Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide

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Abstract The nature and origin of the reactive oxygen species (ROS) involved in the early part of Ultraviolet-B (UV-B)-induced signaling pathways were investigated in *Arabidopsis thaliana* using a range of enzyme inhibitors and free radical scavengers. The increase in *PR-1* transcript and decrease in *Lhcb* transcript in response to UV-B exposure was shown to be mediated through pathways involving hydrogen peroxide (H_2O_2) derived from superoxide ($O_2^{\bullet-}$). In contrast, the up-regulation of *PDF1.2* transcript was mediated through a pathway involving $O_2^{\bullet-}$ directly. The origins of the ROS were also shown to be distinct and to involve NADPH oxidase and peroxidase(s). The up-regulation of *Chs* by UV-B was not affected by ROS scavengers, but was reduced by inhibitors of nitric oxide synthase (NOS) or NO scavengers. Together these results suggest that UV-B exposure leads to the generation of ROS, from multiple sources, and NO, through increased NOS activity, giving rise to parallel signaling pathways mediating responses of specific genes to UV-B radiation.

Key words: Gene expression; Nitric oxide; Reactive oxygen species; Signal transduction; Ultraviolet-B

1. Introduction

Depletion of the stratospheric ozone layer is leading to an increase in solar ultraviolet-B (UV-B: 280–320 nm) radiation reaching the Earth's surface [1,2]. We are studying the molecular mechanisms of plant responses to UV-B stress (reviewed in [3]) and in particular, changes in gene expression. These include down-regulation of photosynthetic genes [4–6] and up-regulation of genes for flavonoid biosynthesis [5] and antioxidant enzymes [4,7]. In addition, the expression of a number of pathogenesis-related genes, the acidic PR genes and the defencin gene, *PDF1.2*, have also been shown to increase in response to UV-B exposure [6,8]. This overlap in induced gene expression could be due to the production of reactive oxygen species (ROS) by both stresses (see [4,9–12]). The rise in ROS levels leads to increases in levels of salicylic acid (SA), ethylene and jasmonic acid (JA), which are important for regulation of gene expression and subsequent resistance/tolerance to both pathogen infection and UV-B exposure (see [12,13] and references within).

Although an important role for ROS has been established in UV-B signal transduction (see [3,12]), the origin of these ROS remains elusive. A number of sources of ROS during plant-pathogen infections have been proposed, including peroxidases, lipoxygenases and oxalate oxidase [10,11]. However, a large body of evidence indicates that a plasma membrane-bound multi-component enzyme system, NADPH oxidase, analogous to the mammalian phagocyte oxidase, is the likely source in plants [9,14,15]. Plant homologs of this enzyme have been identified in *Arabidopsis* [16] and rice [17].

It has been shown that the induction of the gene encoding chalcone synthase, *Chs*, is not mediated by ROS, however, clear roles for calcium and calmodulin have been demonstrated [18,19]. This indicates that additional early signaling pathways are likely to be involved in responses to UV-B radiation. Nitric oxide (NO) has been implicated as a potential second messenger during the hypersensitive response (HR), exerting effects that are both complementary and agonistic to those of H_2O_2 [20]. NO is a free radical which is synthesized from L-arginine in a reaction involving NO synthase (NOS) [20–22]. The activity of NOS was found to rise in response to pathogen infection and inhibitors of this enzyme compromised the HR response to bacterial infection in *Arabidopsis* leaves, preventing the up-regulation of *Chs* and phenylalanine ammonia lyase (*PAL*) [21,22]. There have been no studies reported on the role of NO in plant responses to UV-B radiation.

In this paper we have used a pharmacological approach to dissect and study the role of different, but parallel, early signaling components during the response to UV-B radiation. The results show, for the first time, that multiple components, including ROS of different biochemical origin, and NO, act in parallel pathways to mediate the responses of specific genes to UV-B radiation.

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Abbreviations: chs, chalcone synthase; CAT, catalase; DDC, *N,N*-diethyldithiocarbamate; DPI, diphenyleneiodonium; JA, jasmonic acid; IM, imidazole; *Lhcb*, light harvesting complex binding proteins; L/D-NAME, N^G-monomethyl-l-arginine; NO, nitric oxide; NOS, NO synthase; *PAL*, phenylalanine ammonia lyase; *PDF1.2*, defencin; *PR-1*, pathogenesis-related-1; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide; ROS, reactive oxygen species; SA, salicylic acid; SHAM, salicylhydroxamic acid; SOD, superoxide dismutase; UV-B, ultraviolet-B

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2. Materials and methods

2.1. Plant material and experimental treatments

The conditions for growth, UV-B and control (UV-A) treatments of *Arabidopsis* plants were as described previously [6].

The 4 week old plants were sprayed as previously described [6] with 250–1000 μM diphenyleneiodonium (DPI) 20–100 mM imidazole (IM), 1–10 mM salicylhydroxamic acid (SHAM), 5–50 mM Tiron, 10–100 mM *N*,*N*-diethyldithiocarbamate (DDC), 8–10 K units catalase (CAT), 1–2 K units superoxide dismutase (SOD), 1–20 mM *S*-nitrosoglutathione (GSNO), 5–100 μM *S*-nitroso-*N*-penicillamine (SNAP), 10–20 μM 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide (PTIO), 50–200 mM N^G-monomethyl-1-arginine (L- and D-NAME). All chemicals were obtained from Sigma-Aldrich. The plants were sampled half way through the light period of the 2nd day of treatment (30 h) unless otherwise stated.

In all experiments, whole *Arabidopsis* plants were used and not, as in previous studies, cell suspension cultures. Therefore, for all chemicals used, a range of concentrations (see above) were tested. The results presented are the concentrations which were sufficient to produce a response, indicating successful uptake of the chemical by the leaves, but were well below those resulting in changes in quantity of RNA recovered or *18S rRNA* (control) transcript levels or leading to any visible effects on the plants. All experiments were repeated independently at least four times.

2.2. Measurement of transcript levels

After illumination, at least three whole rosettes were harvested into liquid nitrogen and RNA isolated as described previously [8]. The yield of total RNA was not affected by any of the treatments. Equal amounts of RNA (20 μg) were applied to lanes of a 1.5% agarose-formaldehyde gel. Equal loading was confirmed by staining with ethidium bromide (data not shown) as well as hybridization to the constitutive *18S rRNA* DNA probe [8]. After electrophoresis, RNA was blotted onto nylon and hybridized to the radiolabelled probes as described in [8]. Washes, autoradiography and quantification of blots are as described previously [8]. Blots presented are results obtained from one of four replicate experiments and are representative of the results obtained. Relative amounts of radioactivity bound to specific bands were quantified using a phosphorimager SI (Molecular Dynamics Ltd, Bucks, UK) and data presented as described in figure legends.

2.3. NADPH oxidase activity assay

Activity of NADPH oxidase was measured spectrophotometrically as described previously [7]. The assay mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7), 150 μM NADPH (Boehringer Mannheim), 10 μM KCN and 50 μg of membrane protein. The reaction was initiated by the addition of protein and the decrease in A_{340} was followed for 2 min.

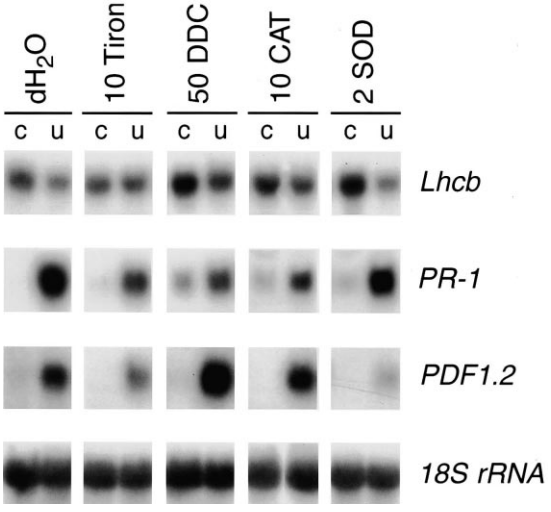

Fig. 1. Autoradiographs of *Lhcb*, *PR-1*, *PDF1.2* and *18S rRNA* transcript levels in wild type plants sprayed with water (dH_2O), 10 mM Tiron, 50 mM DDC, 10 units ml^{-1} CAT or 2 units ml^{-1} SOD prior to treatment with (u) or without (c) UV-B radiation for 2 days. The amount of radioactivity was quantified using a phosphorimager and is presented in the table

Chemical	Concentration	Action	Relative transcripts (percent transcripts in plants+UV-B/transcripts in plants−UV-B)		
			Lhcb	*PR-1*	*PDF1.2*
dH_2O	–	control	**18** (1.2)	**1644** (102)	**445** (40.1)
Tiron	5 mM	$O_2^{\bullet-}$ scavenger	52 (2.6)	1212 (122)	263 (6.2)
	10 mM		**94** (8.5)	**250** (56.5)	**154** (2.6)
DDC	25 mM	Inhibitor of SOD	38 (4.4)	371 (40.1)	710 (85.6)
	50 mM		**66** (3.5)	**185** (25.3)	**985** (74.3)
CAT	8 units ml^{-1}	$H_2O_2 \rightarrow H_2O$	39 (5.1)	548 (58.0)	450 (45.6)
	10 units ml^{-1}		**47** (4.4)	**354** (67.4)	**480** (68.5)
SOD	1 unit ml^{-1}	$O_2^{\bullet-} \rightarrow H_2O_2$	21 (2.8)	1524 (190)	275 (28.1)
	2 units ml^{-1}		**25** (1.8)	**1489** (173)	**130** (18.9)

The data shown have been corrected for loading differences by using counts obtained with *18S rRNA*. Although a range of concentrations were tested, the results presented are the concentration of chemicals which produced the most effect in at least one transcript studied without causing any visible effects on the plants or altering *18S rRNA* transcript levels. Values in bold correspond to results from concentrations illustrated on the blots. These results are presented as percent transcript levels in UV-B-treated plants compared with plants treated under the same conditions (chemical treatments) but in the absence of UV-B (100% indicates no difference in transcript levels between plants treated with and without UV-B). The dH_2O treatment indicates the effect of UV-B alone (control). Values are means (S.E.M.) of four independent experiments.

3. Results and discussion

3.1. Role of hydrogen peroxide and superoxide radicals in UV-B signaling

Previous studies have shown a role for ROS in regulation of gene expression in response to UV-B radiation, but no attempt has been made to determine the nature of these ROS (see [3,6,8]). In order to identify the ROS involved, plants were sprayed with different concentrations of various ROS scavengers prior to exposure to UV-B. The effects of these inhibitors on transcripts encoded by the photosynthetic gene, light harvesting complex binding proteins (*Lhcb*), two pathogenesis-related genes, *PR-1* and *PDF1.2* were determined and results are presented in Fig. 1. UV-B exposure, in plants treated with water as a control, resulted in the decrease in *Lhcb* transcripts (18% of levels in non-UV-B treated plants) and an increase in *PR-1* (1644%) and *PDF1.2* (445%) transcripts (Fig. 1) as previously reported [8]. Tiron, a scavenger of $O_2^{\bullet-}$ [23], significantly reduced the effect of UV-B on all three genes (Fig. 1), at both 5 and 10 mM indicating that the first ROS species generated by exposure to UV-B is $O_2^{\bullet-}$. DDC, an inhibitor of SOD [24] which leads to accumulation of $O_2^{\bullet-}$, markedly reduced the increase in *PR-1* transcripts and decrease in *Lhcb* transcripts in response to UV-B exposure but resulted in a greater increase in *PDF1.2* transcripts. These observations are consistent with the results obtained after the addition of SOD, which reduced the effect of UV-B on *PDF1.2*, and of CAT, which reduced the effect of *Lhcb* and *PR-1* transcripts (Fig. 1). Taken together, these results indicate that up-regulation of *PR-1* and down-regulation of *Lhcb* genes by UV-B radiation is regulated by H_2O_2 derived from $O_2^{\bullet-}$ while *PDF1.2* gene is up-regulated by $O_2^{\bullet-}$ and not by H_2O_2.

The effect of CAT and SOD, unlike the other treatments used, is likely to be through their activity at the surface of cells, since they are unlikely to penetrate the plasma membrane.

These results indicate that the first ROS species generated by UV-B is $O_2^{\bullet-}$ and are consistent with previous studies using ascorbic acid [4,6,8] and gain of function experiments using 3-AT, a CAT inhibitor, known to generate H_2O_2 [4,6]. Superoxide appears to be involved directly in the up-regulation of *PDF1.2* because inhibition of SOD (resulting in accumulation of $O_2^{\bullet-}$) by DDC resulted in a greater accumulation of this transcript on exposure to UV-B and removal of $O_2^{\bullet-}$ by addition of SOD reduced the effect of UV-B (Fig. 1). Involvement of $O_2^{\bullet-}$ in gene induction for pathogen responses has been reported [15,25] but not in responses to UV-B radiation. In contrast, H_2O_2 generated from dismutation of $O_2^{\bullet-}$ is the ROS involved in up-regulation of *PR-1*, as both CAT and DDC treatment reduced the effect of UV-B on this gene (Fig. 1). Regulation of *PR-1* and *PDF1.2* by ROS has been demonstrated in pathogen responses (see [9,26]) but little is known about the nature of these ROS (see [15]). The primary source of H_2O_2 involved in UV-B-induced down-regulation of *Lhcb* is $O_2^{\bullet-}$ as DDC and CAT reduced the impact of UV-B on this

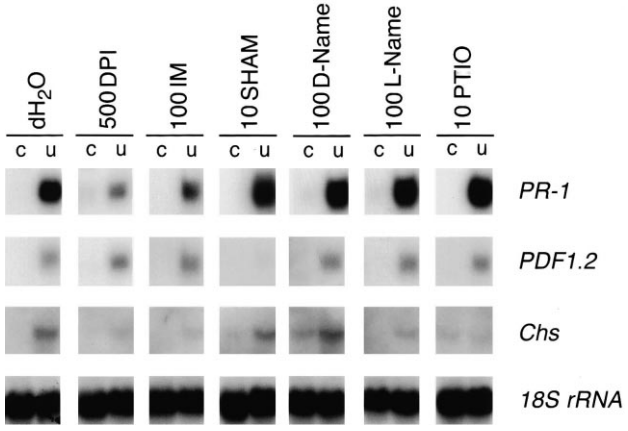

Fig. 2. Autoradiographs of *PDF1.2*, *PR-1*, *Chs* and *18S rRNA* transcript levels in wild type plants sprayed with water (dH_2O), 500 μM DPI, 100 mM IM, 100 mM D-NAME, 100 mM L-NAME, 20 μM PTIO or 5 mM SHAM prior to treatment with (u) or without (c) UV-B radiation for 2 days. Quantitative results are presented in the table

Chemical	Concentration	Action	Relative transcripts (percent transcripts in plants+UV-B/ transcripts in plants−UV-B)		
			PR-1	*PDF1.2*	*Chs*
dH_2O	–	Control	**1644** (102)	**445** (40.1)	**680** (21.9)
DPI	250 μM	Inhibitor of NADPH ox/ NOS	412 (32.6)	450 (32.3)	334 (16.2)
	500 μM		**240** (18.5)	**482** (45.5)	**154** (23.8)
IM	50 mM	Inhibitor of NADPH ox	848 (144.4)	412 (30.1)	313 (25.6)
	100 mM		**524** (93.5)	**440** (21.3)	**192** (44.3)
SHAM	5 mM	Inhibitor of peroxidases	1712 (155.1)	206 (27.4)	648 (65.3)
	10 mM		**1768** (114.4)	**128** (18.3)	**698** (58.2)
D-NAME (control)	100 mM	Inactive isomer of L-NAME	**1798** (100.7)	**460** (53.4)	**702** (33.7)
L-NAME	50 mM	NO scavenger	1865 (93.6)	452 (44.0)	402 (55.0)
	100 mM		**1822** (124.5)	**469** (16.1)	**228** (35.8)
PTIO	5 μM	Inhibitor of NOS	1706 (170)	476 (19.0)	296 (14.5)
	10 μM		**1815** (145)	**425** (37.3)	**133** (12.0)

(see legend for Fig. 1 for more detail). Values in bold correspond to results from concentrations illustrated on the blots.

transcript (Fig. 1). In agreement with previous studies [18] none of the ROS scavengers influenced the effect of UV-B on the levels of the *Chs* transcript (data not shown).

3.2. Biochemical origin of ROS generated in response to UV-B radiation

The origin of the ROS that are generated in response to pathogen infection is controversial. Using inhibitor a number of studies have demonstrated a key role for NADPH oxidase (see [9,14,15]). In order to investigate the role of this enzyme in responses to UV-B exposure, its activity was measured in plants treated with supplementary UV-B. The activity of NADPH oxidase increased steadily from 2.7 (±0.5 (S.E.M.)) μmol h^{-1} mg $protein^{-1}$, at the beginning of the experiment, to 8 (±0.7) μmol h^{-1} mg $protein^{-1}$ after 6 h of UV-B exposure, rising to 15.4 (±1.4) μmol h^{-1} mg $protein^{-1}$ after 30 h. There was no significant change in activity of this enzyme in control UV-A-treated plants over the same time period. The increase in activity in response to UV-B exposure indicates a potential role for this enzyme in the generation of $O_2^{\bullet-}$ in response to UV-B, as previously reported [7], and thus the increase in ROS that are involved in the regulation of our marker genes.

DPI and imidazole are inhibitors of mammalian NADPH oxidase [27,28] and have been used effectively to inhibit this enzyme in plant systems (see [9,10] and references within). Long and Jenkins [18] have also reported that DPI can inhibit the increase in *Chs* transcripts in *Arabidopsis* cell cultures in response to UV-B exposure. Fig. 2 illustrates *PR-1*, *PDF1.2* and *Chs* transcript levels from plants sprayed with DPI and IM prior to UV-B treatment for 2 days. Both compounds reduced the effect of UV-B on *PR-1* and *Chs* transcripts but had no influence on the increase in *PDF1.2* transcripts (Fig. 2a,b) or the decrease in *Lhcb* transcripts (data not shown). The results indicate a role for NADPH oxidase in generation of ROS involved in up-regulation of *PR-1*, but not of *PDF1.2*, or in down-regulation of *Lhcb*, in response to UV-B radiation.

Recent studies have shown a role for cell wall peroxidases in the synthesis of $O_2^{\bullet-}$ in response to pathogen infection [10,29] although their direct involvement in regulation of gene expression has not been studied. In order to assess the potential role of peroxidases as ROS-generators in response to UV-B exposure, plants were sprayed with the peroxidase inhibitor, SHAM [10,29] (Fig. 2). SHAM did not affect the UV-B induced increase of either *PR-1* or *Chs* transcripts (Fig. 2) or decrease in *Lhcb* transcripts (data not shown), but effectively reduced the increase in *PDF1.2* transcripts (Fig. 2). Thus, the production of the ROS involved in the regulation of *PDF1.2*, but not *PR-1*, *Chs* and *Lhcb* genes, is likely to involve the activity of peroxidase(s).

The absolute specificity of each inhibitor used in this study can always be questioned, but our data do show clearly that the sources of ROS involved in regulating *PR-1*, *Lhcb* and *PDF1.2* are distinct.

3.3. Role of NO in UV-B signaling

Recent studies have suggested that NO plays an important role in plant growth and development, signal transduction and disease resistance. In pathogen responses, NO has been shown to trigger the induction of defense-related genes such as *Chs* and PAL and to be important for limiting the spread and growth of bacterial infection [19]. In addition, although induction of *Chs* by UV-B appears to be through an ROS-independent pathway, DPI can inhibit the induction of this gene (Fig. 2; [18]). Previous studies have indicated that DPI can also inhibit the activity of NOS [30] and this contradiction may be due to the involvement of NO, and not ROS, in regulation of *Chs* expression. Therefore, in order to determine whether NO is involved in up-regulation of *Chs* in response to UV-B exposure, plants were sprayed with PTIO, a scavenger of NO [31], or L-NAME, an inhibitor of NOS, or its inactive form D-NAME as a control [32] (Fig. 2). The presence of PTIO or L-NAME, but not D-NAME, prevented the induction of *Chs* expression (Fig. 2), indicating that up-regulation of *Chs* by UV-B requires NO. Thus the effect of DPI on *Chs* induction, in response to UV-B radiation, is likely to be through the action of DPI on NOS [30], and not NADPH oxidase. Consistent with these findings, two compounds known to generate NO, GSNO and SNAP, were found to lead to an increase in *Chs* transcript levels in the absence of UV-B (Fig. 3), but had no effect on any other of the transcripts studied (data not shown). Thus these results show that NO is also important in regulating gene expression in response to UV-B. Further studies are, however, needed to indicate the importance of NO in resistance to UV-B radiation.

In contrast, neither PTIO nor L-NAME affected the up-regulation of *PR-1* transcript, as previously illustrated in response to pathogens [22]. This result, in combination with the SHAM data, indicates that both DPI and IM action on *PR-1* expression (Fig. 2) is likely to be through inhibition of

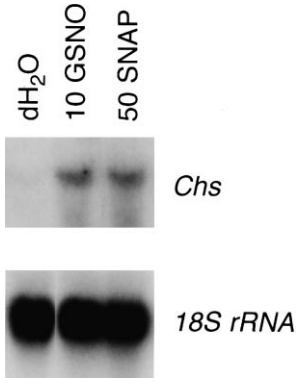

Fig. 3. Autoradiographs of *Chs* and *18S rRNA* transcript levels in wild type plants sprayed with water (dH_2O), 10 mM GSNO, 50 μM SNAP in the absence of supplementary UV-B. Quantitative results are presented in the table

Chemical	Concentration	Relative transcripts (percent transcripts in treated plants/transcripts in control plants)
Control	–	**100**
GSNO	5 mM	198 (17.7)
	10 mM	**324** (45.6)
SNAP	25 μM	267 (25.6)
	50 μM	**415** (74.3)

(see legend for Fig. 1 for more detail). The results are presented as percent transcript levels in plants treated with the chemical compared with plants treated under the same conditions but with 0.1% DMSO as the control (100% indicates no difference in transcript levels between plants treated with and without the chemical). Values are means (S.E.M.) of four independent experiments. Values in bold correspond to results shown in blot.

NADPH oxidase activity. Neither PTIO or L- and D-NAME affected the activity of NADPH oxidase (data not shown).

4. Conclusions

Our results strongly indicate that there are multiple sources of ROS produced in response to UV-B exposure (summarized in Fig. 4). We have shown that UV-B exposure leads to production of $O_2^{\bullet-}$ and that these anions are directly involved in the up-regulation of *PDF1.2*. On the other hand, H_2O_2 derived from $O_2^{\bullet-}$ mediates the up-regulation of *PR-1* and the down-regulation of *Lhcb* (Fig. 1). The source of $O_2^{\bullet-}$ involved in *PR-1* induction is NADPH oxidase, while it is peroxidase that generate the $O_2^{\bullet-}$ involved in up-regulation of *PDF1.2* (Fig. 2). Furthermore, the origin of ROS involved in regulation of the photosynthetic genes is clearly distinct from these two sources and our future work will be directed at identifying these alternative sources of ROS.

Signaling components involved in up-regulation of the *Chs* gene in response to UV-B exposure have been studied in some detail but the current data suggest involvement of NO (Figs. 2 and 3). An inhibitor of NOS, L-NAME, but not the inactive isomer, D-NAME, was effective at preventing the induction of *Chs* expression (Fig. 2) indicating that the source of NO in response to UV-B exposure is most likely to be NOS. To our knowledge this is the first study indicating a role for NOS and NO in UV-B responses and further work will be needed to test the role of NO in other responses to UV-B radiation. Future work will be directed to investigating how NO fits into the previously identified calcium- and calmodulin-dependent pathway involved in regulation of *Chs* by UV-B radiation [18,19].

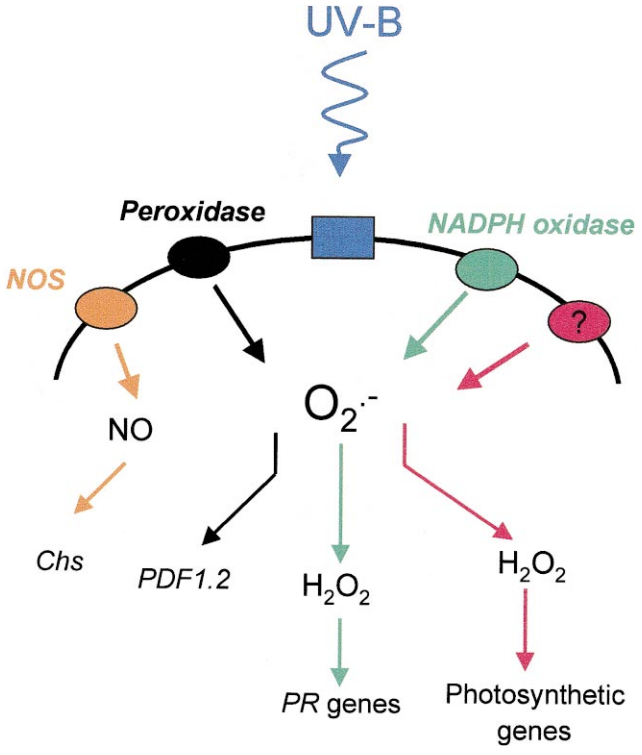

Fig. 4. A scheme of biochemical origin and nature of ROS and role of NO in regulation of gene expression in response to UV-B. $O_2^{\bullet-}$ = superoxide; H_2O_2 = hydrogen peroxide.

Previous studies have shown a role for ethylene, JA and SA in UV-B signaling [6,8] and in this paper we have identified NO, $O_2^{\bullet-}$ and H_2O_2 as important early upstream signaling components. In recent years our understanding of how UV-B regulates gene expression has increased greatly. As a result, it has become increasingly clear that some effects of UV-B are caused by non-specific absorption and hence damage, but others, for example effects on gene expression, are specific and mediated through a series of signal pathways. UV-B is likely to be perceived through a number of UV-B receptors, leading to increases in the activity of at least two enzymes involved in ROS generation, NADPH oxidase and a peroxidase, and NOS which leads to NO generation. These early signaling compounds are then likely to feed into a network of pathways which lead to the regulation of different sets of genes important in defense and tolerance to UV-B radiation (Fig. 4). Future studies will be directed at identifying further components within these various UV-B-induced signal transduction pathways.

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