# An increase in the concentration of abscisic acid is critical for nitric oxide-mediated plant adaptive responses to UV-B irradiation

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## Summary

- Here, the link between UV-B stimulus and the abscisic acid (ABA)-induced nitric oxide (NO) synthesis pathway was studied in leaves of maize (*Zea mays*).
- The ABA concentration increased by 100% in UV-B irradiated leaves. Leaves of *viviparous 14* (*vp14*), a mutant defective in ABA synthesis, were more sensitive to UV-B-induced damage than those of the wild type (wt). ABA supplementation attenuated UV-B-induced damage in both the wt and *vp14*. The hydrogen peroxide ($H_2O_2$) concentration increased in the irradiated wt, but changed only slightly in *vp14*. This increase was prevented by diphenylene iodonium (DPI), an inhibitor of NADPH oxidase (pNOX).
- NO was detected using the fluorophore 4,5-diamino-fluorescein diacetate (DAF-2DA). DAF-2DA fluorescence increased twofold in UV-B-irradiated wt leaves but not in *vp14* leaves. $H_2O_2$ and NO production was restored in *vp14* plants supplied with 100 μM ABA. Catalase, DPI and the NO synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME) partially blocked UV-B-induced NO accumulation, suggesting that $H_2O_2$ as well as NOS-like activity is required for a full plant response to UV-B. NO protects against UV-B-induced cell damage.
- Our results suggest that UV-B perception triggers an increase in ABA concentration, which activates pNOX and $H_2O_2$ generation, and that an NOS-like-dependent mechanism increases NO production to maintain cell homeostasis and attenuate UV-B-derived cell damage.

## Introduction

Plants are sessile organisms that need to absorb sunlight to grow and develop. As a consequence, they are inevitably exposed to ultraviolet (UV) radiation (200–400 nm), which represents almost 7% of the electromagnetic radiation emitted from the sun.

The vast majority of UV-C (200–280 nm) and UV-A (320–400) radiation is 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 (Frohnmeyer & Staiger, 2003). 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 (Frohnmeyer & Staiger, 2003).

Nevertheless, ROS are signalling molecules that modulate various plant responses to abiotic and biotic stresses (for a review, see Apel & Hirt, 2004). Hydrogen peroxide ($H_2O_2$) is one of the most extensively studied ROS involved in signalling. It has been reported that $H_2O_2$ functions as a second messenger mediating the systemic expression of various defence-related genes in tomato (*Solanum lycopersicum*) and Arabidopsis (Alvarez *et al.*, 1998; Orozco-Cardenas *et al.*, 2001).

The antioxidant response during water stress is also linked to $H_2O_2$ production, which is regulated by the phytohormone abscisic acid (ABA) (Jiang & Zhang, 2002b) . However, several studies on plant–pathogen interactions have characterized ABA as a negative regulator of the plant defence response. It was reported that drought stress led to increased concentrations of ABA in Arabidopsis leaves and compromised the ability of the

plant to prevent disease symptoms induced by an avirulent strain of *Pseudomonas syringae* pv. tomato (Mohr & Cahill, 2003). ABA accumulation also suppresses the salicylic acid (SA)-dependent defence pathway in tomato (Audenaert *et al.*, 2002), and antagonizes the jasmonic acid (JA)/ethylene (ET)-dependent defence pathways in Arabidopsis (Anderson *et al.*, 2004). By contrast, UV-B treatment promotes resistance to the biotrophic pathogen *Hyaloperonospora parasitica* in Arabidopsis (Kunz *et al.*, 2008). Moreover, treatments with ABA suppressed the expression of defence-related genes, such as plant defensin 1.2 (PDF1.2), which are up-regulated by UV-B (Mackerness *et al.*, 2001), and it was recently shown that UVBox$^{ANAC13}$, a novel *cis*-regulatory element for UV-B-induced transcription, is activated by UV-B, but not by ABA, osmotic, salt, heat or cold stress (Safrany *et al.*, 2008). All these results suggest that ABA generated by water stress may have the opposite effect to ABA generated by UV-B.

Nevertheless, UV-B and water stress could trigger similar responses, such as nitric oxide (NO) induction (An *et al.*, 2005; He *et al.*, 2005; Zhang *et al.*, 2007). NO is a ubiquitous bioactive molecule, produced in plants by enzymatic and nonenzymatic routes. NO can be enzymatically synthesized from nitrite by nitrate reductase (NR) and also by nitrite reductase (NiR), and there is also considerable evidence of an L-arginine-dependent NO synthase (NOS) activity in plants (see Wilson *et al.*, 2008 for a review). NO orchestrates a wide range of processes in plants. For example, NO acts as a signal in disease resistance (Delledonne, 2005), regulates stomatal closure (Garcia-Mata & Lamattina, 2001) and stimulates germination (Beligni & Lamattina, 2000). NO has also been proposed to be a broad-spectrum anti-stress molecule (Lamattina *et al.*, 2003; Lamattina & Polacco, 2007), because it confers protection against diquat, UV-B, drought, and salt stress (Garcia-Mata & Lamattina, 2001; Beligni & Lamattina, 2002; Zhao *et al.*, 2004; Shi *et al.*, 2005). Furthermore, NO modulates the expression of cell cycle regulatory genes (Correa-Aragunde *et al.*, 2006) and may be involved in signalling mechanisms activating mitogen-activated protein (MAP) kinase pathways (Pagnussat *et al.*, 2004; Zhang *et al.*, 2006, 2007) and increasing cyclic GMP (cGMP) concentrations (Durner *et al.*, 1998). It was recently shown that NO also regulates phospholipid signalling during stomatal closure and plant defence responses (Laxalt *et al.*, 2007; Distéfano *et al.*, 2008).

Thus, in this work, we investigated downstream signals that control the plant response to UV-B irradiation. Evidence supports a link between UV-B perception, ABA-induced signals and plant responses directed to counteract UV-B-induced cell damage.

## Materials and Methods

### Plants and growth conditions

Seeds from near-isogenic lines of wild-type maize (*Zea mays* L.) and the ABA synthesis-deficient mutant *vp14* (vp14-2274) were supplied by the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana, IL, USA). After surface sterilization with 0.5% hypochlorite for 20 min, seeds were washed and germinated on water-saturated filter paper at 25°C in darkness. Germinated seedlings were grown on soil:vermiculite (3 : 1, v/v) in an environment-controlled chamber with a 14 : 10 h photoperiod under light at 120 μmol photons $m^{-2}$ $s^{-1}$ at 25°C. Fourteen-day-old healthy seedlings were used in the experiments.

Sunflower (*Helianthus annuus* L.) genotype HAR2 was grown in an environment-controlled chamber with a 14 : 10 h photoperiod under light at 120 μmol photons $m^{-2}$ $s^{-1}$ at 25°C. Healthy leaves at the anthesis stage were used for experiments.

### ABA and NO donor treatments

For ABA supplementation, seedlings were excised at the base of the stem and placed in distilled water for 1 h to induce acclimatization. Water was then replaced with 100 μM ABA 24 h before irradiation. For sunflower experiments, the 'NO' sections of sunflower leaves were sprayed with 100 μM sodium nitroprusside (SNP) 24 h before irradiation. An 'old' SNP solution was obtained by maintaining a 100 μM SNP solution for 48 h in the light in an open tube to eliminate NO. This solution was sprayed onto leaves 24 h before irradiation as a control to examine the possibility of effects of ferrocyanide, nitrite and nitrate. Cut pieces of leaves were used for chlorophyll quantification.

### UV-B treatments

White light was supplemented with UV-B Philips tubes (TL100W/12; Philips, Amsterdam, the Netherlands) filtered with 0.13-mm-thick cellulose diacetate. A spectral irradiance of 3.3 W $m^{-2}$ was determined with an Ultraviolet Meter Model 3D (Solar Light Co., Pennsylvania, PA, USA). The time of irradiation is indicated in each figure.

### ABA quantification

Wild-type maize seedlings were irradiated with 3.3 W $m^{-2}$ of UV-B. At the times indicated, the second leaf of each seedling was immediately frozen in liquid $N_2$, lyophilized and powdered, and ABA analyses were carried out using the radioimmunoassay (RIA) method as described by Steinbach *et al.*, (1997). The results are the mean ± SD for four plants with three replicates per treatment.

### $H_2O_2$ quantification

$H_2O_2$ was quantified according to Bellincampi *et al.*, (2000) based on the peroxide-mediated oxidation of $Fe^{2+}$, followed by the reaction of $Fe^{3+}$ with xylenol orange (*o*-cresolsulfonephthalein 3′,3″-bis(methylimino)diacetic acid; sodium salt). Determinations were made on the second leaves of each seedling 60 min

after the end of UV-B irradiation (the same time-point as used for ABA quantification). When used, 100 μM diphenylene iodonium (DPI) was added 24 h before UV-B irradiation. The results are the mean ± SD for four plants with three replicates per treatment. The content of $H_2O_2$ was calculated based on a standard curve.

### Chlorophyll quantification

One hundred mg of leaves was grounded in liquid $N_2$, and extracted in 1 ml of 80% acetone overnight in the dark at 4°C. After centrifugation at 12 000 ***g*** for 10 min, the total chlorophyll concentration was determined in the supernatant according to the formula $A_{652}/34.2 = \text{mg Chl ml}^{-1}$, where $A_{652}$ is the absorbance at 652 nm (Arnon, 1949).

### NO detection and fluorescence quantification

NO was measured in the second leave 24 h after the end of irradiation using a 100 μM concentration of the specific fluorescent probe fluorophore 4,5-diamino-fluorescein diacetate (DAF-2DA), which was loaded into the leaves for 1 h; the leaves were then thoroughly washed to remove excess probe. The production of green fluorescence (515–555 nm) under these conditions was attributable to NO. Images were visualized in a Nikon Eclipse E200 (Nikon, Melville, NY, USA). Pictures show general phenomena representative of at least four individual experiments. Images acquired from the microscope were analysed using IMAGEJ 1.3 software (National Institutes of Health (NIH, Bethesda, MD, USA)).

For the characterization of UV-B-induced NO production, plants were excised at the base of the stem and placed in distilled water for 1 h to eliminate wound stress. The water was then replaced with 100 μM DPI, 500 U $\text{ml}^{-1}$ catalase, 100 μM NG-nitro-L-arginine methyl ester (L-NAME) or 100 μM c-PTIO which were added 24 h before UV-B irradiation.

### Cellular damage

Maize leaves were harvested and cut into 25-mm pieces. They were then washed in deionized water to remove surface-adhered electrolytes and placed in Petri dishes with 15 ml of deionized water at 25°C for 3 h. Electrical conductivity in the bathing solution ($C_1$) was determined using a Hanna HI8733 conductimeter (Sigma). Samples were then heated at 80°C for 2 h and 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 80°C. Relative ion leakage (%) = $(C_1/C_2) \times 100$.

### Statistical treatment

One-way ANOVAs were performed. Values represent mean ± SD for four plants per treatment.

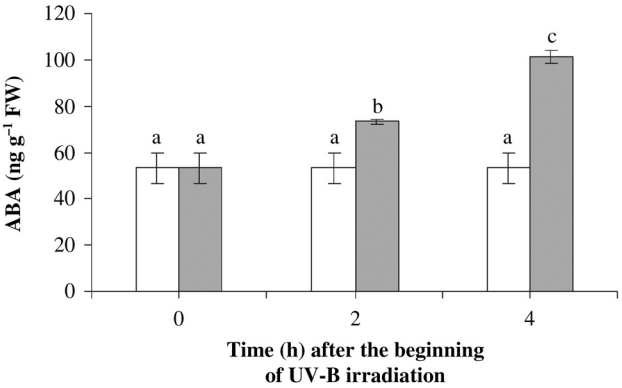


**Fig. 1** Abscisic acid (ABA) concentration increases in UV-B-irradiated maize (*Zea mays*) seedlings. Maize seedlings were irradiated with 3.3 W $\text{m}^{-2}$ of UV-B for 3 h, and the ABA content was assayed in the second leaf by radioimmunoassay at the beginning of irradiation, at 2 h after the beginning of irradiation and 1 h after the end of irradiation. White bars, control; grey bars, UV-B irradiated seedlings. Data are the mean ± SD for four plants with three replicates per treatment. Means with different letters differ at $P < 0.05$ according to one-way ANOVA. FW, fresh weight.

## Results

In order to determine whether there is a link between ABA and plant response to UV-B stress, we measured the ABA concentration in leaves of maize seedlings irradiated with 3.3 W $\text{m}^{-2}$ of UV-B for 3 h. Figure 1 shows that the ABA concentration in maize leaves was 50 ng $\text{g}^{-1}$ fresh weight (FW) at time zero. After 2 h of irradiation, ABA increased to 70 ng $\text{g}^{-1}$ FW, and reached 100 $\text{ng}^{-1}$ FW after 4 h (1 h after the end of the irradiation). These results demonstrate that the ABA concentration increased in response to a high dose of UV-B.

To investigate whether this ABA increase was directed towards counteracting the deleterious effect of the UV-B, we irradiated maize seedlings of the wild type (wt) and of *vp14*, a mutant deficient in ABA synthesis (Tan *et al.*, 1997). Figure 2(a) shows that wt and *vp14* maize leaves were injured by UV-B irradiation. However, *vp14* leaves seemed to be more sensitive than wt to UV-B. Interestingly, both *vp14* and wt maize leaves become less sensitive to UV-B when supplemented with 100 μM ABA before irradiation. Figure 2(b) shows that ion leakage, as a measure of cellular damage, was increased as a consequence of irradiation in both wt and *vp14*, this damage being lower in wt. When wt and *vp14* seedlings were supplemented with 100 μM ABA and then irradiated, ion leakage was reduced in both wt and *vp14* maize leaves. These results demonstrate that either endogenously increased or exogenously applied ABA confers a certain degree of protection to maize leaves from the deleterious effects of UV-B irradiation.

A cause–effect relationship between ABA and $H_2O_2$ production has been described. Water stress-increased or exogenously added ABA is an important inducer of $H_2O_2$ production in maize leaves (Jiang & Zhang, 2002b; Hu *et al.*, 2006; Zhang

**Table 1** Hydrogen peroxide ($H_2O_2$) is increased as a consequence of UV-B-triggered abscisic acid (ABA) induction

| $H_2O_2$ (μg $g^{-1}$ FW) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | UV-B | UV-B + DPI | DPI | ABA | ABA + DPI | ABA + UV-B |
| wt | 15.8 ± 1.3 (100%) | 26.1 ± 1.6 (170%) | 16.8 ± 0.8 (107%) | 15.1 ± 1.1 (96%) | 21.7 ± 1.3 (137%) | 17.6 ± 0.9 (111%) | 29.3 ± 2.0 (185%) |
| *vp14* | 13.0 ± 0.8 (100%) | 15.9 ± 1.1 (122%) | 14.3 ± 1.3 (110%) | 13.2 ± 1.5 (101%) | 20.2 ± 0.9 (147%) | 16.2 ± 0.8 (125%) | 26.8 ± 1.3 (206%) |

Detached maize (*Zea mays*) leaves from wild-type (wt) and *viviparous 14* (*vp14*) seedlings were irradiated with 3.3 W $m^{-2}$ of UV-B for 3 h, and $H_2O_2$ was quantified 1 h after the end of irradiation. Where indicated, maize leaves were treated with 100 μM ABA, 100 μM diphenylene iodonium (DPI) or 100 μM ABA + 100 μM DPI. Treatment started 24 h before irradiation. Data shown are mean ± SD for three plants with three replicates per treatment. Numbers in parentheses are percentages of variation in $H_2O_2$ content between UV-B-treated and non-irradiated (none) leaves of *vp14* and wt maize.

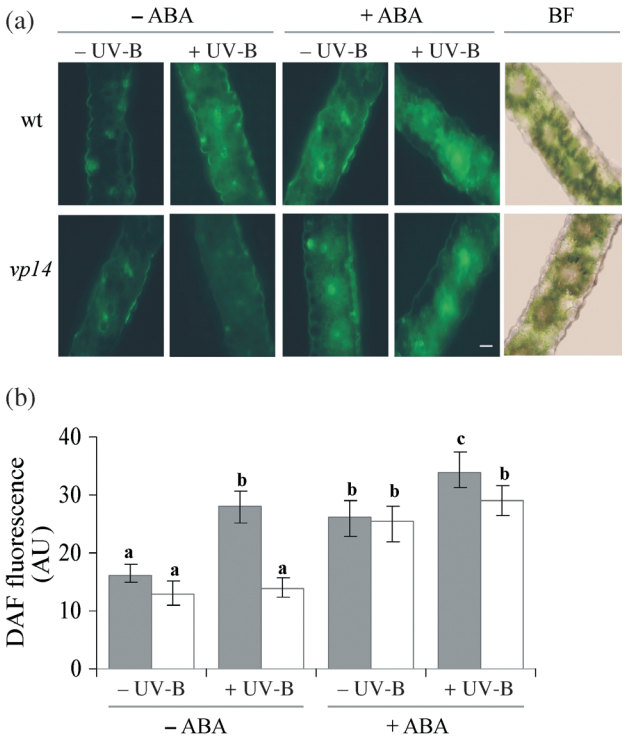


**Fig. 2** Abscisic acid (ABA) protects maize (*Zea mays*) seedlings against UV-B-induced damage. Wild-type and *viviparous 14* (*vp14*) maize seedlings were excised at the base of the stem and placed in distilled water for 1 h to eliminate wound stress. Where indicated, water was replaced with 100 μM ABA 24 h before irradiation. Then, plants were UV-B-irradiated for 3 h. (a) Representative images of the second leaves of plants, 3 d after UV-B irradiation. Bar, 1 cm. (b) Cellular damage was evaluated as ion leakage in the leaves shown in (a) (see the Materials and Methods). Wild type, grey bars; *vp14*, white bars. Values represent mean ± SD for four plants, with three replicates per treatment. Means with different letters differ at $P < 0.05$ according to one-way ANOVA. BF, bright field.

*et al.*, 2007). However, there is evidence that $H_2O_2$ production is reduced by ABA in germinating seeds, and in ABA-deficient tomato plants supplied with exogenous ABA (Schopfer *et al.*, 2001; Asselbergh *et al.*, 2007). To determine the effect of UV-B-triggered ABA induction on the endogenous $H_2O_2$ concentration, we measured $H_2O_2$ in irradiated maize seedlings of wild type (wt) and *vp14*. Table 1 shows that the $H_2O_2$ concentration increased to 170% in wt leaves 60 min after the end of the irradiation, but was only slightly modified in the *vp14* mutant, indicating that ABA could be mediating the UV-B induced $H_2O_2$ increase. The UV-B-induced increase of $H_2O_2$ was prevented in wt by DPI, a mammalian NADPH oxidase inhibitor, indicating that plant NADPH oxidase (pNOX) is involved in this ABA-mediated $H_2O_2$ production (Table 1). DPI had no effect on either basal or UV-B-irradiated *vp14* $H_2O_2$ production. Table 1 also shows that $H_2O_2$ increased to reach similar values when the wt and the *vp14* mutant were supplemented with 100 μM ABA. The $H_2O_2$ increase mediated by ABA supplementation was dependent on pNOX activity because DPI blocked this increase. UV-B + ABA increased $H_2O_2$ to the same extent in wt and *vp14*, confirming that the *vp14* phenotype reverted to the wt phenotype under ABA treatment.

It has been reported that NO is increased as a consequence of UV-B irradiation (An *et al.*, 2005), but the signal activating this response is still unknown. He *et al.* (2005) showed that NO synthesis promoted by endogenous $H_2O_2$ has an important role in UV-B-induced stomatal closure in *Vicia faba*. More recently, it was demonstrated that NO generation in maize leaves is triggered by ABA-induced $H_2O_2$ production (Zhang *et al.*, 2007). To investigate whether the generation of NO could be a consequence of the UV-B-induced ABA increase, maize leaves from different treatments were loaded with the permeable fluorophore DAF-2DA. This probe allows specific detection of NO in plant cells (Garcia-Mata & Lamattina, 2002; He *et al.*, 2005; Bright *et al.*, 2006; Zhang *et al.*, 2007).

Figure 3(a) shows that nonirradiated leaves of wt and *vp14* displayed low amounts of DAF-2DA green fluorescence. By contrast, when these seedlings were UV-B-irradiated, DAF-2DA green fluorescence accumulated in wt but not in *vp14*.

Fluorescence quantification in Fig. 3(b) shows that non-irradiated wt and *vp14* mutants displayed similar amounts of NO. However, while UV-B was able to induce a twofold increase in NO in wt, it was ineffective in the *vp14* mutant. Thus, the UV-B-induced NO increase relies on the presence of ABA, as *vp14* did not respond to the UV-B stimulus. Moreover, the NO concentration increased to similar values when wt

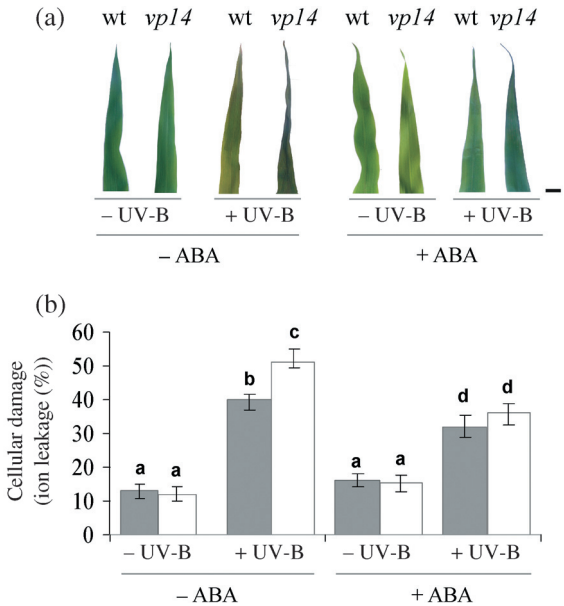


**Fig. 3** Abscisic acid (ABA) is required for nitric oxide (NO) production in UV-B-irradiated maize (*Zea mays*) seedlings. (a) Fluorescent micrographs of maize leaves: wild-type (wt) and *viviparous 14* (*vp14*) maize seedlings were UV-B-irradiated for 3 h, and NO was detected 24 h after irradiation as green fluorescence in cross-sections of the leaves, using the NO-specific fluorescent probe 4,5-diamino-fluorescein diacetate (DAF-2DA). Images were visualized in an Elite Nikon microscope. Where indicated, 100 μM ABA was added 24 h before irradiation as in Fig 2. Bright field (BF) images were included to facilitate morphological interpretation. Bar, 30 μm. (b) Quantification of fluorescence: images acquired from the microscope were analysed using IMAGEJ 1.3 software (NIH). wt, grey bars; *vp14*, white bars. Data shown are the mean ± SD for four plants, with three replicates per treatment. Means with different letters differ at $P < 0.05$ according to one-way ANOVA.

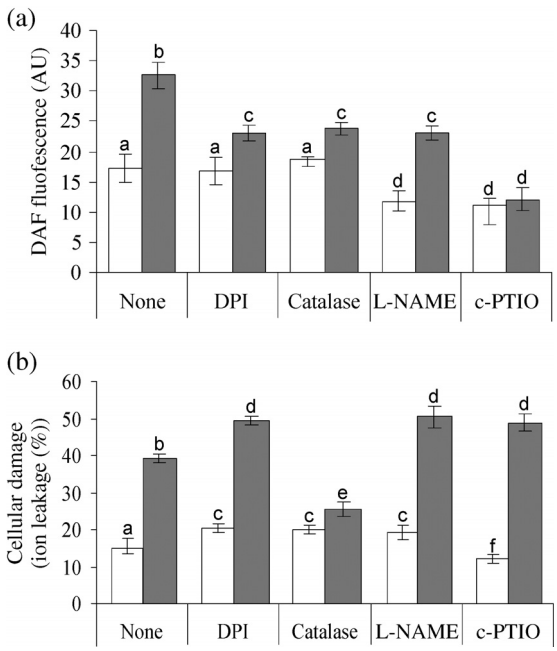


**Fig. 4** Characterization of UV-B-induced nitric oxide (NO) production. Wild-type (wt) maize (*Zea mays*) seedlings were irradiated with 3.3 W $m^{-2}$ of UV-B for 3 h. Where indicated, 100 μM diphenylene iodonium (DPI), 500 U $ml^{-1}$ catalase, 100 μM NG-nitro-L-arginine methyl ester (L-NAME) or 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was added 24 h before UV-B irradiation as in Fig. 2. Control, white bars; UV-B-irradiated seedlings, grey bars. (a) Quantification of fluorescence: NO was detected 24 h after irradiation as green fluorescence in cross-sections of leaves, using 4,5-diamino-fluorescein diacetate (DAF-2DA). Images acquired from the microscope were analysed using IMAGEJ 1.3 software (NIH). Values represent the mean ± SD for four plants per treatment. Means with different letters differ at $P < 0.05$ according to one-way ANOVA. (b) Cellular damage was evaluated as ion leakage 3 d after the end of irradiation. Values represent the mean ± SD for four plants per treatment. Means with different letters differ at $P < 0.05$ according to one-way ANOVA.

and *vp14* seedlings were supplemented with 100 μM ABA (Fig. 3a,b), confirming that ABA supplementation can restore NO production in the *vp14* mutant. Figure 3(b) also shows that NO did not significantly increase in ABA-supplemented wt or *vp14* seedlings after UV-B irradiation, indicating that the effects of both stimuli, ABA and UV-B, were noncumulative with respect to NO production.

Taken together, these results confirm that the accumulation of endogenous NO in maize leaves in response to UV-B radiation is ABA-dependent and is paralleled by increased tolerance to high doses of UV-B radiation.

To better characterize the mechanism of UV-induced NO production in wt maize, seedlings were pretreated with chemicals that interfere with ROS production before UV-B irradiation. Nonirradiated seedlings were used as a control. Figure 4(a) shows that pretreatment with DPI reduced the DAF-2DA fluorescence intensity induced by UV-B by > 30%. Similar results were obtained when seedlings were pretreated with catalase (a $H_2O_2$-degrading enzyme) and L-NAME (an NO synthase inhibitor). UV-B-induced NO production was drastically reduced when seedlings were pretreated with c-PTIO (a specific NO scavenger). Even though the treatment with L-NAME did not completely prevent the UV-B-induced NO production, results strongly suggest that a NOS-dependent pathway is required to generate NO as a response to UV-B irradiation. Figure 4(b) shows that cellular damage increased when seedlings were pretreated with DPI, L-NAME and c-PTIO before UV-B irradiation. This was coincident with the reduction of the NO concentration caused by these compounds. Catalase reduced the NO concentration (Fig. 4a) but also reduced cellular damage (Fig. 4b), probably because catalase degrades $H_2O_2$ and reduces the ROS concentration to a level at which cellular damage is also reduced.

Several reports have presented evidence that exogenous addition of NO is able to protect cyanobacteria, green alga (*Chlorella pyrenoidosa*), maize, bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) from UV-B radiation (Chen *et al.*, 2003; Zhang *et al.*, 2003; An *et al.*, 2005; Shi *et al.*, 2005; Qu *et al.*,

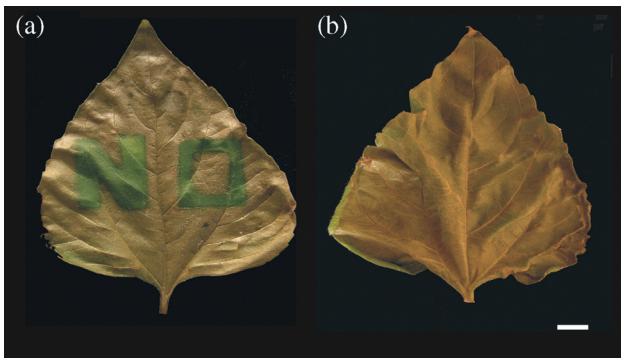


**Fig. 5** Nitric oxide (NO) protects sunflower (*Helianthus annuus*) leaves against UV-B irradiation. An 'NO' section of sunflower leaves was sprayed with a 100 μM concentration of the NO donor sodium nitropruside (SNP) (a) or a 100 μM concentration of 'old' SNP (b). Twenty-four hours later, leaves were subjected to 8 h of high UV-B intensity (3.3 W $m^{-2}$). The image is representative of three individual experiments and was taken 4 d after the end of irradiation. Bar, 1 cm.

2006; Xue *et al.*, 2007). It was proposed that the protective effect of exogenous NO was mediated through increases in the activities of antioxidant enzymes, and modification of the chemical properties of cell wall polysaccharides.

To complement our results, we assayed a dicotyledonous plant from the Compositae family. *Helianthus annuus* (sunflower), a UV-sensitive plant, was treated with exogenous NO and subjected to high UV-B irradiation. Sunflower leaves were sprayed with a 100 μM concentration of the NO donor SNP, marking the letters NO. Twenty-four hours later, leaves were subjected to 8 h of high UV-B intensity (3.3 W $m^{-2}$). Figure 5(a) shows that, 5 d after irradiation, UV-B protection was observed in the NO-sprayed section; the chlorophyll concentration was 0.86 mg $g^{-1}$ FW in the NO-sprayed section, and 0.46 mg $g^{-1}$ FW in the nonsprayed section. When leaves were sprayed with old SNP solutions (containing no NO but nitrate, nitrite and ferrocyanide) no protection was observed (Fig. 4b), and the chlorophyll content was reduced in both sections. Nonirradiated leaves showed no differences between sprayed and nonsprayed regions (not shown). These results indicate that the use of exogenous NO could be a generalized mechanism of plant protection against the deleterious effects of UV-B.

## Discussion

In this work, we have investigated the molecular basis of plant adaptive responses to UV-B irradiation. The results presented here show that maize leaves respond to UV-B irradiation by increasing the concentration of ABA, $H_2O_2$ and NO, and that ABA is required for the NO-mediated attenuation of deleterious effects produced by UV-B.

This report shows that an increase in the concentration of ABA is one of the earliest responses involved in the signalling pathways triggered by UV-B in maize leaves. The ABA concentration increased by 100% in maize leaves after 3 h of UV-B irradiation. In the same period, $H_2O_2$ and NO concentrations increased by 70% and 100%, respectively, in an NADPH oxidase-dependent manner. These results are in agreement with those obtained in maize leaves submitted to water stress (Jiang & Zhang, 2002b; Zhang *et al.*, 2007). This is an important point, because an ABA increase may have different effects in different experimental systems, and under different types of stress. Firstly, plant cells possess different types of ABA receptors inside and outside the cell. ABA receptors have different structures and localizations (i.e. nuclear, plastid and plasma membrane), enabling ABA to work simultaneously and independently at multiple sites in the cell, and probably to drive different responses at each site (Hirayama & Shinozaki, 2007). Secondly, the sites of ABA perception may differ in response to different environmental cues, and may be organ-, tissue- or even cell-specific, and developmental stage-specific. Thirdly, the effect of ABA may be modified by its interaction with other stress hormones such as SA, JA and ET (Mauch-Mani & Mauch, 2005). Overall, an ABA increase may have no effects if antagonist hormones are present at higher concentrations, or if the cellular context produces activation of a different signalling cascade downstream of ABA.

Here, we present the first genetic and pharmacological evidence showing that either endogenously increased or exogenously applied ABA protects maize leaves against UV-B irradiation. Our results were obtained using the *vp14* maize mutant, which is defective in ABA synthesis. The *VP14* gene encodes a 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme (Tan *et al.*, 1997). Epoxycarotenoids are cleaved by NCED to yield xanthoxin, which is then modified by a short-chain dehydrogenase/reductase (SDR1) to form abscisic aldehyde. Aldehyde oxidase (AO) converts this substrate to ABA. UV-B irradiation affected *vp14* leaves more severely than wt leaves. Interestingly, both *vp14* and wt maize leaves become less sensitive to UV-B when supplemented with 100 μM ABA before irradiation (Fig. 2).

The data here obtained, working with wt and *vp14* irradiated plants, provide the first quantitative evidence that ABA could mediate the UV-B-induced $H_2O_2$ increase. This increase was prevented by DPI, an NADPH oxidase inhibitor, indicating that pNOX is also involved in this process (Table 1).

It has been reported that ABA-induced $O_2^-$ and $H_2O_2$ production is inhibited by DPI under conditions of water stress (Jiang & Zhang, 2002a,b, 2003), and that UV-B-induced $H_2O_2$ production is reduced in the pNOX Arabidopsis double mutant *atrboh D/F* (Kalbina & Strid, 2006). Interestingly, this mutant is more sensitive to UV-B than wt plants (Kalbina & Strid, 2006), and displays diminished ABA-regulated NO synthesis and stomatal closure (Bright *et al.*, 2006). Catalase diminished NO in wt irradiated plants (Fig. 4), indicating that $H_2O_2$ is involved in UV-B-induced NO synthesis. However, although $H_2O_2$ production increases in response to ABA and

UV-B, this $H_2O_2$ increase does not always result in NO accumulation. Frohnmeyer & Staiger (2003) propose a model in which UV-B may induce nonspecific $H_2O_2$ accumulation, and this $H_2O_2$ accumulation may not result in NO production.

Here we have shown that UV-B was able to induce an increase in NO in wt maize seedlings, but was ineffective in the *vp14* mutant (Fig. 3). However, NO was increased in wt or *vp14* plants when they were supplemented with 100 µM ABA. Thus, the UV-B-induced NO increase relies on the presence of ABA. The ABA-related NO source is a matter of debate. It has been proposed that either ABA- or UV-B-induced NO is mainly produced by NOS-like activity (Zhang *et al.*, 2003, 2007; An *et al.*, 2005; Zhou *et al.*, 2005; Qu *et al.*, 2006). However, other pharmacological and genetic data suggest that nitrate reductase (NR) is the major source of NO in guard cells in response to ABA-mediated $H_2O_2$ synthesis (Bright *et al.*, 2006). Our results indicate a contribution of NOS-like activity in the UV-B-induced NO increase (Fig. 4a). Nevertheless, as the NOS inhibitor L-NAME was ineffective in completely blocking UV-B-induced NO production, we cannot rule out the presence of other NO sources. The role of NR could be difficult to evaluate in our experimental system because tungstate, an inhibitor of NR, is also an ABA synthesis inhibitor.

The results presented here demonstrate that high endogenous NO production induced by UV-B correlates with a high level of cell protection against UV-B. When the NO concentration is high, UV-B-induced cell damage is reduced (Figs 2, 3). When NO was scavenged by c-PTIO, or reduced as a consequence of $H_2O_2$ degradation or pNOX and NOS inhibition, UV-B-dependent cell damage was increased (Fig. 4). In agreement with these results, when NO was elevated by treatment with the exogenous NO donor SNP, UV-B protection was increased (Fig. 5).

Our results support the model explaining plant responses to elevated doses of UV-B radiation shown in Fig. 6. This model proposes that UV-B perception by plants triggers a signalling cascade that leads to an increase in ABA concentration, followed by activation of pNOX and $H_2O_2$ generation. In turn, through a mechanism regulated in part by NOS-like activity, there is an enhancement of NO production to maintain cell homeostasis and to protect cells from the deleterious effects of oxidative stress. In addition, as plants are able to emit and absorb NO, they may also use exogenous NO as a protection strategy against elevated doses of UV-B.

NO can effectively protect plants from UV-B in several ways: it has been demonstrated that NO may be a ROS scavenger in UV-B-irradiated plants (Zhang *et al.*, 2003), but NO has also been suggested to act as a signal in response to UV-B stress. NO may fulfil this function by enhancing the activity of antioxidant enzymes (Shi *et al.*, 2005) or by modifying the chemical properties of cell wall polysaccharides through changes in glucanase activities and the protein content of cell walls (Zhang *et al.*, 2003; An *et al.*, 2005; Qu *et al.*, 2006). Interestingly, it has been shown that the induction of the gene encoding chalcone synthase (*Chs*) was co-ordinately regulated by NO and UV-B (Mackerness *et al.*, 2001). *Chs* is the main regulator of the synthesis of flavonoids, which are recognized as UV-absorbing and antioxidant compounds.

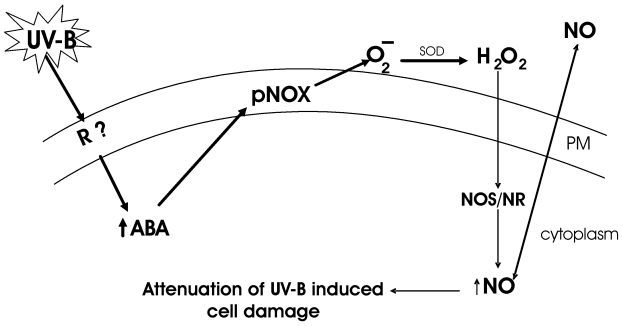


**Fig. 6** Simplified scheme of UV-B abscisic acid (ABA)–nitric oxide (NO) interactions. Plant UV-B perception by a putative receptor (R?) increases the ABA concentration. ABA activates NADPH oxidase (pNOX), which produces $O_2^-$ in the apoplast. This $O_2^-$ is dismuted to $H_2O_2$ by a superoxide dismutase (SOD). $H_2O_2$ crosses the plasma membrane into the cytoplasm and enhances NO production through a mechanism regulated in part by nitric oxide synthase (NOS) and possibly by nitrate reductase (NR) activity. In addition, plants may also incorporate atmospheric NO. As a result, evidence supports NO as a key player in ABA-induced plant cell defence mechanism to overcome damage produced by UV-B. PM, plasma membrane.

Plant responses to abiotic and biotic stimuli rely on a complex network of metabolic pathways. In this context, we propose the UV-B–ABA–NO pathway as one of the components of this network. An example of this complexity is provided by microarray and reverse transcriptase–polymerase chain reaction (RT-PCR) analyses performed on *UV resistance locus 8* (*uvr8*) and wt UV-B-irradiated Arabidopsis plants. Brown *et al.* (2005) have shown that the transcriptional regulator UVR8 mediates the UV-B-induced expression of a range of genes, many of which, including *Chs*, play important roles in UV protection. In addition, Casati & Walbot (2004) reported significant variations in the maize transcriptoma profile after 8 h of UV-B irradiation. It would be interesting in the future to investigate what proportion of UV-B-induced gene expression is dependent on the ABA–NO pathway.

As stated above, it has been demonstrated that NO can be produced and emitted by plants as a consequence of UV irradiation (Hari *et al.*, 2003). Our results support the hypothesis that UV-B-induced ABA production can trigger NO synthesis by plants. A higher intensity of UV-B radiation will produce a rise in the tropospheric NO concentration as a result of emission from vegetation. As a consequence, the NO concentration will increase in high-UVB-irradiated regions. We propose that this increased NO production and emission by plants will help them, and neighbouring plants, to counterbalance the deleterious effects of UV-B radiation.

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