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Research paper

Heme oxygenase up-regulation under ultraviolet-B radiation is not epigenetically restricted and involves specific stress-related transcriptions factors

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ABSTRACT

Heme oxygenase-1 (HO-1) plays a protective role against oxidative stress in plants. The mechanisms regulating its expression, however, remain unclear. Here we studied the methylation state of a GC rich HO-1 promoter region and the expression of several stress-related transcription factors (TFs) in soybean plants subjected to ultraviolet-B (UV-B) radiation. Genomic DNA and total RNA were isolated from leaves of plants irradiated with 7.5 and 15 kJ m-2 UV-B. A 304 bp HO-1 promoter region was amplified by PCR from sodium bisulfite-treated DNA, cloned into pGEMT plasmid vector and evaluated by DNA sequencing. Bisulfite sequencing analysis showed similar HO-1 promoter methylation levels in control and UV-B-treated plants (C: 3.4 ± 1.3%; 7.5: 2.6 ± 0.5%; 15: 3.1 ± 1.1%). Interestingly, HO-1 promoter was strongly unmethylated in control plants. Quantitative RT-PCR analysis of TFs showed that GmMYB177, GmMYBJ6, GmWRKY21, GmNAC11, GmNAC20 and GmGT2A but not GmWRK13 and GmDREB were induced by UV-B radiation. The expression of several TFs was also enhanced by hemin, a potent and specific HO inducer, inferring that they may mediate HO-1 up-regulation. These results suggest that soybean HO-1 gene expression is not epigenetically regulated. Moreover, the low level of HO-1 promoter methylation suggests that this antioxidant enzyme can rapidly respond to environmental stress. Finally, this study has identified some stress-related TFs involved in HO-1 up-regulation under UV-B radiation.

1. Introduction

Soybean (Glycine max.) is economically the most important crop in the world. Its growth and yield is affected by different abiotic stresses such as drought, salinity, and ultraviolet-B (UV-B) exposure. UV-B radiation (280–320 nm), which is filtered through the stratospheric ozone layer, has gained a lot of interest due to the reduction in the ozone concentration that increases the amount of biologically active radiation reaching the earth's surface [1]. Despite it represents a small proportion of total radiation, enhanced UV-B has shown severe effects on plant growth and development including decreased biomass formation, reduced photosynthetic rates, impaired chloroplast function, and damage to DNA [2]. UV-B exposure also increases the formation of reactive oxygen species (ROS) leading to oxidative stress [3]. To counteract the toxicity of ROS, plants have developed complex non-enzymatic and enzymatic defense systems [4]. The enhancement of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) can thus increase plant tolerance to different stress factors.

Heme oxygenase (HO) catalyzes the oxidative degradation of heme to biliverdin IXa (BV), carbon monoxide (CO), and free iron (Fe^{2+})[5]. In mammals, HO has well-known antioxidant properties and its induction constitutes an important cellular defense mechanism against oxidative damage [6,7]. In plants, HO was originally thought to

Abbreviations: APX, ascorbate peroxidase; CAREs, cis-acting regulatory elements; CAT, catalase; HO, heme oxigenase; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TFs, transcription factors; UV-B, ultraviolet-B radiation

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participate in the biosynthetic pathway leading to phytochrome chromophore formation [8]. More recently, several studies demonstrated that HO is also induced to confer protection against different abiotic stresses, such as heavy metals [9–12], salinity [13], and UV-B radiation [14,15]. We have previously shown that nitric oxide (NO) and H_2O_2 participate in the signaling pathway required for HO-1 up-regulation under stress conditions [14–16]. Mechanisms mediating HO-1 expression, however, are poorly understood.

One of the most immediate responses to stress is the regulation of the temporal and spatial expression patterns of specific stress genes, an important part of the plant stress tolerance mechanisms [17,18]. Stress gene induction occurs mainly at the level of DNA transcription, which is regulated by an extensive network of transcription factors (TFs) [18]. These TFs often belong to large gene families which share the binding to DNA on specific cis-acting regulatory elements (CAREs), short conserved motifs of 5–20 nucleotides usually found at the 5′ end of promoter genes. In this way, TFs play a crucial role in the conversion of stress signal perception to stress-responsive gene expression. The interaction of TFs with CAREs present in the promoter region of target stress-responsive genes activates different signaling pathways that act together in enhancing plant tolerance to severe environmental conditions [17,19,20]. Of note, physical bind of TFs to CAREs can be altered by epigenetic modifications of DNA which constitute an additional regulatory mechanism that influences the expression of the underlying genes [21]. Cytosine DNA methylation is a stable epigenetic mark associated with gene silencing. Thus, changes in DNA methylation patterns can modulate gene expression during plant development and tolerance to stress [22–24].

To better understand the mechanisms mediating HO-1 up-regulation in soybean plants subjected to UV-B radiation, here we studied: 1) the GmHO-1 promoter region to identify CAREs binding sites; 2) the expression of several stress-related TFs with specific binding sites at the HO-1 promoter; and 3) the methylation state of a GC rich GmHO-1 promoter region.

2. Materials and methods

2.1. Plant material and treatments

Soybean (Glycine max. L.) seeds were germinated and grown using Hoagland nutrient solution [25] in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h. After 3 weeks of growth, plants were subjected to ultraviolet radiation using a UV-B lamp (UVM-57 chromato-Vue, UVP, San Gabriel, CA, USA) (290–320 nm) at an irradiance of 5.2 W m^{-2} ultraviolet light at plant level. UV-B was filtered through 0.13 mm thick cellulose acetate filter (to avoid transmission below 290 nm) for UV-B treatments or through 0.13 mm thick Mylar Type S filter (absorbing radiation under 320 nm) for control treatments. The UV-B doses employed in this study were adjusted by exposure of plants during 25 and 50 min to the illumination source and corresponded to 7.5 and 15 kJ m^{-2} according to Caldwell normalization [26]. These UV-B doses were chosen from previous results obtained using the same plant model [14,15]. After irradiation plants were left to recover during different times (0, 2, 4, or 8 h) to evaluate the time course of the response. Leaves samples were used for determinations. In vitro experiments were carried out to study the effect of exogenously added hemin, a strong HO-1 inductor. Leaf discs (12 mm diameter, 0.3 g) were floated abaxial side down in petri dishes containing 50 mM phosphate buffer (pH 7.4) supplemented with 10 μM hemin for 4 h before the recovery time course analysis.

2.2. Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as previously described [27]. Fresh control and treated leaves (0.3*g*) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 × *g* for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% butylated hydroxytoluene (BHT) in ethanol was added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000 × *g* for 15 min and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3. Enzyme preparation and assay

Extracts for determination of catalase (CAT) and ascorbate peroxidase (APX) activities were prepared from 0.5*g* of leaves homogenized under ice-cold conditions in 5 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1*g* PVP, and 0.5% (v/v) Triton X-100. Extraction buffer for APX activity also contained 5 mM ascorbic acid. The homogenates were centrifuged at 10,000*g* for 30 min and the supernatant fraction was used for the assays. Protein concentrations were determined using the Bradford micromethod assay (BioRad). Catalase activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. Catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} [28]. Ascorbate peroxidase activity was measured in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The H_2O_2-dependent oxidation of ascorbate was followed monitoring the absorbance decrease at 290 nm ($e = 2.8$ mM^{-1} cm^{-1}) [29]. Heme oxygenase activity was assayed as previously described using a reaction mixture containing 10 μM hemin, 0.15 mg ml^{-1} bovine serum albumin, 50 μg ml^{-1} spinach ferredoxin, 0.025 U ml^{-1} spinach ferredoxin-NADP + reductase and 100 μM NADPH [8].

2.4. Real-time quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen), treated with RNase-free DNase I (Promega), and reverse transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen). Quantitative RT-PCR was performed using soybean specific primers (Table S1) on a StepOne real-time PCR system (Applied Biosystems). The threshold cycle (Ct) values were normalized against the reference gene 18S, which has shown to be stable under several UV-B settings [14]. Results were calculated using the Relative Quantification (ΔΔCt) method [30] and presented as the fold change in gene expression normalized and relative to the untreated control.

2.5. DNA methylation analysis

Soybean leaf genomic DNA was extracted with CTAB lysis buffer and purified using a silica membrane binding based method (NucleoSpin® Plant II, Macherey-Naguel). Bisulfite conversion was performed using the MethylCode Bisulfite Conversion Kit (Invitrogen) and approximately 300 bp of a GC rich genomic HO-1 promoter region was amplified by PCR with primers designed to recognize the bisulfite-converted DNA only (Table S1). PCR products were cloned into the pGEMTeasy vector (Promega), and 8–15 individual clones were sequenced for each sample. Conversion efficiency was > 98% for each bisulphite-treated sample. Sequencing data were analyzed with CyMATE software [31].

2.6. Statistics

Continuous variables are expressed as mean ± SD. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

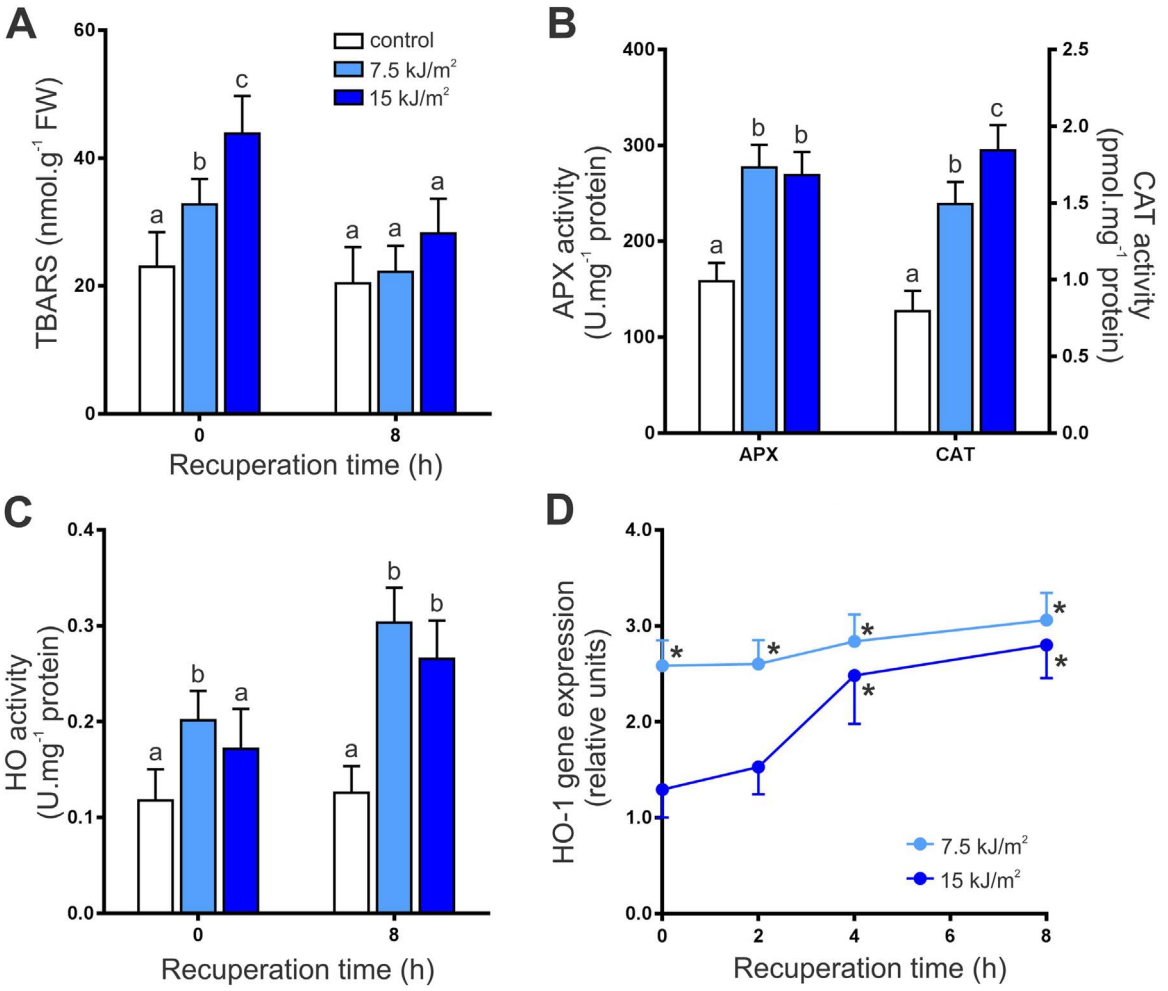

Fig. 1. *Oxidative stress parameters and HO up-regulation under UV-B stress.* Soybean plants were irradiated with different UV-B doses (7.5 or 15 kJ m^{-2}) and allowed to recover for up to 8 h. Leaves samples were used for the assays. (A) Lipid peroxidation evaluated as TBARS formation. Values are the mean of five independent experiments and bars indicate SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. (B) Classical antioxidant enzymes activities after 8 h of recuperation. One unit of ascorbate peroxidase forms 1 nmol of ascorbate oxidized per min under assay conditions. Catalase activity was expressed as pmol mg^{-1} protein. Values are the mean of five independent experiments and bars indicate SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. (C) Heme oxygenase enzyme activity. One unit of the enzyme forms 1 nmol of biliverdin per min under assay conditions. Values are the mean of five independent experiments and bars indicate SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. (D) HO-1 gene expression was determined by qRT-PCR. Transcript level of untreated plants was normalized against 18S and expressed as 1 unit. Data are means of five independent experiments and bars indicate SD. *$P < 0.05$ vs control according to Tukey's multiple range test.

3. Results

3.1. Oxidative stress parameters and HO up-regulation in leaves of soybean plants subjected to UV-B radiation

TBARS formation significantly increased by 42% and 90% in 7.5 and 15 kJ m^{-2} UV-B-irradiated soybean plants compared with controls, respectively (Fig. 1A). After 8 h of recuperation, plants treated with both UV-B doses showed TBARS levels comparable to controls. At this time point, APX and CAT activities were significantly augmented inferring that the antioxidant enzyme system responded to UV-B-induced oxidative damage (Fig. 1B). The enzyme HO presented a similar behavior. As shown in Fig. 1C, HO activity rapidly increased and showed an enhancement of more than 2-fold after 8 h of recuperation for both UV-B treatments. As we have previously reported, 7.5 and 15 kJ m^{-2} UV-B-irradiated plants overexpressed the HO-1 gene demonstrating that changes in HO activity were preceded by an up-regulation of HO-1 transcript levels (Fig. 1D). The expression of the constitutive gene (18S) was unaffected throughout all experiments (not shown).

3.2. UV-B radiation mediates HO-1 up-regulation by enhancing the expression of several transcription factors involved in stress responses to abiotic stress

To further understand the mechanism by which UV-B radiation regulates HO-1 gene expression, we analyzed the soybean HO-1 promoter region starting approximately 1800 bp upstream of the open reading frame and including 200 bp of the first codon sequence. We found different consensus sequences for the binding of several transcription factor gene families related to abiotic stress tolerance, including GmNAC, GmMYB, GmWRKY, and GmGT (Supp Fig. 1). We selected members representing each of these families based on previous reports studying their roles in soybean stress responses (Table 1). These TFs act as transcriptional activators which are differentially regulated at gene expression level in response to abiotic stresses. Thus, we analyzed the expression profile of these TFs by qRT-PCR in leaves of soybean plants subjected to both 7.5 and 15 kJ m^{-2} UV-B radiation doses (Fig. 2). Expression of GmMYB177 and GmMYBJ6 was significantly induced by UV-B after 4 h of recuperation (Fig. 2A, B). Expression of GmWRKY21 was induced but GmWRKY13 was not affected by UV-B radiation (Fig. 2C, D). Both GmNAC members studied

Table 1
Selected transcription factors with binding sites in soybean HO-1 promoter region.

Family	Gene	Cis-element	Reference
MYB	GmMYB177 GmMYBJ6	MBSI (AACGG)	Liao et al., [37] Yang et al., [38]
CBF/DREB NAC	GmDREBa,b,c GmNAC11 GmNAC20	DRE (CCGAC) CGTG/A	Li et al., [39] Hao et al., [40]
WRKY	GmWRKY13 GmWRKY21	W-Box (TTGAC)	Zhou et al., [42]
Trihelix/GT	GmGT-2A	BoxII-GT1 (GGTTAA)	Xie et al., [43]

here were strongly induced by UV-B at all recuperation time points (Fig. 2E, F). The expression of GmDREB was not affected while GmGT2A showed a slight increase under UV-B treatments (Fig. 2G, H).

We also investigated the expression profile of these TFs after hemin treatment in the absence of UV-B radiation. Hemin is a potent and specific HO inducer which was used at a dose that provided a 3–4-fold increase in HO-1 gene expression (data not shown). As shown in Fig. 3, hemin was able to enhance the expression of several TFs inferring that they may directly mediate HO-1 up-regulation. We constructed a heat map using the expression data to facilitate the identification of patterns of gene expressions (Fig. 4). GmDREB and GmWKRY13 did not respond to the different treatments indicating that they are not likely involved in UV-B stress responses nor in HO-1 up-regulation by hemin. The expression profile found for GmMYB177, GmMYBJ6, GmWKRY21, GmNAC11, GmNAC20 and GmGT2A was similar between both UV-B doses. The up-regulation of these TFs under UV-B irradiation suggest that they may be required to trigger mechanisms mediating UV-B tolerance. In addition, the differential gene expression patterns infer that they may function at different stages of the UV-B stress response. Hemin treatment induced the expression of GmMYB177, GmWKRY21, GmNAC11, GmNAC20 and GmGT2A providing further support for their role in HO-1 up-regulation under enhanced UV-B radiation. Interestingly, GmMYBJ6 did not respond to hemin but it was induced only after 4 h of recuperation in UV-B-irradiated plants. These results indicate that GmMYBJ6 is not likely involved in the up-regulation of HO-1 under UV-B.

3.3. HO-1 promoter is strongly unmethylated in soybean plants

DNA methylation represents an epigenetic mechanism that represses gene expression by preventing the binding of TFs. The DNA methylation profile of the HO-1 promoter was not previously studied and may be an important mechanism to regulate HO response to abiotic stress. We first analyzed the HO-1 promoter region (2000 bp) for CpG island prediction and frequency of possible methylation sites using the MethPrimer software [32]. We identified a CpG island of 226 bp and designed primers to study this particular promoter region by bisulfite DNA sequencing (Supp Figs. 2 and 3). The bioinformatic analysis showed that this region contained a total of 84 possible cytosine methylation sites, including 28 class 1 methylation sites (CGN, 33%), 10 class 2 methylation sites (CHG, 12%) and 46 class 3 methylation sites (CHH, 55%). Bisulfite sequencing analysis showed similar HO-1 promoter methylation levels in control and UV-B-irradiated plants (C: 3.4 ± 1.3%; 7.5 kJ m^{-2}: 2.6 ± 0.5%; 15 kJ m^{-2}: 3.1 ± 1.1%) (Fig. 5). Interestingly, the fact that HO-1 promoter was poorly methylated in untreated soybean plants indicates that the transcriptional up-regulation of this enzyme is not epigenetically restricted.

4. Discussion

According to traditional concepts, the essential role attributed to HOs in plants is their participation in the biosynthetic pathway leading to phytochrome chromophore formation [8]. In the last decade, however, numerous studies demonstrated that, as it occurs in mammals, HO also participates in cell protection against oxidative stress in plants [9,10,14,33]. We have previously shown that HO and its product BV are key components of the antioxidant defence system. BV produced by the HO-catalysed reaction is an efficient scavenger of ROS [9]. This mechanism provides protection against oxidative damage in several abiotic stresses, such as cadmium [9–11], salinity [13], and UV-B [14,15]. In addition, other studies have found that CO, another product of heme degradation by HO, is an important signaling molecule for the tolerance mechanisms against cadmium and salt stress [34,35]. The up-regulation of HO-1 transcripts usually precedes the enhancement of HO protein expression inferring that HO activity is mainly regulated at the transcriptional level under stress conditions [11,14]. Experiments carried out in UV-B-irradiated soybean plants showed that ROS production mediates HO-1 up-regulation. In agreement with this notion, an antioxidant pre-treatment efficiently blocks this response, while treatment with H_2O_2 increases HO-1 transcript levels in the absence of UV-B [14]. Nitric oxide can also up-regulate HO-1 expression, but a certain balance between NO and ROS is required to trigger the full UV-B response [15]. Nevertheless, the signaling pathway leading to HO-1 up-regulation during stress conditions is still poorly understood.

Molecular and genomic analyzes have shown that stress-responsive gene expression is mediated by different transcriptional regulatory pathways that represent a crucial part of the plant response to abiotic and biotic stresses [17,20]. It is well-known that ROS production under stress conditions results in changes of the nuclear transcriptome. ROS sensors are activated to induce signaling cascades that change gene expression by modifying the activity of specific transcription factors [36]. The expression of stress-responsive genes are mainly controlled by several classes of TFs, such as members of the MYB, NAC and WRKY families, through binding of the corresponding cis-acting regulatory elements (CAREs) [17]. In the present study, we have analyzed the soybean HO-1 promoter sequence and found that it contains several CAREs specific for the binding of different families of stress-related transcription factors. Of note, this finding supports the novel role attributed to HO in oxidative stress defence in plants. The families of TFs that potentially regulate the expression of HO-1 include MYB, CBF/DREB (dehydration-responsive element binding), NAC (NAM, ATAF1/2, CUC1), WRKY (TFs containing highly conserved WRKY domain) and Trihelix/GT (DNA binding proteins specific for GT-elements) (Table 1, Fig. S1). Most of these TFs were not previously investigated under UV-B stress and thus, we decided to use this experimental model employing both UV-B doses that provided a significant increase in HO-1 expression [14]. MYB-type TFs represent one of the largest families in plants and contain the conserved MYB DNA-binding domain. Up to date, only a few soybean MYB TFs genes related to abiotic stress were reported [37]. Three stress-related soybean MYB genes, GmMYB76, GmMYB92, and GmMYB177 were involved in tolerance to salt and cold stress [37]. Here we found that GmMYB177 and GmMYBJ6 were significantly induced by UV-B after 4 h of recuperation. This result is in agreement with a previous report showing that GmMYBJ6 was induced by UV-B radiation, drought, and high-salt treatment [38]. Moreover, GmMYBJ6 is expressed only in leaves and up-regulates certain flavonoid biosynthetic genes providing UV-B tolerance [38]. Interestingly, GmMYBJ6 was not induced by hemin and its pattern of expression following UV-B irradiation suggests that it may not be required for HO-1 up-regulation. Although salt, drought, and cold stresses can induce the expression of GmDREB in leaves of soybean seedlings [39], this TF did not respond to UV-B nor hemin treatments inferring that it is not likely involved in UV-B tolerance or in the modulation of HO-1 gene expression. NAC-type proteins constitute a plant-specific TF family. A recent report identified two GmNAC genes (GmNAC11 and GmNAC20) involved in soybean tolerance to salt stress [40]. Both GmNAC11 and GmNAC20 genes were significantly induced under UV-B radiation, thus our results provide

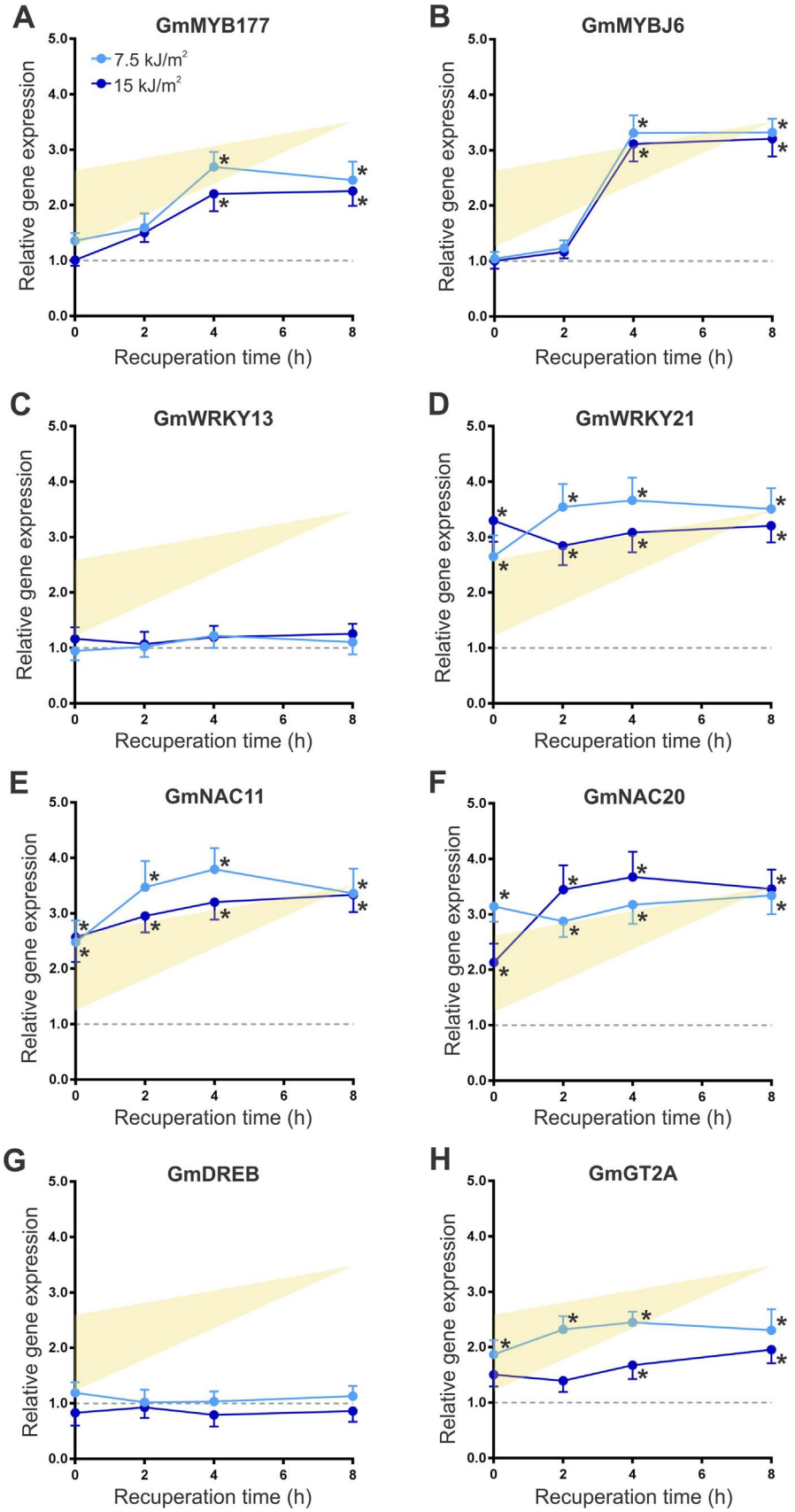

Fig. 2. *Time-course analysis of gene expression data from different stress-related transcription factors under UV-B stress:* GmMYB177 (A), GmMYBJ6 (B), GmWRKY13 (C), GmWRKY21 (D), GmNAC11 (E), GmNAC20 (F), GmDREB (G), GmGT2A (H). Soybean plants were irradiated with different UV-B doses (7.5 or 15 kJ m^{-2}) and allowed to recover for up to 8 h. Leaves samples were collected at time 0, 2, 4 and 8 h after irradiation. Gene expression was measured by qRT-PCR and relative quantitation was calculated using $2^{-\Delta\Delta Ct}$ method and 18S as endogenous control. Control treatment is expressed as 1 unit (dashed line) and HO-1 gene expression pattern is represented to facilitate the comparisons (light yellow triangle). Data are means of five independent experiments and bars indicate SD. *P < 0.05 vs control according to Tukey's multiple range test.

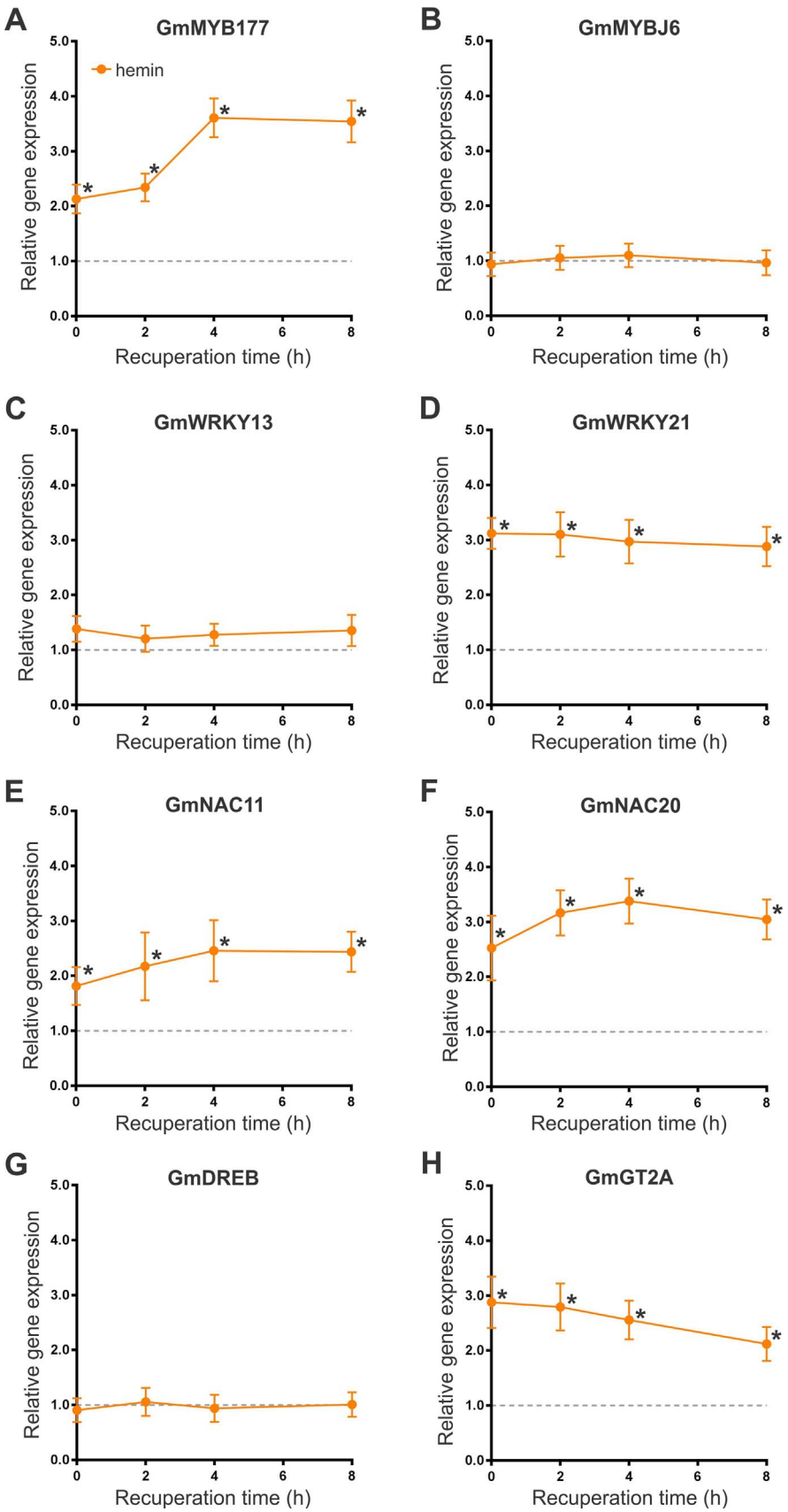

Fig. 3. *Time-course analysis of gene expression data from different stress-related transcription factors after induction with hemin:* GmMYB177 (A), GmMYBJ6 (B), GmWRKY13 (C), GmWRKY21 (D), GmNAC11 (E), GmNAC20 (F), GmDREB (G), GmGT2A (H). Soybean leaf discs were treated with 10 μM hemin for 4 h in the absence of UV-B. Samples were analyzed at time 0, 2, 4 and 8 h after hemin treatment. Gene expression was measured by qRT-PCR and relative quantitation was calculated using $2^{-\Delta\Delta Ct}$ method and 18S as endogenous control. Control treatment is expressed as 1 unit (dashed line). Data are means of five independent experiments and bars indicate SD. *P < 0.05 vs control according to Tukey's multiple range test.

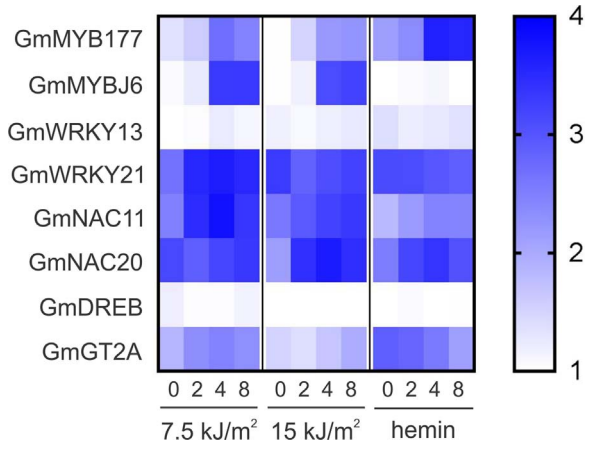

Fig. 4. *Unclustered heatmap visualization of gene expression values for the different stress-related transcription factors analyzed in UV-B-treated and hemin-treated soybean plants.* Each colored pixel represents a specific gene expression value at each time-point. Blue represents relative increases in gene expression compared with untreated plants (expressed as 1 unit).

more evidence about the potential role of GmNACs in plant stress tolerance. Plant WRKY-type TFs regulate the expression of target genes containing the W-box binding sequence and have been involved in developmental processes and responses to biotic and abiotic stresses. The expression of WRKY genes is induced in *Arabidopsis* under drought, cold and high-salinity stresses [41]. A recent report found that the soybean members GmWRKY13, GmWRKY21 and GmWRKY54 have differential effects on abiotic stress tolerance [42]. GmWRKY21 improves tolerance to cold stress, whereas plants over-expressing GmWRKY13 showed increased sensitivity to salt and mannitol stress. Here we found that the expression of GmWRKY21 but not GmWRKY13 was induced in soybean plants subjected to UV-B radiation. Hemin treatment showed similar results. These data suggest that GmWRKY21 may participate in HO-1 up-regulation under UV-B. Members of Trihelix family, also known as GT factors, play important roles in light-regulated processes by binding a light-responsive element named Box II/GT1 box. However, a recent study has identified two soybean GT factors (GmGT-2A and GmGT-2B) that may be implicated in abiotic stress responses as their over-expression improves tolerance to salt, freezing and drought stresses [43]. As expected for TFs involved in light signaling pathways, GmGT-2A was up-regulated in leaves of soybean plants subjected to UV-B radiation. GmGT-2A was also induced by hemin treatment suggesting that it may mediate HO-1 gene expression. Altogether, these data show that several soybean TFs mainly associated with tolerance to salt, cold and drought stresses may also participate in plant responses to UV-B radiation. More specifically, we found here that the expression of GmMYB177, GmMYBJ6, GmWRKY21, GmNAC11, GmNAC20 and GmGT2A is significantly induced in leaves of UV-B-irradiated soybean plants. These results provide more evidence about the participation of these TFs in mechanisms mediating abiotic stress tolerance. The specific role of each TFs under UV-B stress needs to be further investigated. Noteworthy, some of these TFs, such as GmWRKY21, GmNAC11, GmNAC20 and GmGT2A were enhanced by hemin in the absence of UV-B radiation. The fact that they respond to a specific HO inducer and their corresponding CAREs are present in the soybean HO-1 promoter region further support their role as regulators of HO-1 gene expression. This is an interesting finding because these TFs are known to mediate the expression of stress-related genes in response to different abiotic stresses in which HO has shown a protective effect against oxidative damage [9–11,13–15,34,35]. Additional studies are warranted to better establish which families of TFs identified here are more effective in enhancing HO-1 gene expression.

Epigenetic modifications of DNA and histones, the core components of chromatin, are stable marks that modulate transcriptional regulation [21]. In plants, cytosine DNA methylation can occur in all sequence contexts and represents an important mechanism to regulate the silencing of repetitive sequences, genomic imprinting, and stable gene silencing. At gene promoters, DNA methylation leads to transcriptional repression by blocking the binding of TFs. However, little is known about how DNA methylation patterns changes in response to environmental modifications. In addition, only a few reports have studied the role of epigenetic changes on the expression of specific genes involved in stress tolerance. On most cases, the up-regulation of stress-responsive genes has been associated with a reduction of DNA methylation in their promoter regions [44,45]. Interestingly, global DNA demethylation enhanced the expression levels of genes encoding enzymes of the flavonoid biosynthetic and antioxidative pathways and hence improved tolerance to salt stress in tobacco [46]. It was also found that changes in DNA methylation can activate the expression of stress-related TFs, such as GmMYB and GmDREB members, in soybean plants subjected to salinity stress [47]. Moreover, the accumulation of flavonoids in maize plants adapted to high levels of UV-B radiation is mediated by a MYB TF

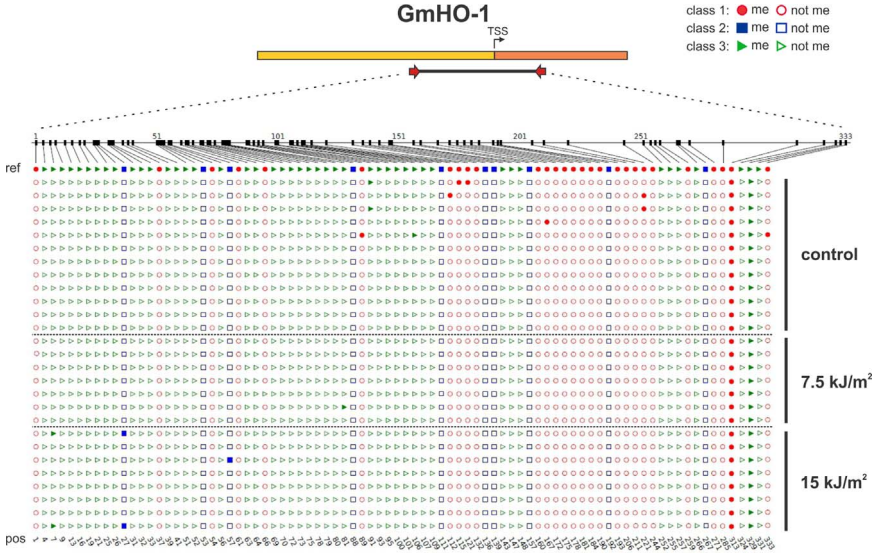

Fig. 5. *Bisulfite DNA methylation analysis at the HO-1 promoter.* Bisulfite sequencing analysis of cytosine methylation is shown at HO-1 promoter in leaves of soybean plants subjected to UV-B irradiation. Up to fifteen individual clones were sequenced to determine the methylation status of the promoter in each treatment. 5-methylcytosines in the CG (class 1), CHG (class 2), and CHH (class 3) contexts were analyzed and displayed using CyMATE [31]. Abbreviations: TSS, transcription start site.

which is mainly controlled by DNA methylation [48]. These data provide evidence suggesting that changes in DNA methylation patterns at specific regions are important in the regulation of stress responses. Noteworthy, the role of epigenetic mechanisms in regulating the expression of HO-1 promoter was not previously studied. For these reasons, we evaluated changes in the DNA methylation pattern of the soybean HO-1 promoter during UV-B exposure. We found that HO-1 promoter is strongly unmethylated in control plants and UV-B radiation does not significantly modify DNA methylation at this region. The fact that HO-1 promoter is not significantly methylated infers that TFs are readily able to enhance HO-1 gene expression. Consequently, soybean HO-1 gene expression does not appear to be epigenetically regulated. This observation can also explain why HO can be rapidly induced to counteract the oxidative stress generated by different stressors [10,11,14] and suggests that its regulation is mainly mediated by TFs. Whether this notion also applies to other antioxidant enzymes, such as SOD, CAT and APX, is still unknown as their DNA methylation patterns have not been studied yet.

In conclusion, we have identified specific members from different stress-related TFs families which are induced by UV-B radiation and may participate in plant responses to this stressor. These TFs were associated with tolerance to different abiotic stresses but not previously linked to UV-B. In addition, our data demonstrate that GmWRKY21, GmNAC11, GmNAC20 and GmGT2A may also mediate HO-1 up-regulation. Finally, soybean HO-1 promoter is strongly unmethylated explaining the mechanism by which this antioxidant enzyme can rapidly respond to oxidative stress.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.03.028.

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