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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 $P \leq 0.05$.