper stems showing a peak at 16:00 h on day 31 in all treatments, after six hours of UV-B and/or 22 h of LT exposure (Fig. 2). *MYB* exhibited 18.2- and 17.4-fold higher expression in LT and LT+UV-B treatments, respectively; whereas in UV-B treatment, *MYB* expression was slightly lower showing 14.9-fold higher relative expression. In the case of the other sampling times, *MYB* was not induced by UV-B treatments remaining similar to control condition (10:00 h, day 31), while in LT and LT+UV-B treatments, *MYB* showed a tendency to increase for most of sampling times. This finding clearly indicates that *MYB* is strongly induced after six hours of UV-B and/or 22 h of LT exposure alone or in combination.

Considering the possible activation of the anthocyanin biosynthetic pathway in bell pepper stems triggered by *MYB* upregulation, we decided to evaluate the expression of *F3H* and *F3´5´H*, the early structural genes of anthocyanin biosynthesis belonging to the general flavonoid pathway (Aza-González *et al.* 2012). Interestingly, *F3H* was strongly induced under LT+UV-B treatment, starting on 11:00 h on day 31 showing 6-fold higher expression, and increased continuously until early morning (04:00 h, day 32) reaching 11-fold higher expression, and then *F3H* levels decreased at 11:00 h on day 32 (Fig. 3). Interestingly, LT showed a similar expression pattern to LT+UV-B treatment, showing 3.4-fold increase of *F3H* at 16:00 h on day 31 (6 h UV-B/22 h of LT exposure), exhibiting a peak at 04:00 h (6.6-fold) and decreasing at 11:00 h on day 32. Meanwhile, *F3H* levels showed a tendency to be increased by UV-B

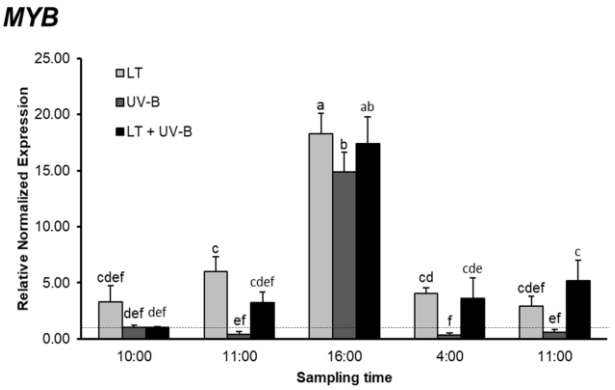

Fig. 2: Relative gene expression of *MYB* gene in *C. annuum* stems under low temperature (LT), UV-B radiation and LT+UV-B at day 31 (10:00, 11:00 and 16:00 h) and 32 (04:00 and 11:00 h) of the experiment. Statistically significant differences ($P \leq 0.05$) are indicated with letters. Dotted line indicates the expression level of calibrator (relative expression set as 1) corresponding to UV-B at 10:00 h on day 31

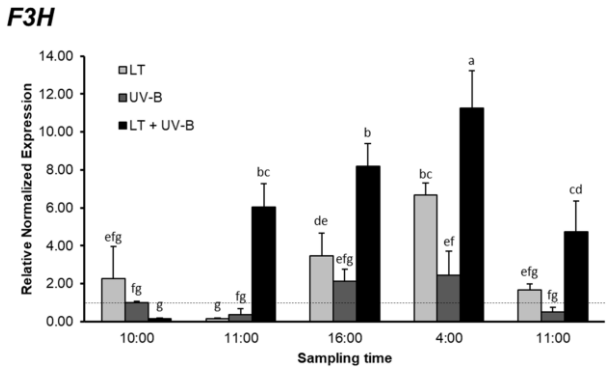

Fig. 3: Relative gene expression of *F3H* (Flavanone 3-beta-hydroxylase) gene in *C. annuum* stems under low temperature (LT), UV-B radiation and LT+UV-B at day 31 (10:00, 11:00 and 16:00 h) and 32 (04:00 and 11:00) of the experiment. Statistically significant differences ($P \leq 0.05$) are indicated with letters. Dotted line indicates the expression level of calibrator (relative expression set as 1) corresponding to UV-B at 10:00 h on day 31

treatment at 16:00 and 04:00 h on day 31 and 32, respectively (Fig. 3). On the other hand, *F3´5´H* gene was

upregulated mainly under LT treatment starting on 11:00 h (14.4-fold) and showing a peak at 16:00 h (27.2-fold) on day 31; moreover, *F3´5´H* expression gradually decreased until reaching a basal level at 11:00 h on day 32 (Fig. 4). Strikingly, LT+UV-B treatment induced the expression of *F3´5´H* at lower levels in comparison to LT treatment; despite *F3´5´H* levels were increased since 11:00 h on day 31, they remained similar in later sampling times being not statistically significant. Again, UV-B treatment did not modulate *F3´5´H* expression in bell pepper stems (Fig. 4).

Based on our previous findings, the temporal expression of late genes of anthocyanin biosynthetic pathway, *DFR* and *ANS*, was also conducted under LT, UV-B and LT+UV-B treatments. Interestingly, *DFR* was strongly upregulated only in LT treatments showing a dramatic increase starting from 11:00 (110.3-fold) and maintained at 16:00 (119.3-fold) on day 31 until early morning at 04:00 h (109.6-fold) on day 32. Then, *DFR* levels in pepper stems decreased at 11:00 h on day 32, but no significant differences were found in comparison with earlier sampling times (Fig. 5). Besides, stems exposed to LT+UV-B displayed a slight but no significant increase in *DFR* after exposure of stress treatment. In contrast, no significant changes in *DFR* gene expression were found in bell pepper stems exposed to UV-B treatment, which remained at basal levels over all sampling times. On the other hand, the relative expression of *ANS* was significantly increased (3.2-fold) at 11:00 h on day 31 in LT treatment, and then *ANS* expression dropped at basal levels in later sampling times (Fig. 6). Interestingly, in the case of UV-B treatment, *ANS* was slightly upregulated at 04:00 and 11:00 h (1.9- and 2-fold increase, respectively) on day 32, suggesting a late response after UV-B exposure. In contrast, combined LT+UV-B treatment did not induce significant changes in *ANS* expression at analyzed sampling times.

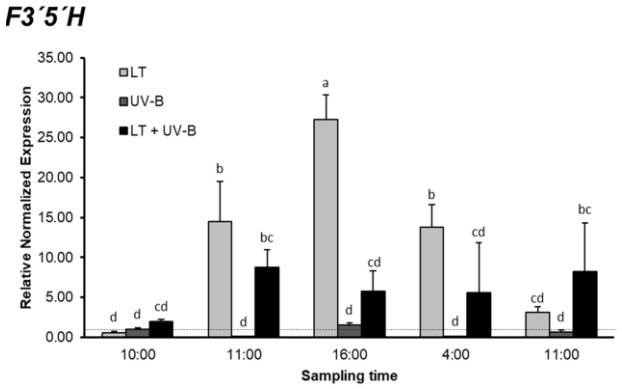

Fig. 4: Relative gene expression of *F3´5´H* (Flavonoid-3', 5'-hydroxylase) gene in *C. annuum* stems under low temperature (LT), UV-B radiation and LT+UV-B at day 31 (10:00, 11:00 and 16:00 h) and 32 (04:00 and 11:00) of the experiment. Statistically significant differences ($P \leq 0.05$) are indicated with letters. Dotted line indicates the expression level of calibrator (relative expression set as 1) corresponding to UV-B at 10:00 h on day 31

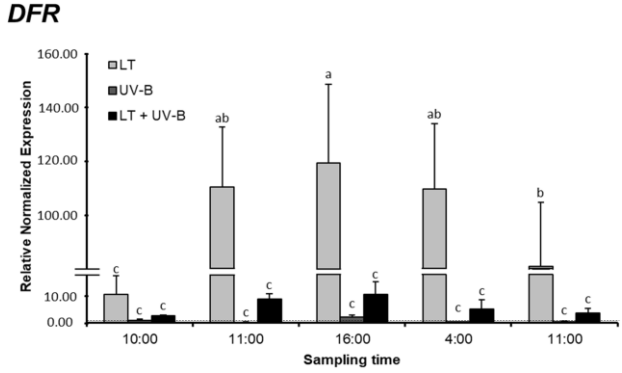

Fig. 5: Relative gene expression of *DFR* (Dihydroflavonol-4-reductase) gene in *C. annuum* stems under low temperature (LT), UV-B radiation and LT+UV-B at day 31 (10:00, 11:00 and 16:00 h) and 32 (04:00 and 11:00) of the experiment. Statistically significant differences ($P \leq 0.05$) are indicated with letters. Dotted line indicates the expression level of calibrator (relative expression set as 1) corresponding to UV-B at 10:00 h on day 31`

Discussion

Previous studies have shown that bell pepper is highly sensitive to LT and UV-B radiation (León-Chan *et al.* 2017; Rodríguez-Calzada *et al.* 2019) showing changes in some phytochemicals, where anthocyanin accumulation has been mostly studied in leaves and fruits under developmental and stress-induced responses, while little has been addressed in other tissues such as stems (Lightbourn *et al.* 2007; Stommel *et al.* 2009). Modulation of the regulatory and structural genes represents the first level of regulation by which anthocyanin biosynthesis is modulated in response to developmental or environmental clues. The MYB transcription factors are one of the key regulators of anthocyanin biosynthesis during abiotic stress conditions and development for several plants (Hichri *et al.* 2011; Wang *et al.* 2016). Herein, *MYB* was upregulated showing a peak at 16:00 h on day 31 independently of LT, UV-B or combined LT+UV-B treatments and then was diminished, which suggest the induction of *MYB* stress-induced responses that may include some genes belonging to the anthocyanin biosynthetic pathway. In ever-red leaf crabapple was shown that MYB10 positively regulated the transcription of *F3H*, *F3´H* and *ANS* at LT (15°C) demonstrating the direct interaction of *MYB10* on *F3H* promoter (Tian *et al.* 2015). Meanwhile, *DFR* expression has been correlated with *MYB* transcription and cold stress resistance in *Brassica rapa* (Ahmed *et al.* 2014). Interestingly, CaMYB-silenced bell pepper leaves exhibited dramatically reduced expression of anthocyanin biosynthetic genes such as *F3H*, *F3´5´H*, *DFR* and *ANS*, indicating MYB as positive regulator of anthocyanin pathway, and associated to resistance against *Phytophthora capsici* (Zhang *et al.* 2015). In fact, MYB has been reported as subunit of the MBW complex, which includes bHLH and

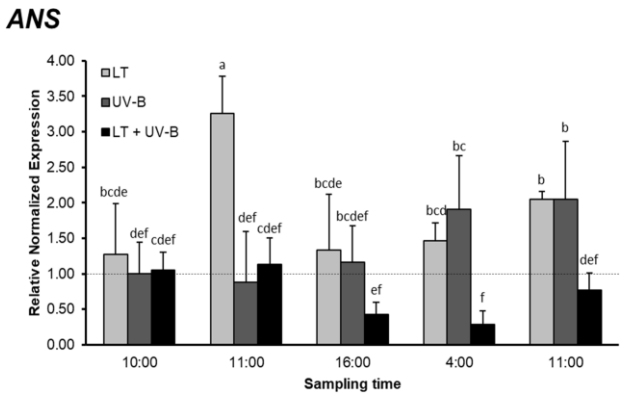

Fig. 6: Relative gene expression of *ANS* (anthocyanidin synthase) gene in *C. annuum* stems under low temperature (LT), UV-B radiation and LT+UV-B at day 31 (10:00, 11:00 and 16:00 h) and 32 (04:00 and 11:00) of the experiment. Statistically significant differences ($P \leq 0.05$) are indicated with letters. Dotted line indicates the expression level of calibrator (relative expression set as 1) corresponding to UV-B at 10:00 h on day 31

WDR transcription factors, participating in flavonoid regulation by abiotic factors, such as light and LT, through the activation of late biosynthesis genes like as *ANS* and *DFR* (Li *et al.* 2012; Zhang *et al.* 2012; Rouholamin *et al.* 2015; Xu *et al.* 2015). In tomato, the *AH* gene encoding a bHLH protein possess a MYB-interacting region; interestingly, *AH* induced anthocyanin accumulation and increased expression levels of *F3'5'H*, *DFR* and *ANS* under LT and development supporting the role of MBW complex in anthocyanin regulation (Qiu *et al.* 2016). Despite *MYB* was suddenly upregulated at 11:00 h, however, the expression of *F3H*, *F3´5´H*, *DFR* and *ANS* were induced earlier over exposure to LT or combined LT-UV-B, showing a weak correlation with *MYB* expression opening the possibility that other factors, even including other members of MYB family in bell pepper, are responsible of anthocyanin gene modulation in response to LT and/or UV-B.

Regarding anthocyanin biosynthetic genes, strikingly the early genes *F3H* and *F3´5´H* were highly modulated by LT and combined LT+UV-B treatments, while UV-B exposure did not change expression level of these genes. In other species such as *Reaumuria soongorica*, *F3H* expression level and enzyme activity are induced in response to UV-B radiation and drought stress correlating with anthocyanin accumulation (Liu *et al.* 2013). Anthocyanins are synthesized by an extension of the flavonoid pathway, which is also involved in the biosynthesis of isoflavonoids and flavonols (Winkel, 2006). In this regard, upregulation of *F3H* but barely induction of *F3´5´H* under LT+UV-B treatment seem to suggest that F3H could be mostly redirected toward the production of other flavonoids than anthocyanins, like flavonols, to cope with the combined effect of LT and UV-B radiation in bell pepper. In fact, flavonols such as quercetin, kaempferol, apigenin and luteolin have been reported to accumulate in response to LT and UV-B radiation exposure (Jaakola and Hohtola 2010; León-Chan *et al.* 2017). Moreover, dihydroxylation of the flavonoids produced during LT and LT+UV-B stress may constitute an important defense factor in *C. annuum* stems according to the increase of *F3´5´H* expression. In this regard, León-Chan *et al.* (2017) reported higher concentration and antioxidant activity of dihydroxylated flavonoid luteolin-7-glucoside in comparison to monohydroxylated apigenin-7-glucoside caused by LT and UV-B radiation in leaves of *C. annuum.*

On the other hand, it was noted that *F3´5´H* showed the highest expression levels and exhibited a similar pattern than the late anthocyanin biosynthetic gene *DFR* when plants were exposed to LT, exhibiting a gradual increase until reaching a peak at 16:00 h on day 31, which coincides with the highest expression level of *MYB*. DFR is a pivotal enzyme in anthocyanin biosynthesis that use NADPH as cofactor to catalyze the transformation of dihydroflavonols into leucoanthocyanidins (Wang *et al.* 2013; Ahmed *et al.* 2014), which are subsequently converted into anthocyanidins by ANS (Ahmed *et al.* 2014). *DFR* expression has been positively correlated to anthocyanin accumulation in different genotypes and LT treatment in *Punica granatum* and Japanese Parsley (Hasegawa *et al.* 2001; Rouholamin *et al.* 2015). In contrast, *ANS* expression was only increased at 11:00 h on day 31 and 4:00 and 11:00 h on day 32 in *C. annuum* plants exposed to LT and UV-B radiation, respectively, displaying a very different expression pattern than *F3´5´H* and *DFR* genes. Moreover, the temporal expression pattern of *ANS* over all treatments also suggests that *ANS* is a MYB-independent regulated gene. However, we cannot rule out a possible regulation by other MYB factors due to at least other three genes have been identified in *C. annuum* in addition to *MYB(A)* analyzed here, which corresponds to the locus *A* previously described (Li *et al.* 2011; Borovsky *et al.* 2004). The late increase in *ANS* expression only at second day of UV-B treatment (day 31) indicates that *ANS* modulation requires more time to respond to UV-B stress than LT. *ANS* expression has been strongly associated with cold stress tolerance and anthocyanin accumulation in *Brassica rapa* being regulated by MYB transcription factor (Ahmed *et al.* 2014). However, because of differences on *DFR* and *ANS* expression, it is possible that most of DFR products are destined to the synthesis of proanthocyanidins instead of anthocyanins in *C. annuum* stems exposed to LT. The biosynthesis of proanthocyanidins is carried out by leucoanthocyanidin reductase (LAR), which takes as substrate the leucoanthocyanidins produced by *DFR* (Wang *et al.* 2013). Collectively, all our results provide new insights about the transcriptional regulation of anthocyanin biosynthesis genes in response to LT and UV-B alone or in combination, highlighting a differential modulation of flavonoid-anthocyanin pathway in response to stress conditions in bell pepper stems.

Conclusion

The MYB transcription factor is suddenly induced by exposure to LT, UV-B or combined LT+UV-B treatments but it did not appear to activate the expression of *F3H*, *F3´5´H*, *DFR* and *ANS* genes in *C. annuum* stems due to there is a weak correlation because these genes were induced earlier than *MYB*. The increased expression of *MYB*, *F3´5´H*, *DFR* and *ANS* caused by LT suggests that anthocyanin biosynthesis in bell pepper stems is more influenced by LT than UV-B radiation. The highest expression of *F3H* in LT+UV-B treatment may suggest the biosynthesis of other flavonoids, such as flavonols, to protect plants from LT+UV-B, because there is no correlation with *ANS* and *DFR* expression. On the other hand, the increased *DFR* expression in comparison to *ANS* by LT stress suggest an enhancement toward proanthocyanidin biosynthesis over anthocyanidins, taking as substrate leucoanthocyanidins that are produced by *DFR*. Collectively, all our results provide new findings about modulation of anthocyanin biosynthetic genes in response to LT and UV-B alone or in combination, highlighting a differential modulation of flavonoid-anthocyanin pathway in response to stress conditions in bell pepper stems.

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