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Nitric oxide and flavonoids are systemically induced by UV-B in maize leaves

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ABSTRACT

Flavonoids are UV-B absorbing compounds whose concentration, increase in plant cells stimulated by UV-B irradiation. In this work, we characterized the systemic accumulation of flavonoids in maize seedlings irradiated with 3.3 W m^{-2} UV-B. Results indicate that both nitric oxide (NO) and flavonoids are systemically induced in UV-B-irradiated maize seedlings. Maize leaves pre-treated with the specific NO scavenger cPTIO, do not accumulate NO and flavonoids in response to UV-B. Whereas NO and flavonoids are accumulated in the mesophyll cells near to the leaf side receiving the UV-B irradiation, they are distributed in all tissues displaying the systemic response. Flavonoids and NO co-localize in UV-B irradiated maize leaves analyzed by images from epifluorescence microscopy. Chalcone synthase (CHS) and chalcone isomerase (CHI) genes are involved in the flavonoid biosynthetic pathway and their expression is systemically induced by UV-B in a NO dependent pathway. Finally, a functional approach demonstrates that maize leaves expressing the systemic response to UV-B show low cellular damage measured as ion leakage when they are challenged by a second round of irradiation.

1. Introduction

Plants are sessile organisms dependent on sunlight to grow and develop. As a consequence, they are inevitably exposed to ultraviolet radiation UV (200–400 nm), emitted from the sun. The vast majority of UV-C (200–280 nm) and UV-A (320–400 nm) radiations are absorbed by atmospheric gases. UV-B radiation (280–320 nm) is absorbed by stratospheric ozone but a small proportion is transmitted to the Earth's surface [1]. High doses of UV-B light induce the production of reactive oxygen species (ROS), causing damage to proteins, lipids and DNA, and affecting the cell integrity, morphology, and physiology of plants [1]. In parallel, ROS are also signaling molecules that modulate various plant responses to abiotic stresses like UV-B (for a review, see Apel and Hirt [2]).

Nitric oxide (NO) is a reactive nitrogen species (RNS), a gaseous signal molecule involved in plant responses to various stresses and proposed as a broad-spectrum anti-stress compound [3–5]. NO confers protection against the herbicide diquat, drought, and salt stress [6–8]. In UV-B stress, the NO produced is able to protect the cells from the deleterious effects of oxidative stress contributing with the antioxidant response, maintaining the cell redox homeostasis [9].

Flavonoids are molecules that protects against the oxidative damage caused by UV-B [10,11].

These metabolites exert antioxidant activity mainly through two ways: (i) Due to their lower redox potentials (0.23–0.75 V), they are able to reduce highly oxidizing free radicals with redox potentials in the range 2.13–1.0 V as O_2^{-}, H_2O_2, and $OH^{\bullet}$. Moreover, flavonoids may also efficiently chelate trace metals, limiting $OH^{\bullet}$ formation, and (ii) flavonoids inhibit several enzymes involved in ROS generation (see [12] and references therein). These secondary metabolites are able to absorb UV radiation reducing the risk of ROS generation [13–15]. Flavonoids occur not only in the vacuoles and the walls of the epidermical cells [16], but also in vacuoles of mesophyll cells and in chloroplast [17]. Plants exposed to several stresses, such as wounding and pathogen attack, respond with a systemic flavonoids and phytoalexin production [18–20]. Moreover, flavonoids are transported long distances from roots and distributed to whole plant [21]. As a consequence, flavonoids are optimally located to reduce light-induced oxidative damage in the site of ROS production [15]. The biosynthesis of flavonoids is regulated by the combined action of transcription factors (TFs) [22,23]. The maize *P* gene (*ZmP*) encodes a Myb-like TF that activates flavonoid biosynthetic genes as chalcone synthase (CHS),

Abbreviations: A, absorbance; AU, arbitrary units; BF, bright field; DAF-FM-DA, 4,5-diamino-fluorescein diacetate; dai, days after irradiation; CC, completely covered; CHS, chalcone synthase; CHI, chalcone isomerase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.; DPBA, diphenylboric acid-2-aminoethyl ester; DFR, dihydroflavonol reductase; NO, nitric oxide; PC, partially covered; ROS, reactive oxygen species; TF, transcription factors; U, uncovered; UV, ultraviolet radiation; ZMP, maize *P* gene.

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chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) [24,25]. Interestingly, it has been shown that the induction of some of these genes may be co-ordinately regulated by UV-B and NO [26,27].

NO and flavonoids have been separately reported as signal molecules in systemic response to stress [28,29]. It was reported that injection of NO donors into tobacco leaves reduces the size of lesions caused by tobacco mosaic virus in nontreated leaves [30], indicating that NO may function as a systemic signal. Moreover, it was shown that NO production increases systemically in tomato plants in response to powdery mildew infection [31].

Recently, it has been demonstrated that exposure of just the top of maize leaf to UV-B irradiation alters substantially the transcriptome, proteome and metabolome of both irradiated and shielded organs [32,33].

Since the information related to the systemic response to UV-B in plants is scarce, in this study were investigated whether NO as well as flavonoids may be systemically induced in response to UV-B.

2. Materials and methods

2.1. Plant growth conditions and treatments

Maize (*Zea mays* N107B, W23) seeds were supplied by the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana). After surface sterilization with 0.5% (v/v) hypochlorite for 20 min, seeds were washed and germinated on water-saturated filter paper at 25 °C in the dark. Germinated seedlings were grown on soil:vermiculite (3:1, v/v) at 25 °C in an environment-controlled chamber at a light intensity of 120 μmol photons m^{-2} s^{-1} and a 14/10 h light/dark photoperiod. Fourteen days-old healthy seedlings were used in the experiments, and analyses were performed in the second leaf of these plants. When indicated, the second leaf of the seedling was sprayed with H_2O or 100 μM cPTIO, 24 h before UV-B irradiation.

2.2. UV-B treatment

Maize seedlings were exposed to 3.3 W m^{-2} of UV-B radiation for 8 h, which is equivalent to 10 times the sunlight UV-B intensity. The UV-B dose used to irradiate plants was compared with sunlight UV-B. The sunlight UV-B was measured with the same device and data was the average of ten values measured at noon in the week of 19th to 23rd July 2010, at 38°00′ south latitude 57°33′ west longitudes at the sea level. The spectral irradiance was determined with an Ultraviolet Meter Model 3D (Solar Light Co, USA). The UV-B light tubes (Philips TL100W/12) used were covered with 0.13 mm thick cellulose diacetate and supplemented with white light tubes.

In maize seedlings, the second leaf of the seedlings remained uncovered (U), or was completely covered (CC) or partially covered (PC) during the irradiation. To cover the leaves we used polyester filters (PE, 100 mm clear polyester plastic; Tap plastics).

2.3. NO fluorescence

NO was measured according to Tossi et al. [9,27]. The second leaf was excised and placed in distilled water for 1 h. After that, the leaf was loaded with 100 μM 4,5-diaminofluorescein diacetate (DAF-FM-DA) for 1 h, and thoroughly washed with 20 mM Hepes buffer (pH 7.5) to remove excess of probe. Then, leaves were cut and green fluorescence (515–555 nm) was visualized in a Nikon Eclipse E200 microscope. Images were analyzed using IMAGEJ 1.3 software (NIH). Whole leaf area of the micrography was used for quantification of fluorescence in green channel. Fluorescence was expressed in arbitrary units (AU).

2.4. Flavonoid quantification

Flavonoids were extracted according to Bieza and Lois [34]. One hundred mg of leaves were grounded in liquid N_2 and flavonoids were extracted for 2 h at 4 °C with 400 μL of methanol, centrifuged for 10 min at 10,000 × *g*, and the supernatant was quantified at $A_{330\,nm}$ in an Ultrospec 1100 *pro* spectrophotometer.

2.5. In situ flavonoid staining

For flavonoid detection, the second leaf was excised and cut in cross sections. Samples were stained for 20 min with saturated 0.25% (w/v) diphenylboric acid 2-amino ethyl ester (DPBA) [35] with 0.02% (v/v) Triton X-100 and observed with an epifluorescent microscope (Nikon Eclipse E200).

2.6. Cellular damage: ion leakage (%)

Maize leaves were harvested and cut into 25 mm^2 pieces. Then, they were washed in deionized water to remove surface-adhered electrolytes and placed in tubes with 15 mL of deionized water at 25 °C for 2 h. Electrical conductivity in the bathing solution was determined (*C*1) using a Hanna HI8733 conductimeter. After that, samples were autoclaved and total conductivity was read again in the bathing solution (*C*2). Relative ion leakage was expressed as a percentage of the total conductivity after heating at 121 °C using the formula: relative ion leakage (%) = $C1/C2 \times 100$.

2.7. RT-PCR analysis

One hundred milligrams of maize leaves were used to obtain RNA samples. Total RNA was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD) and treated with DNAse I (Promega, Madison, WI). Two μg of total RNA were used for reverse transcription with an oligo dT primer and M-MLV reverse transcriptase (Promega) in a reaction volume of 20 μL. PCR reactions were performed using 2 μL of a 5-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer and 1 U of Taq DNA polymerase (Invitrogen) in a 20 μL reaction volume. To verify the exponential phase of PCR amplification, a different number of amplification cycles ranging from 20 to 34 were tested for each cDNA template. cDNA was amplified by PCR using the primers described in Table 1. The relative abundance of actin was determined and used as internal standard. The number of cycles of the PCR reactions was adjusted for each transcript. PCR products were analyzed on agarose gels and stained with SyBr safe.

2.8. Chemicals

2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was purchased in Molecular probes (Eugene, OR, USA); 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM-DA) from Molecular probe Invitrogen; diphenylboric acid-2-aminoethyl ester (DPBA) from SIGMA (St. Louis, MO, USA.)

2.9. Statistical treatment

One-way Anova test were performed. Values represent mean ± s.d. per treatment. Letters indicate statistical differences at $P \leq 0.05$.

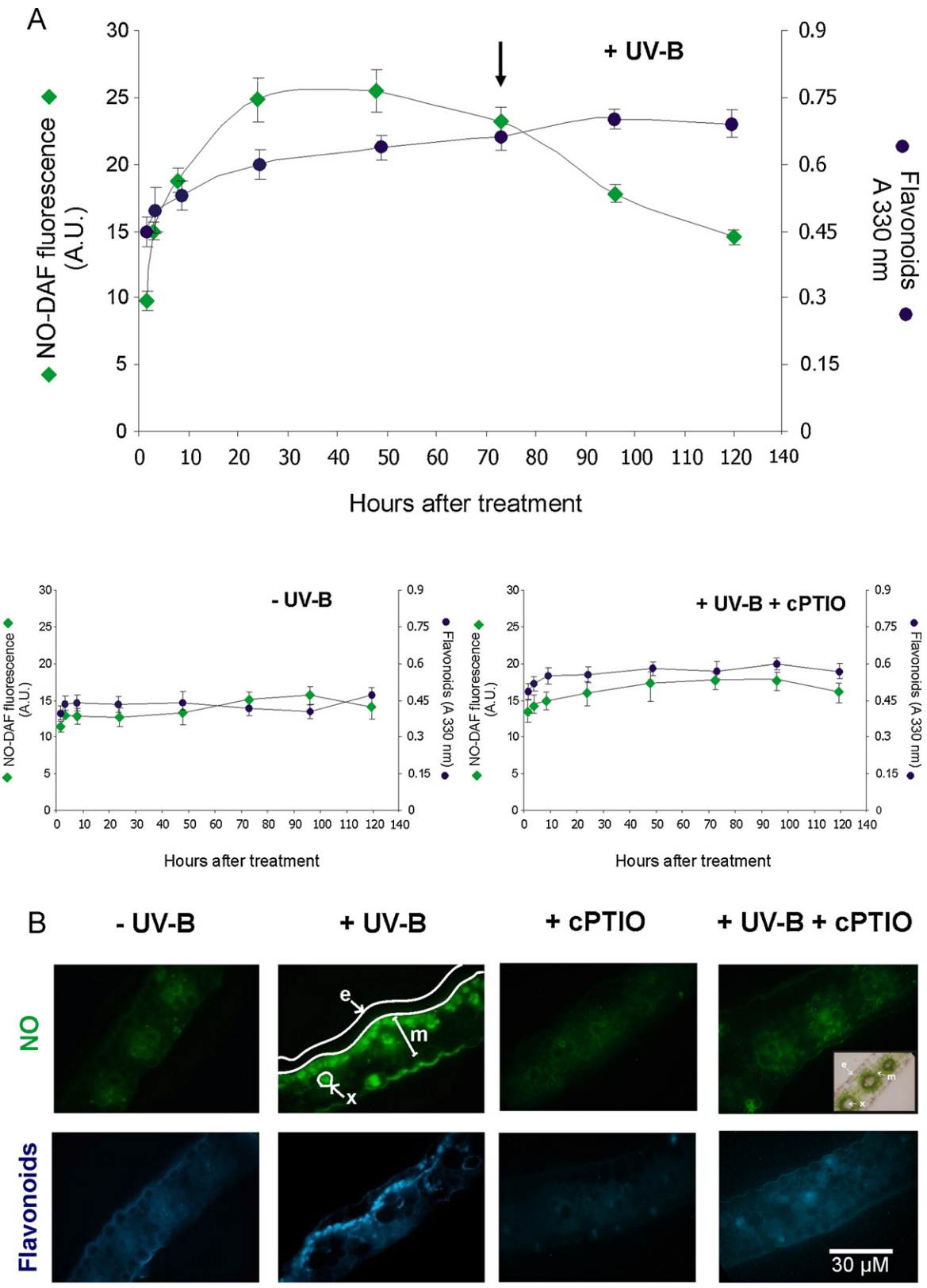

Fig. 1. Time course accumulation and localization of nitric oxide (NO) and flavonoids in maize leaves exposed to UV-B. The second leaf of maize seedling was sprayed with H_2O or 100 μM cPTIO, 24 h before UV-B irradiation. Then, the seedling was irradiated with white light (−UV-B) or white light supplemented with 3.3 W m^{-2} of UV-B for 8 h. (A) Time course of NO and flavonoids accumulation. The presence of NO was monitored in the second leaf using the fluorescent probe DAF-FM-DA. The probe was internalized by vacuum and leaves were cut in cross sections. Images were visualized in an Eclipse E 200 Nikon microscope and were analyzed using IMAGEJ 1.3 software (NIH). Fluorescence was expressed as arbitrary units (AU). Flavonoids were determined as indicated in Materials and methods. Error bars represent SE of means. Data shown are the mean ± s.d. of three plants with three replicates per treatment. (B) NO and flavonoids localization in maize leaves. Fluorescent micrographs were taken at the time point indicated by black arrow in part (A) leaves were irradiated in the adaxial side. NO and flavonoids were detected using the probes DAF-FM-DA and DPBA, respectively. Since DAF-FM-DA and DPBA probes emit in the same wavelength the flavonoids color was changed from green to blue for better comprehension. Bright field images (BF) were included for leaf morphology interpretation. e, epidermis; m, mesophyll; x, xylem. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
The nucleotide sequence of primers used in the reverse transcriptase-PCR.

cDNA	Primer forward	Primer reverse	Size PCR predict (bp)
ZmP	5′ ACCGCCGGGCCTGACGCAACC 3′	5′ CTCCGGCCCGCCCCACAGATG 3′	523
CHS	5′GGAGGTGCCGAAGCTGGG 3′	5′ GCGGCGGAGACGAGCTGG 3′	385
CHI	5′ CGCCGGGCCGCTCCATCCT 3′	5′ CCGCCCGCTCCTCGCCTCAG 3′	298
Actin	5′CC(C/A)AA(G/A)GCC(A/C/T)ACAG(A/G)GAGAAA(A/G)TGAC 3′	5′ TTCATGAT(G/T)GA(G/A)TTGTA(C/G/T)GT(G/T)G 3′	612

3. Results

3.1. NO is involved in both accumulation and localization of UV-B-induced flavonoids

To perform a time course of NO and flavonoids occurrence in UV-B irradiated maize leaves, NO was detected by microscopy in cross sections using the probe diaminofluorescein-FM diacetate (DAF-FM-DA). Flavonoids were analyzed in methanolic extracts by absorbance at 330 nm. Fig. 1A shows that NO increased 2.5 folds after UV-B irradiation, attaining a maximum level 24 h after irradiation. This result was confirmed by NO quantification using assay kit Promega Griess Reagent System [27] (Supplementary Fig. S1). In turn, flavonoids increased gradually, reaching a plateau (1.6 fold) after 96 h irradiation. Pre-treatment of the second leaf with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), prevented both NO and flavonoids UV-B-induced accumulation.

To see if NO production co-localizes with flavonoids, NO and flavonoids were detected three days after irradiation (dai) using the fluorescence probes DAF-FM-DA and diphenylboric acid-2-aminoethyl ester (DPBA), respectively. Fig. 1B shows that NO and flavonoids were detected at basal levels in non-irradiated maize leaves. Interestingly, after UV-B irradiation, huge NO and flavonoids amounts were localized in parenchymal cells close to the upper irradiated epidermis. cPTIO pre-treatment prevented both the NO and flavonoid increase induced by UV-B, indicating that flavonoid localization is NO-mediated. These results suggest that NO is involved in both accumulation and localization of UV-B-induced flavonoids.

3.2. NO and flavonoids are systemically induced in response to UV-B

Several reports have presented evidences supporting the influence of NO and flavonoids in systemic responses against pathogen attack and wounding [18,30,19,20,31]. Thereby, a series of experiments were designed to determine whether NO as well as flavonoids may be systemically induced in response to UV-B (experimental design is shown in Fig. 2). The second leaf of maize seedlings was uncovered (U) or was partially covered (PC) or completely covered (CC) and the whole seedling exposed to UV-B. UV-B absorbing polyester filters (PE, 100 mm clear polyester plastic; Tap plastics) were used to cover the second leaf.

Fig. 3A shows that when the seedlings were UV-B-irradiated, the NO-specific fluorescence was increased almost two fold in the apical part of U, PC, and CC second leaves, indicating that the NO increase was not dependent of direct UV-B irradiation. This accumulation was prevented by pre-treatment with cPTIO. This result was confirmed by NO quantification using assay kit Promega Griess Reagent System [27] (Supplementary Fig. S1). Flavonoid content was also analyzed in methanolic extracts obtained from the second leaf three dai. Fig. 3B shows that A_{330} was increased 63% in U, 45% in PC and 42% in CC in the apical part of the second leaf. A_{330} diminished when leaves were pretreated with cPTIO before UV-B irradiation.

Interestingly, Fig. 4 shows that in U leaves, NO and flavonoids were localized in the mesophyll cells of the leaf-side exposed to UV-B, whereas in PC and CC leaves, NO and flavonoids were distributed along the whole leaf. In PC leaves, NO-fluorescence was lightly increased in bundle-sheath cells.

These results indicate that both NO and flavonoids are systemically induced by UV-B in maize leaves, and that UV-B determines the NO and flavonoid localization near the surface of the leaves receiving the UV-B irradiation.

3.3. NO mediates the systemically UV-B-induced expression of phenylpropanoid genes

It has been previously demonstrated that UV-B irradiation induces *ZmP*, *CHS* and *CHI* expression in a NO-dependent manner in maize leaves [27]. Thus, it was interesting to see if the systemic induction of flavonoids is due to the activation of the biosynthetic key enzymes in the site of the phenylpropanoid accumulation and to know whether NO is required. The systemic expression of those genes was analyzed by RT-PCR in the experimental system. Fig. 5 shows that *ZmP*, *CHS* and *CHI* were strongly induced by UV-B in the apical segments of the U, PC and CC second leaf of maize seedlings. This is coincident with the increase of both NO and flavonoid induced by UV-B irradiation (Fig. 3A and B). Fig. 5 also shows that the UV-B-induced expression of *ZmP*, *CHS* and *CHI* was partially prevented by the pretreatment with cPTIO, suggesting that NO mediates the systemically UV-B-induced expression of *ZmP*, *CHS* and *CHI*.

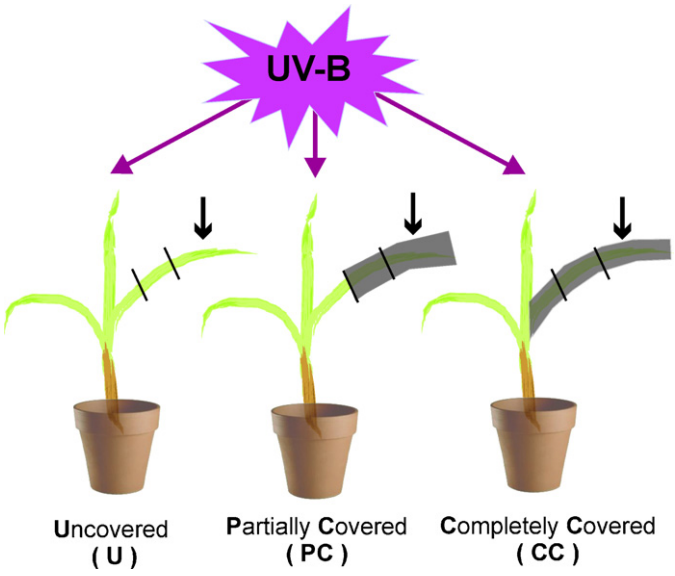

Fig. 2. Simplified scheme of the designed experimental system to analyze the systemic response to UV-B in leaves. The second leaf of fourteen days-old maize seedlings remained uncovered (U) or were partially covered (PC) or completely covered (CC) with polyester filter that prevent UV-B of reaching the leaf surface. Seedlings were then exposed to 3.3 W m^{-2} UV-B for 8 h. Black arrows indicate the apical segment of the leaf used for the analysis of the systemic response.

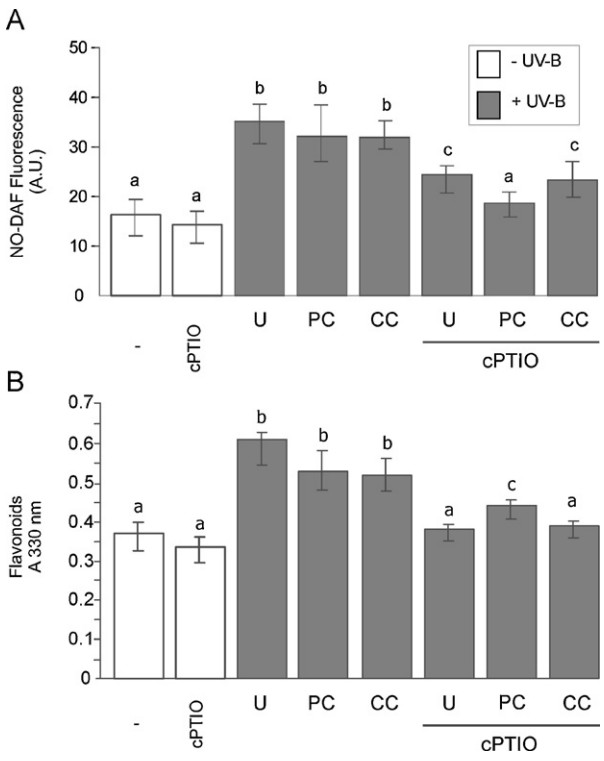

Fig. 3. NO and flavonoids are systemically induced in response to UV-B irradiation in maize seedlings. The second leaf of maize seedling was sprayed with H_2O or 100 μM cPTIO, 24 h before UV-B irradiation. Then, second leaves remained uncovered (U), or were partially covered (PC), or completely covered (CC) and the seedlings were exposed to 3.3 W m^{-2} UV-B for 8 h. (A) NO was detected three days after irradiation (dai) using the specific probe DAF-FM-DA in cross-sections of the apical part of second leaves. Images were acquired with the microscope Eclipse E200 Nikon, and analysed using IMAGEJ 1.3 software. The fluorescence was expressed as arbitrary units (A.U.). (B) Flavonoids were extracted from the second leaf apical part 3 dai as indicated in section 2. Data shown are the mean ± s.d. of four plants, with three replicates per treatment. Different letters indicate significant differences between treatments at $P \leq 0.05$ according to one-way ANOVA.

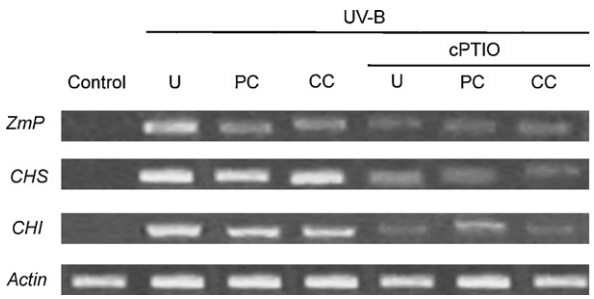

Fig. 5. UV-B irradiation induces the systemic expression of the flavonoid biosynthetic genes in a NO-dependent manner. The second leaf was treated as indicated in Fig. 2. One hour after the irradiation, the transcripts levels of *ZmP CHS* and *CHI* were analyzed by RT-PCR. Experiments were performed at least three times with similar results. *ZmP*: maize *P* gene; *CHS*: chalcone synthase; *CHI:* chalcone isomerase.

3.4. UV-B-induced systemic response exerts a partial protection against a second irradiation

A preliminary approach was conducted to prove if systemic response could prevent the deleterious effects produced by additional UV-B exposures. Maize seedlings holding U, PC and CC second leaves were UV-B irradiated as shown in Fig. 2 (1st irradiation). Cell damage produced by UV-B was measured as relative ion leakage.

Fig. 6 shows that 24 h after the 1st irradiation, the U leaves were severely affected by UV-B, displaying a high ion leakage (almost 50%). Ion leakage in PC and CC leaves was below 20%, not significantly different to non-irradiated leaves. cPTIO treatment did not provoke relevant cellular damage in non-irradiated leaves. Flavonoid concentration was low in non-irradiated leaves and cPTIO treated leaves. U, PC and CC leaves presented a slight flavonoids increase. Five days after the 1st irradiation, the second leaves of PC and CC were uncovered and all the seedlings were UV-B-irradiated for a second time (2nd irradiation). After 24 h, ion leakage in U leaves remained in 50%. Interestingly, ion leakage in PC and CC leaves was lower (30%). At this time, flavonoids were significantly increased in both U, PC and CC leaves. However, if NO and flavonoids were reduced by cPTIO pre-treatments, ion leakage was increased to 50%.

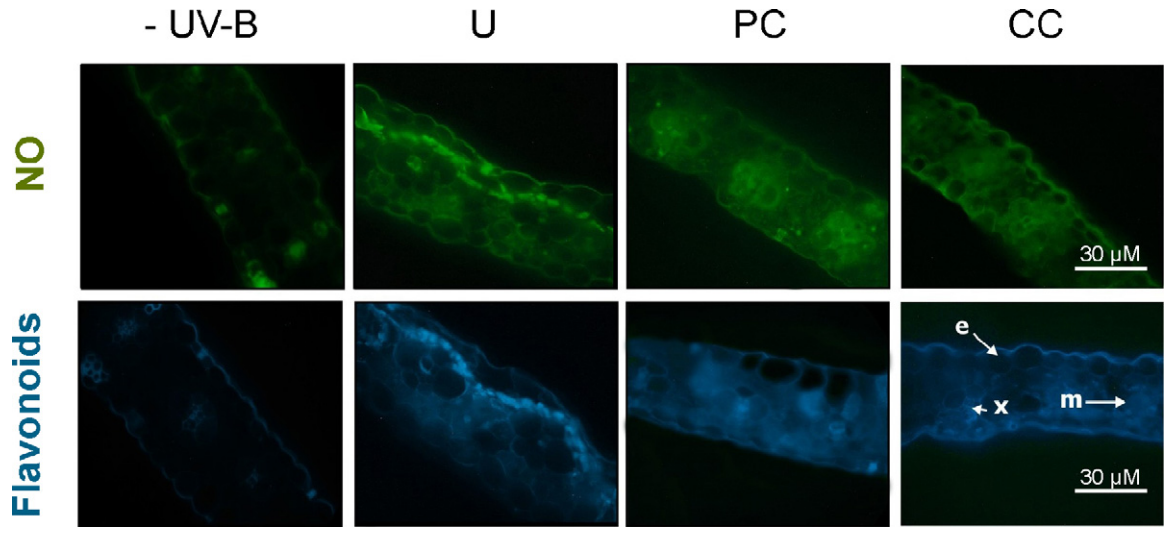

Fig. 4. The side of the leaves exposed to UV-B irradiation determines the NO and flavonoids localization. The second leaf of maize seedling remained uncovered (U) or was partially covered (PC) or completely covered (CC) and seedlings were exposed to 3.3 W m^{-2} UV-B for 8 h. NO and flavonoids were detected in the apical segment of the second leaf using the probes DAF-FM-DA and DPBA, respectively. Probes were internalized by vacuum and leaves were cut in cross sections to be analyzed. Images were visualized in an Eclipse E 200 Nikon microscope three dai. e, epidermis; m, mesophyll; x, xylem. –UV-B: non-irradiated seedlings.

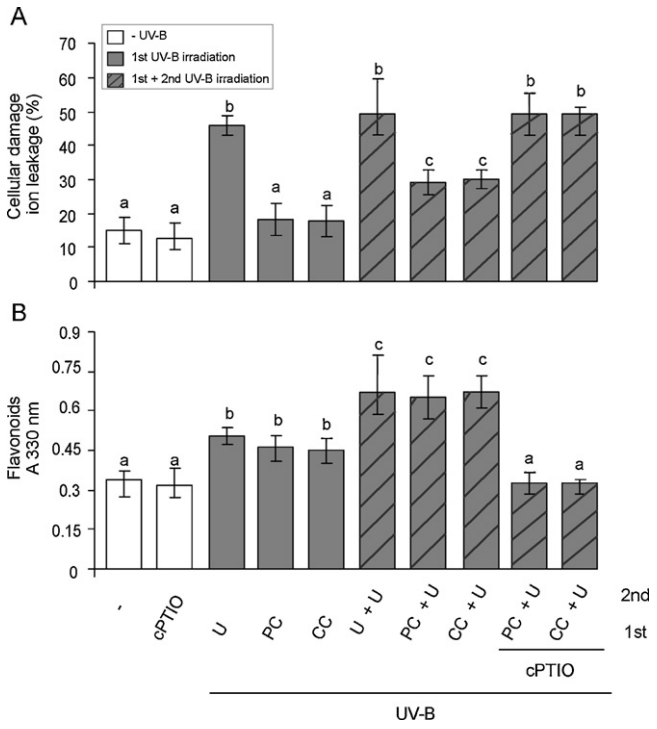

Fig. 6. Systemic response to UV-B irradiation in maize seedlings reduces the cellular damage induced by a second irradiation. The second leaf of maize seedling was sprayed with H_2O or 100 μM cPTIO, 24 h before UV-B irradiation and then was kept uncovered (U), partially covered (PC) or completely covered (CC) as shown in Fig. 2, and seedlings exposed to 3.3 W m^{-2} UV-B for 8 h (1st irradiation, grey bars). After 5 day of the first exposition, the PC and CC leaves were uncovered (PC + U and CC + U respectively) and seedlings exposed again a second round of 3.3 W m^{-2} UV-B for 8 h (2nd irradiation, striped bars). Non-irradiated seedlings: white bar. (A) Cellular damage was determined 24 h after the 1st and the 2nd irradiation, and quantified as ion leakage (see Material and methods) in the apical part of the second leaves. (B) Flavonoids were extracted from the second leaf apical part 24 h after the 1st and the 2nd irradiation as indicated in Material and methods. Data shown are the mean ± s.d. of four plants, with three replicates per treatment. Different letters indicate significant differences between treatments at $P \leq 0.05$ according to one-way ANOVA.

These results indicate that the UV-B-induced systemic response contribute to enhance the battery of the tools leading to the reduction of cell damage caused from further irradiations.

4. Discussion

Plants use inducible mechanisms to defend themselves from environmental concerns. Limited and confined injuries induce rapid resistance both locally and in organs that are not yet damaged. Casati et al. [32] have shown that transcriptome, proteomic and metabolomic changes occurs in both shielded and UV-B irradiated maize organs, demonstrating that there is a systemic response triggered by UV-B light. Furness and Upadhyaya [36] suggested the existence of a systemic signal component in plants in response to UV-B. They found morphological changes in roots of common agricultural weeds in which the aerial part were exposed to UV-B. Here, we demonstrate that NO and flavonoids are part of the plant systemic response when they receive UV-B irradiation.

Similar results were obtained using two different methods for NO quantitation. Although DAF fluorescence and Griess reactions may have interferences, those are different. By example, high nitrate content in plants may interfere with Griess method, but not with DAF [37]. Moreover, it was reported that nitrate was similarly distributed between leaves and roots of barley plants and its concentration was not significantly influenced by UV-B [38]. It is reasonable to assume that our experimental system is not significantly affected by nitrate.

In a previous work, we determined that NO counteracts the deleterious effects produced by UV-B in maize seedlings by inducing flavonoids production and ROS scavenging [27]. On the other hand, Pitervoká et al. [31] reported that NO is local and systemically produced in tomato in response to powdery mildew infection. Here, we present substantial evidence supporting that in UV-B-stressed maize leaves: (i) NO production is required for flavonoids increase, (ii) UV-B triggers NO and flavonoids accumulation and they are localized in the same cells and (iii) NO and flavonoids are systemically induced and flavonoids accumulation relies on the NO-dependent activation of flavonoid biosynthetic genes.

NO can be produced and emitted by plants as a consequence of UV irradiation [39]. It has been demonstrated that NO functions as a volatile signal by stimulating germination of dormant Arabidopsis seeds [40]. Interestingly, there is evidence that systemic responses in plants are facilitated by volatile signals. MeJA and MeSA are potent vascular and airborne inducers of defence responses [41,42], mediating long-distance resistance in the same or neighboring plants. Based on this knowledge, we drew the hypothesis that NO could also act as a volatile signal molecule in plant response to UV-B.

Here we show that a primary UV-B exposure induces a systemic response in non-irradiated leaves that enable them to alleviate the damage caused by a second UV-B exposure. Although flavonoid levels are similar in U, PC and CC after 2nd irradiation, ion leakage is higher in U leaves. After 1st irradiation, oxidative stress is immediately produced in U leaves because NO and flavonoids are at basal levels ([9,27] and Fig. 1A). Then, NO and flavonoids increased gradually in U, PC and CC (Figs. 1A and 6A and B). Thus, at the beginning of 2nd irradiation (five days after the 1st irradiation), cell damage was not diminished in U leaves, and NO and flavonoid increase may not counteract the stress produced by further irradiations. Nevertheless, if NO and flavonoids were increased before irradiation (as in PC and CC leaves), plants may deal with oxidative stress. The systemic increase of flavonoids concentration was 41% in covered leaves that not received direct UV-B irradiation. Bieza and Lois [34] reported that the UV-B resistant Arabidopsis *uvt1* mutant has 50% more flavonoids than wt. This suggests that the increase of systemic flavonoids concentration observed in PC and CC leaves are in the range and are sufficient to protect maize plants from a second exposure to UV-B.

Exposure to biotic or abiotic stress factors makes plants more resistant to subsequence exposures. This behavior indicates the capacity of plants for "memory" [43] and it agrees with our observation concerning the UV-B-induced flavonoids increase sustained for at least 96 h (Fig. 1A). This flavonoids increase may be part of a short memory response since plants with high flavonoids levels are more resistant to UV-B stress [34].

In UV-B-irradiated plants, flavonoids are often present in epidermal cell layers [1] in the adaxial side of UV-stressed leaves [15]. Here it is demonstrated that flavonoids and NO are mostly detected in adaxial side mesophyll of uncovered leaves exposed to UV-B and in entire mesophyll of leaves expressing the systemic response. In PC leaves, NO-fluorescence was lightly increased in bundle-sheath cells. NO is able to travel short distances through vascular system. Uncover part of PC leaves produce an increased of NO that could be transport to cover part through xylem.

Previous reports have shown that *ZmP* expression is regulated by UV-B in maize leaves [44,45]. Later, it was demonstrated that NO is required for the *ZmP* expression [27].

In this work, results indicate that *ZmP*, *CHS* and *CHI* are systemically upregulated by UV-B and that NO is required for inducing their expression. It is not yet known if the NO inducing activity is exclusively transported inside the plant, locally produced or even transported in the air. Moreover, Buer et al. [21] have reported

that flavonoids are capable to move long-distances using ABC-type transporters in Arabidopsis plants. Therefore, even if a high correlation exists between the systemic accumulation of flavonoids and the induction of *ZmP*, *CHS* and *CHI* gene expression, it cannot be ruled out that both flavonoid trafficking and *de novo* synthesis are occurring at the same time.

The results presented in this work demonstrate the presence of a systemic response to UV-B radiation in maize. UV-B perception by maize seedlings triggers a systemic accumulation of NO production by a yet unknown source. NO in turn, up regulates *ZmP* expression and its targets genes *CHS* and *CHI* leading to an increased flavonoid biosynthesis in unirradiated leaf regions. This systemic response to UV-B perception, involving enhanced levels of NO and flavonoids, allows plants to be better armed to counteract the maximum potential damage generated from further expositions to UV-B.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.05.012.

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