Anoxia pretreatment protects soybean cells against H_2O_2-induced cell death: possible involvement of peroxidases and of alternative oxidase

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Abstract Anoxia followed by reoxygenation causes extensive damage to cellular components through generation of reactive oxygen intermediates. We examined cellular responses to oxidative stress after anoxia in cultured soybean or human fibroblast cells. Anoxia pretreatment protected soybean but not fibroblasts against H_2O_2 concentrations that induced programmed cell death in normoxic cells. H_2O_2 removal in anoxia-pretreated soybean cultures was faster. Protection was associated with increased action of alternative oxidase (AOX) and peroxidases. AOX inhibitors abolished the protective effect, while induction of AOX protected normoxic cells against H_2O_2. We propose that during anoxia, plant cells can prepare for reoxygenation injury by up-regulating their antioxidant capacity, and that AOX is involved in this process.

Key words: Hydrogen peroxide; Anoxia; Reoxygenation injury; Oxygen radical; Oxidative stress; Alternative oxidase

1. Introduction

Cycles of hypoxia or anoxia followed by reoxygenation occur frequently in the plant kingdom. Animal cells are protected from these fluctuations by circulation of blood through reoxygenating organs, such as lungs or gills. Organisms lacking such systems can experience wide changes in oxygen availability. In plants, oxygen deficiency develops following heavy rains and is associated with poor draining of the root area. Depletion of oxygen from the surrounding water is primarily caused by microbial respiration and can develop within 24 h [1]. Evaporation and drainage therefore expose plants to a reoxygenation injury.

In the animal systems, it has been suggested that major cell damage occurs during reoxygenation and is caused by the generation of reactive oxygen intermediates (ROI) after return of oxygen [2,3]. Reoxygenation-induced cell death was also described in root tips of soybean seedling [1]. Cycles of anoxia–reoxygenation occur routinely in rice, that is germinated anaerobically and then transferred to air [4]. In plants, the transition from hypoxia to oxidative stress may be very rapid, when combined with other ROI generating stresses, such as pathogen attack or many abiotic stresses [5]. Thus, plants have been under selective pressure to cope with oxidative stress.

Many studies have implicated ROI as a principal cause of programmed cell death (PCD) in animal, plant and even yeast cells [6–8]. In contrast to animal systems, little work has been done on the reoxygenation injury in plants. Increased survival of *Iris pseudacorus* [9] and of soybean seedlings [10] against flooding–reoxygenation cycle was correlated with increased superoxide dismutase (SOD) activity, indicating development of oxidative stress during reoxygenation. More recently, Biemelt et al. have reported activation of antioxidant responses following re-aeration of wheat roots [11]. Generation of superoxide is the main oxygen requiring step in formation of ROI, and is also the first step in the ROI production cascade. Once formed, the subsequent production of H_2O_2 and of other ROI, such as the hydroxyl radical, are oxygen independent.

Here, we examined the sensitivity of cultured soybean cells to reoxygenation injury. We show that pretreatment of cells in anoxic conditions renders them resistant to H_2O_2 by a number of processes, including activation of peroxidases and of alternative oxidase (AOX), a mitochondrial enzyme which reduces molecular oxygen to water [12]. Although AOX was previously thought to function in heat generation in thermogenic flowers, lately it was found that it has a protective role in different stresses [12]. It is noteworthy that induction of AOX activity by pyruvate reduced formation of H_2O_2 in isolated plant mitochondria [13], and that overexpression of AOX lowered the formation of ROI in the mitochondria [14]. Our studies suggest that AOX may have a protective role against oxidative stress following periods of anoxia.

2. Materials and methods

2.1. Cell culture and treatments

Soybean cells Williams82 were maintained as described [15]. Human fibroblasts A431 were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. For anoxia, cells were incubated in a sealed chamber, flushed and filled with argon gas and incubated in their usual conditions. All experiments were repeated at least four times with similar results. Cell death in soybean and human cells was assayed with Evan's blue or trypan blue, respectively [7]. The A_{600} numbers of Evan's blue represent absorbance without background subtraction (which was OD 0.05–0.08). Percentage of dead cells was estimated by microscopic observation of stained cells.

2.2. Enzyme activity measurements

Half life of H_2O_2 was measured by a starch/I_2 procedure [16]. Peroxidase activity was assayed in soybean cell extracts, prepared in 50 mM Tris–acetate buffer (pH 7.5) and 2 mM EDTA. The reaction mixture contained 30 mM NaOAc pH 5, 0.3 mM EDTA, 0.03 mM guiacol and 1 mM H_2O_2; absorbance was measured at 485 nm [17]. Cell wall bound peroxidases were released by incubation in 0.2 M $CaCl_2$ [18]. Peroxidase isozymes were separated by native 10% poly-

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acrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and assayed by enhanced chemiluminescence (ECL) according to the manufacturer's (New England Biolabs) instructions. Total SOD activity was measured by the indirect spectrophotometric method of cytochrome *c* oxidation according to [19].

2.3. RNA analysis

RNA was extracted with Qiagen RNAeasy Plant kit and analyzed by Northern blotting. GST and GPX probes were described in [20], SOD (tobacco cytosolic SOD cDNA) was from B. Zelinskas (Rutgers University), TR (soybean thioredoxin cDNA) was from M. Bhattacharyya (Noble Foundation, OK, USA), LOX (soybean lipoxygenase cDNA) from J. Polacco (University of Missouri).

2.4. Mitochondria isolation and AOX detection

Mitochondria were isolated according to [21]. Briefly, cells were resuspended in 350 mM mannitol, 30 mM MOPS pH 7.5, 1 mM EDTA, 4 mM cysteine, 0.2% bovine serum albumin (BSA), 0.6% polyvinylpolypyrrolidone (0.5 g fresh weight in 10 ml). Cells were homogenized and filtered through four layers of Miracloth™ (Calbiochem, USA). Cell debris was removed by centrifugation for 2 min at $6000\times g$. Mitochondria were pelletted by centrifugation for 5 min at $25\,000\times g$ and washed three times in 300 mM mannitol, 20 mM MOPS pH 7.5, 1 mM EDTA and 0.2% BSA. Proteins were separated by SDS–PAGE, transferred to nitrocellulose (Millipore) and probed with monoclonal anti-AOX antibodies [12]. Blots were developed by ECL with goat anti-mouse conjugated to horseradish peroxidase.

3. Results

3.1. Induction of cell death by oxidative stress following anoxia

Since plants are naturally exposed to anoxia during periods of temporary flooding, we compared resistance of cultured soybean and human A431 cells against post-anoxic oxidative stress. Cells were placed in a sealed chamber and the atmosphere inside was replaced with argon gas. After 16 h incubation, they were re-exposed to ambient air and challenged with 5 (soy) or 0.5 mM (human) of hydrogen peroxide. The H_2O_2 dosage was determined by preliminary experiments in normoxic conditions. The selected concentration caused cell death in approximately 50% of cells in each culture, and was in line with published data [20,22]. Remarkably, soybean cells pretreated in anoxic environment were almost completely protected from the H_2O_2-induced cell death (Fig. 1A). In contrast, increased death occurred in the human fibroblasts (Fig. 1B), which is in agreement with the damage caused by anoxia/reoxygenation described in animal systems [23].

We next examined the time course of protection development during anoxia. H_2O_2-induced cell death declined according to the period of anoxia pretreatment and reached close to 100% protection after 18 h (Fig. 2A), suggesting involvement of active metabolic processes. The 'hardened' state following anoxia treatment remained for at least 1 h after reoxygenation. In order to analyze whether the protection against H_2O_2-induced PCD stemmed from intracellular changes, or resulted from molecules secreted into the medium, we exchanged the culture media between argon and air grown cells. Cells preincubated in anoxic conditions remained resistant to H_2O_2 stimulation, even when incubated in the medium from normoxic cells (Fig. 2B). Conversely, cells cultured under normoxic conditions and then placed into the medium of the anoxia grown cells died. Thus, the protecting factor(s) accumulated inside the cells.

3.2. H_2O_2 scavenging in anoxic versus normoxic cultures

The role of ROI scavenging was tested by measuring the

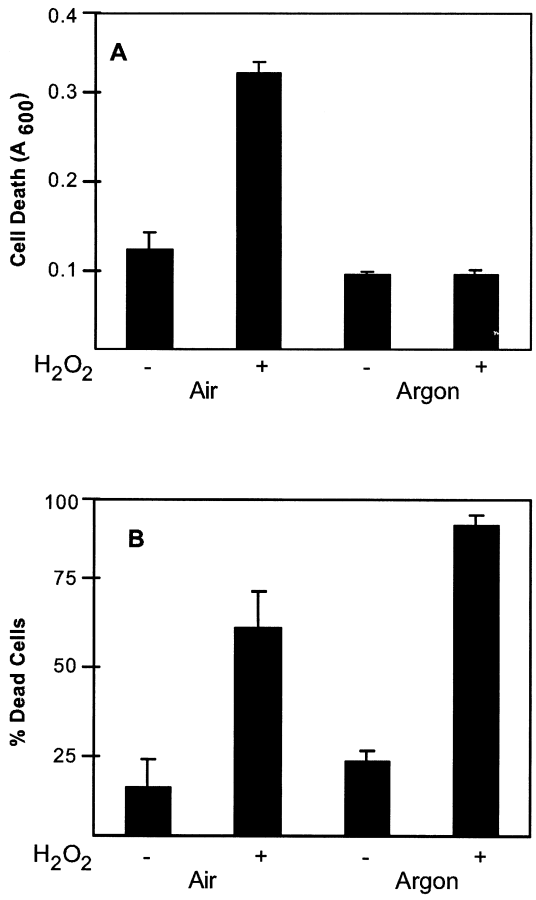

Fig. 1. The effect of anoxia on H_2O_2-induced cell death. Suspension cultured soybean cells (A) or human A431 fibroblasts (B) were preincubated in argon atmosphere and then challenge with 5 mM or 0.5 mM H_2O_2, respectively. Cell death was measured 9 h later with Evan's blue (for soybean cells) or trypan blue (human fibroblasts), longer incubation times did not result in significant increase in cell death. The degree of cell death in plant and human cultures under the normoxic conditions was determined by counting stained cells (100–200 cells) under a microscope. The selected concentrations caused cell death in 50–65% of cells from both species. The degree of cell death in untreated cultures was less than 5%.

H_2O_2 decomposition in cells pretreated in anoxic or normoxic environments (Fig. 3A). The concentration of H_2O_2 in the air grown culture decreased by 10% during the first 2 min, while more than 50% of the H_2O_2 was removed from the anoxic cells, during the same time interval. No difference in H_2O_2 half life was seen in the human fibroblasts (Fig. 3A).

The relative contribution of catalase and peroxidase enzymes in H_2O_2 removal was assayed by addition of specific inhibitors. While addition of up to 5 mM 3-aminotriazole, an inhibitor of catalase [24], had no effect on the rate of H_2O_2 removal, addition of 8 mM sodium azide, an inhibitor of peroxidases [25], inhibited H_2O_2 scavenging by 50% (data not shown), suggesting existence of additional alternative mechanisms for H_2O_2 detoxification.

The involvement of peroxidases was further investigated by analyzing the extracellular (cell wall bound) and intracellular peroxidases activity. Cells were pretreated in anoxic conditions as before and protein extracts from cytosol or cell wall were assayed with guiacol substrate. Approximately 30% increase in peroxidase activity was seen in total and in cell wall

extracts (data not shown). Analysis of individual peroxidase isozymes by native PAGE did not detect induction of new peroxidases during anoxia, although increase in activity of one of the peroxidase bands was seen (Fig. 3B). Measurements of total SOD activity in protein extracts from anoxic and normoxic cells showed insignificant differences (85.5 ± 5.7 and 84.4 ± 6.1 U/mg protein [19], respectively), suggesting that there was no general induction of antioxidant responses during anoxia.

We also analyzed mRNA levels of a number of antioxidant enzymes. No increase in the mRNA of glutathione *S*-transferase (GST), glutathione peroxidase (GPX), TR or SOD was detected. In fact, strongly decreased transcription of GST and GPX genes, and mild reduction in transcription of SOD and TR genes during the anoxia were detected (Fig. 4). On the other hand, lipoxygenase mRNA strongly increased during anoxia, indicating specific changes in regulation of gene expression.

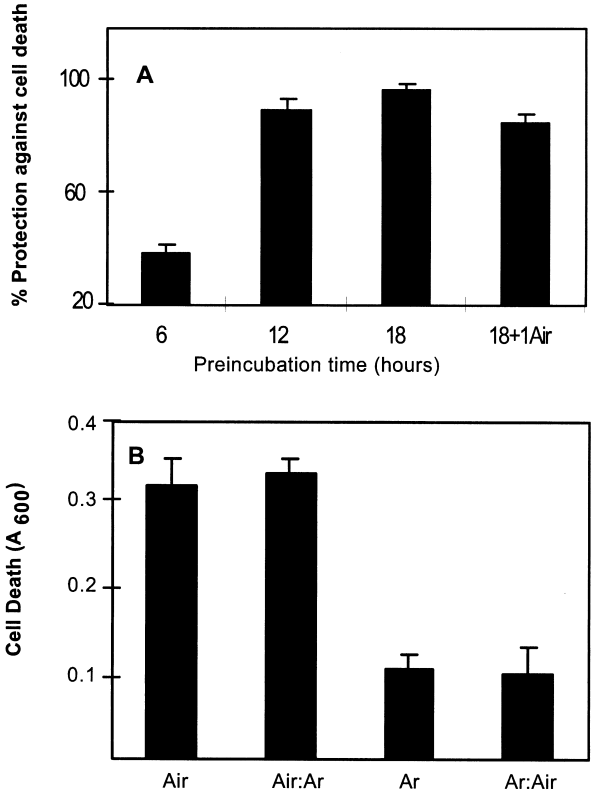

Fig. 2. Timing and localization of anoxia-induced protection against oxidative stress. (A) Development of protection against oxidative stress. Suspension cultured soybean cells were incubated in anoxic atmosphere for the indicated time period and then challenged with 5 mM H_2O_2. Cell death was measured 9 h later. 18+1Air, re-aeration of the anoxic cells for 1 h prior to H_2O_2 challenge. The percentage of dead cells was comparable to Fig. 1. (B) Determination of the anoxia-induced protective activity in cytosol and extracellular medium (apoplast). Soybean cells were incubated in anoxic (Ar) or normoxic (Air) atmosphere. After 16 h, the media of two cultures were exchanged and cells challenged with 5 mM H_2O_2. Cell death was measured 9 h later. Air:Ar, air-preincubated cells in medium from argon-incubated cells; Ar:Air, argon-preincubated cells in medium from air-incubated cells; Air and Ar cultures grown continuously in air or argon.

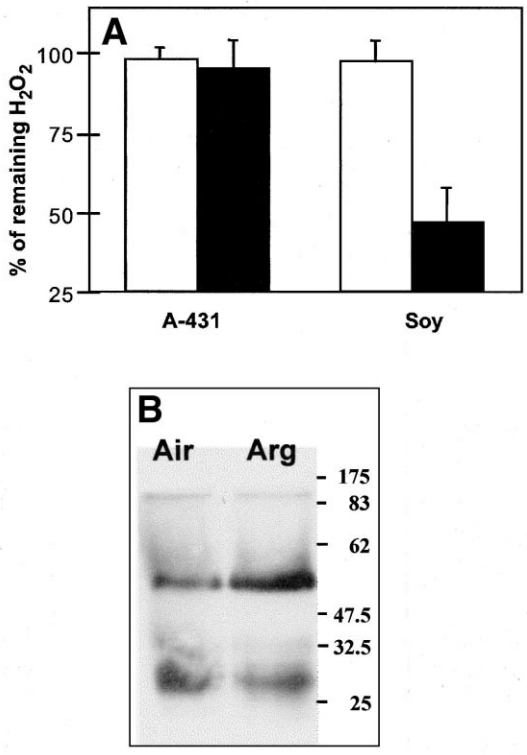

Fig. 3. The effect of anoxia on H_2O_2 scavenging in soybean and human cells. (A) Cultured soybean cells and human fibroblasts were incubated in anoxic (black bars) or normoxic (white bars) atmosphere for 16 h and 1 mM H_2O_2 was then added to the cultures. The concentration of H_2O_2 remaining in the medium was measured after 2 min. A similar trend (although smaller) in H_2O_2 removal between the argon and air-pretreated cultures was also recorded after 5 and after 10 min, not shown. (B) Analysis of total cellular peroxidases. Proteins from cells grown under normoxic or anoxic (Arg) conditions for 16 h were separated by native 10% PAGE, transferred to nitrocellulose and analyzed by ECL.

3.3. Involvement of AOX in protection from PCD caused by oxidative stress

In animal systems, the main site of ROI production during reoxygenation occurs in mitochondria [26]. The plant AOX has been proposed to function in limiting the level of ROI in the mitochondria [27–29]. To test whether AOX was involved in the anoxia-induced protection, we added prior to H_2O_2 treatment two AOX inhibitors, salicylhydroxamic acid (SHAM) or propyl gallate. As shown in Fig. 5A, both SHAM and *n*-propyl gallate completely abolished the protective effect of anoxia. None of the inhibitors, however, affected the cell survival without the H_2O_2 addition, indicating that the degree of oxidative stress did not pass the PCD threshold [20]. A possible H_2O_2 scavenging activity of SHAM was ruled out by testing the half life of H_2O_2 in the presence of SHAM (data not shown). The role of AOX in protection from H_2O_2-triggered cell death was further examined by stimulation of AOX expression in air grown cells with antimycin A (AA) [12], which resulted in strong protection of normoxic cells (Fig. 5B).

Two major control mechanisms of AOX activity are the amount of AOX protein and reducing environment, such as occurs during hypoxia [12]. AOX levels were tested in mitochondria isolated from anoxia-pretreated and from normoxic cells and analyzed together with cytosolic proteins by Western

blotting. AOX protein was detected only in the mitochondria and its expression was stimulated by anoxia (Fig. 5C). This result is in line with increased transcription of AOX gene in cultures treated with AA, or creating a reduced environment by addition of cysteine to culture medium [12].

4. Discussion

While in animals hypoxia or anoxia are always associated with pathological situations, in plants these conditions can be quite common, e.g. during flooding. Oxygen deprivation results in saturation of available electron acceptors and leads to the accumulation of the reduced NAD(P)H molecules, severely reducing the energy balance. Changes in metabolism and in gene expression caused by oxygen deficiency have been documented both in animal and plant cells [30–33]. Many plant genes induced by hypoxia/anoxia participate in energy generation [31,32].

However, much of the damage to cells and tissues occurs during reoxygenation, and is associated with generation of free oxygen radicals [11,26]. In animal systems, conflicting results regarding the requirement for oxygen for PCD were obtained [34,35]. In plants, the relationship between requirement for oxygen and PCD was studied with respect to pathogen-induced hypersensitive response, where it was found that oxygen was required for PCD [36]. These results are in line with studies in plants and animals showing inhibition of PCD by antioxidants [37].

Early studies by Monk and co-workers showed that plant roots exposed to hypoxic conditions induced synthesis of SOD [9]. Recently, these observations were expanded by Biemelt et al. who showed that in wheat roots there is a coordinated activation of antioxidant enzyme activity, such as ascorbate peroxidase that restores the cellular redox state [11]. In order to study plant oxidative stress defences that precede reoxygenation, as opposed to antioxidant defences induced during the reoxygenation period, anoxic cells were treated directly with PCD-inducing concentrations of hydrogen peroxide [7,15]. Thus, the analysis of antioxidant defences in response to reoxygenation, as performed by Biemelt et al. [11], involved mainly the defence responses to oxidative stress formed during the latter period. Our work focused on the antioxidant responses formed during the anoxia period, before the oxidative stress produced during reoxygenation. These defence mechanisms may be instrumental in preventing PCD in the first hours of re-aeration, especially when occurring together with additional ROI producing stresses, such as strong light, chilling or pathogen infection [5,38].

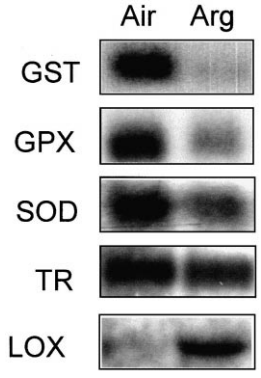

Fig. 4. The effect of anoxia on antioxidant gene expression. Analysis of mRNA levels of antioxidant genes by Northern blotting in cultures incubated in anoxic (Arg) or normoxic (Air) conditions for 16 h. The blots were probed with the following gene fragments: GPX, glutathione peroxidase; SOD, superoxide dismutase; GST, glutathione *S*-transferase; TR, thioredoxin; LOX, lipoxygenase.

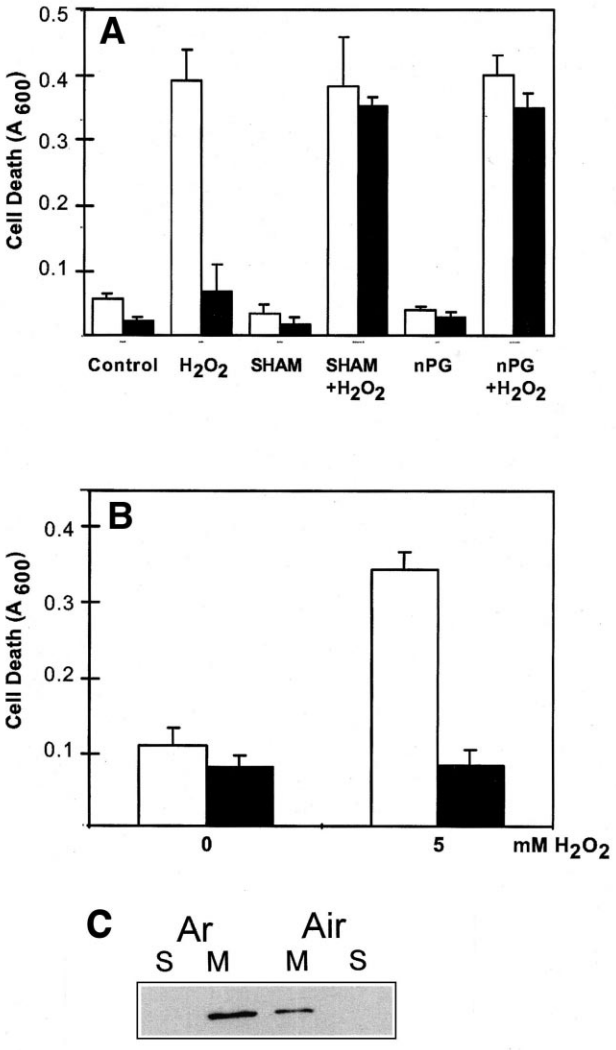

Fig. 5. The involvement of AOX in protection of cells from H_2O_2-induced PCD. (A) Cultured soybean cells were incubated in normoxic (white bars) or anoxic atmosphere (black bars) for 16 h and challenged with 5 mM H_2O_2. SHAM (2 mM) or *n*-propyl gallate (0.25 mM, nPG) were added prior to H_2O_2 and cell death measured after 9 h. (B) Soybean cells were incubated in normoxic conditions (white bars) with 25 μM AA (black bars) for 16 h and then challenged with 5 mM H_2O_2. Cell death was measured 9 h later. (C) Induction of AOX in anoxia-treated cells. Suspension cultured cells were incubated in argon (Ar) atmosphere for 16 h. Equal amounts of extracted proteins from mitochondria (M) and cytosolic supernatant (S) were analyzed by immunoblotting using monoclonal antibodies against AOX.

Our results point to at least two mechanisms induced during anoxia, that provide an overall antioxidant effect, and protect from cell death. Detoxification of H_2O_2 in anoxia-pretreated cells (Fig. 3A) was faster than in normoxic cultures. The measurements of H_2O_2 half life reflect the ability of cells to scavenge ROI, as opposed to tolerance or repair of the ROI-induced damage. The rapid disappearance of H_2O_2 was

in part due to elevated peroxidase activity, but since sodium azide, a strong inhibitor of peroxidases, only partially inhibited the H_2O_2 detoxification, additional mechanisms must be involved. It is noteworthy that while total peroxidase activity increased following anoxia, the transcription of the GPX gene was lower in anoxic cells.

In contrast to earlier studies by Monk et al. [9], we did not observe induction of several antioxidant genes in our system (including SOD, GPX, TR) (Fig. 4), possibly because of differences in experimental systems or in plant species used. The down-regulation of antioxidant genes was probably due to reduction of many biosynthetic activities that take place during anoxia [1]. Interestingly, we found induction of lipoxygenase transcription during anoxia. It is not clear whether the lipoxygenase was activated for lipid breakdown to provide ATP and/or whether it contributed to protection against H_2O_2. It was shown that lipoxygenase reaction product, linoleic acid, reduced H_2O_2 generation in potato mitochondria [39,40].

Substantial protection against H_2O_2 stress after anoxia seemed to be provided by elevated activity of AOX, which is a function of both higher levels of AOX protein (Fig. 5C) and of the reducing conditions that persist in the anoxic cells. The involvement of AOX in protection from H_2O_2-induced cell death is supported by experiments with SHAM and *n*-propyl gallate, two widely used inhibitors of AOX [41], which completely suppressed the anoxia-mediated effect on cell death (Fig. 5A). It should be noted that although at higher concentrations (20 mM) SHAM may also inhibit peroxidases, at a 10-fold lower concentration used here, its effect on peroxidases was minimal. Furthermore, treatment with AA, an inducer of AOX expression [12], under normoxic conditions resulted in a similar protection against H_2O_2-induced PCD.

Hence, during reoxygenation, the electron flow through the cytochrome *c* pathway may become saturated, redirecting the 'overflow' of electrons from the common ubiquinone pool to the alternative pathway [42]. Since the affinity of the soybean AOX towards oxygen is more than 10-fold lower than of the cytochrome oxidase, it is specifically relevant for coping with the overflow from the cytochrome oxidase pathway [43]. Moreover, the release of cytochrome *c* from the mitochondrial membranes that takes place during oxidative stress ([44] and our unpublished results) further increases the flow of electrons through the alternative pathway. Thus, the higher levels of AOX produced during the anoxia 'prepare' the plant cells to cope with electron overflow during reoxygenation. It is noteworthy that exposure of soybean seedlings to anoxia for 5 h resulted in higher resistance to reoxygenation injury than seedlings kept under anoxic conditions for 1 h, suggesting induction protecting processes during the anoxia [1].

Our results are in line with observed induction of AOX gene by H_2O_2, and support its role in oxidative stress [12,45]. Further work is needed to identify the molecular mechanism of how AOX, which was shown to lower ROI generation [14,27], also confers protection against the extracellular H_2O_2-triggered PCD. While under our experimental conditions H_2O_2-induced cell death could be prevented solely by AOX activity, it is reasonable to assume that in a variety of natural situations (associated with reoxygenation), other components of the antioxidant defences, such as induction of specific antioxidant genes or the activation of cell wall peroxidases, will have important roles. As suggested by Crawford and Braendle, tolerance or resistance to anoxia during diverse environmental conditions could be a combined result of a number of strategies [46].

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