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Uncoupling effect of lipid peroxidation in spinach thylakoids exposed to peroxy radicals generated by 2,2'-azobis (2-amidinopropane) dihydrochloride

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Abstract

In this study, we characterized how lipid peroxidation alters the functionality of spinach thylakoids exposed to peroxy radicals generated by the azo compound 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH). Incubation of thylakoids in presence of different concentrations (0 to 200 mM) of AAPH inhibited the formation of ΔpH ($IC_{50} \approx 1.5$ mM) estimated by the quenching of 9-aminoacridine fluorescence (Q9-AA). The Q9-AA inhibition was correlated ($R^2=0.98$) to the extent of lipid peroxidation determined by the accumulation of thiobarbituric acid reactive substances (TBARS). Much higher AAPH concentrations were required to inhibit the maximum (F_v/F_m) and effective ($\Delta F/F_m'$) photochemical efficiencies of photosystem II ($IC_{50} \approx 120$ mM and 50 mM respectively), indicating that moderate lipid peroxidation caused the uncoupling of spinach thylakoids. This was confirmed by the 62 % stimulation of the O_2 uptake rates measured without the artificial uncoupler NH_4Cl when the AAPH concentrations increased from 0 to at 20 mM, reaching similar values to the rates measured in presence of NH_4Cl . Above 20 mM AAPH, the O_2 uptake rates measured with and without NH_4Cl declined similarly to the decrease of $\Delta F/F_m'$. These results suggest that the increased H^+ -leakiness of thylakoid membranes could be one of the primary effects of oxidative stress.

Key words

9-aminoacridine; AAPH; ATP synthesis; oxidative stress; chlorophyll fluorescence; malondialdehyde; non-photochemical quenching; photosynthesis; photoinhibition; reactive oxygen species.

Abbreviations:

AAPH: 2,2'-azobis (2-amidinopropane) dihydrochloride

F_v/F_m : maximum photochemical efficiency of photosystem II

$\Delta F/F_m'$: effective photochemical efficiency of photosystem II

MDA: malondialdehyde

Q9-AA: quenching of 9-aminoacridine fluorescence

PSII: photosystem II

ROS: reactive oxygen species

TBARS: thiobarbituric acid reactive substances

Introduction

Light absorption in the chloroplasts inevitably leads to the production of reactive oxygen species (ROS) due to pigment excitation and electron transfer reactions occurring in presence of high O_2 concentrations (Dietz et al. 2016; Noctor et al. 2018). These ROS can be useful for the plants by safely dissipating energy, thanks to efficient ROS scavenging systems (Noctor and Foyer 1998), and also, by acting as signal molecules involved in metabolic adjustments and plant acclimation to the ambient conditions (Mittler 2002; Apel and Hirt 2004; Foyer et al. 2017). However, a diversity of environmental stresses can exacerbate the effect of high light intensities by increasing the production of ROS to a level that overwhelms the capacity of the scavenging systems. The excess of ROS can affect plant performance by causing oxidative damages to vital cellular components such membranes, enzymes, and genes (Dietz et al. 2016).

Excessive ROS production in the chloroplast results from a cascade of reactions, starting from limitations of the CO_2 assimilation relative to the reducing power generated by the light reactions. The resulting depletion of the terminal electron acceptor $NADP^+$ promotes the Mehler reaction at the photosystem I (PSI) acceptor side, where an electron is transferred to O_2 to form the anion superoxide O_2^- (Asada 2006). This ROS normally dismutates to H_2O_2 , less toxic but which can be transformed via Fenton reaction to the highly toxic hydroxyl radical ($HO\bullet$). Electron leakage to O_2 can also occur at the acceptor side of photosystem II (PSII), where the resulting O_2^- dismutates to H_2O_2 , which is then reduced by the non-heme iron to $HO\bullet$ (Pospíšil 2016). Limitation of CO_2 assimilation relative to the light reactions also causes the slowdowns of pigment de-excitation and electron transfer, thereby increasing the risk of singlet 1O_2 formation in PSII when O_2 interacts with triplet chlorophylls formed either in the antenna directly from excited singlet chlorophyll, or in the reaction center after charge recombination between $Pheo^-$ and $P680^+$ (Dietz et al. 2016). Finally, slow electron transfer on the PSII donor side increases the lifetime of $TyrZ\bullet$ and $P680^+\bullet$. These two strong oxidants can extract electrons from nearby lipids and proteins, creating unstable lipid alkyl radical ($L\bullet$) and protein ($P\bullet$) radicals (Pathak et al. 2017).

One of the primary targets of the chloroplastic ROS is the thylakoid membrane due to its highly polyunsaturated fatty acids prone to lipid peroxidation (Halliwell 1987). $HO\bullet$, the main initiator of lipid peroxidation, can extract a H atom from a methylene group ($-CH_2-$) of a fatty acid to produce an alkyl radical $L\bullet$, which reacts rapidly with O_2 to create a lipid peroxyl radical ($LOO\bullet$) (Yalcinkaya et al. 2019). These radicals were detected for example in thylakoids from plants exposed to atmospheric ozone (Gupta et al. 1991) or UV-B (Hideg and Vass 1996). Then, $LOO\bullet$ can extract a H atom from an adjacent LH, creating another $L\bullet$. This chain of reactions continues until it is terminated with the reduction of $LOO\bullet$ to hydroperoxides ($LOOH$) by α -tocopherol or other antioxidants (Munné-Bosch 2005). Besides $HO\bullet$, 1O_2 can also create a $LOOH$ by adding an O_2 to a C atom adjacent to a double bond of polyunsaturated fatty acid. $LOOH$ can further react to form secondary products (reactive carbonyl species) such as malondialdehyde (MDA), a well-known marker of lipid peroxidation (Pospíšil and Yamamoto 2017; Yalcinkaya et al. 2019).

Lipid peroxidation and its secondary products can affect the functions of the thylakoids firstly by damaging the proteins involved in the photosynthetic electron transport, notably those in the PSII reaction center (D1), in the light-harvesting complex LHCII, and the peripheral proteins on the PSII donor side (Yamauchi et al. 2008; Pospíšil and Yamamoto 2017). Dysfunction of

oxidized proteins can result from subtle conformational changes, or from more extensive damages such as cleavage and aggregation (Pospíšil and Yamamoto 2017). Concomitant lipid peroxidation and inhibition of photosynthetic electron transport are commonly observed in thylakoids from plants exposed to diverse stresses such as cadmium (Prasad and Zeeshan 2005), drought (Zlobin et al. 2019), Mg deficiency (Farhat et al. 2015), and waterlogging (Yan et al. 2018).

Besides electron transport, ATP synthesis is the other fundamental function of the thylakoids that is susceptible to lipid peroxidation. Inhibitions of ATP synthesis have also been observed in plants exposed to a variety of physiological and environmental stresses (Ortiz-Lopez et al. 1991; Tezara et al. 1999; Lawlor and Cornic 2002; Allakhverdiev et al. 2008; Ma et al. 2016). However, only few studies reported lipid peroxidation (MDA accumulation) concurrently to the inhibition of ATP synthesis during oxidative stresses induced by anoxia/re-aeration (Rawlyer et al. 2002), salt (Zheng et al. 2009; Yousuf et al. 2017) and H₂O₂ (Dubovskaya et al. 2007). Furthermore, none of these studies identified the direct cause of ATP synthesis inhibition, which could be the inactivation of the F-ATPase, the inhibition of the photosynthetic electron transport, or the inhibition of the formation of pH gradient (Δ pH) due to an increase of membrane leakiness to H⁺. In that latter case, a loss of membrane integrity due to lipid peroxidation would cause the uncoupling of the thylakoids, i.e. photosynthetic electron transport functioning independently of the activity of ATP synthase. In this condition, H⁺-leaky thylakoid membranes prevent the acidification of the lumen, thereby inhibiting ATP synthesis while allowing electron transport to proceed without the restriction of the plastoquinol oxidation in the cytochrome *b₆f* imposed by the low luminal pH (Dean and Miskiewicz 2003). Besides the inhibition of ATP synthesis, the physiological consequences of this uncoupling would be multiple: the collapse of non-photochemical quenching qN, leading to the overreduction of the electron transport chain, thereby increasing the rates of ROS formation and photoinhibition.

A possible uncoupling effect of lipid peroxidation has not been yet demonstrated in thylakoids. Despite that chloroplasts and mitochondria largely differ in terms of electrons carriers and lipid composition (Schwertner and Biale 1973), such uncoupling in thylakoids is suggested by observations of simultaneous lipid peroxidation and uncoupling in liver mitochondria isolated from mice that received intraperitoneal injection of a peroxy radical initiator, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) (Kanno et al. 1994). This water-soluble azo compound is widely used as a model reagent to mimic oxidative stress due to its ability to generate peroxy radicals at a constant rate at physiological temperatures (Niki 1990). It is used to induce oxidative stress in various *in vivo* and *in vitro* biological systems, such as rat lungs (Gomes et al. 2016), zebrafishes (Wang et al. 2018), chicken embryos (He et al. 2013), human erythrocytes (Zou et al. 2001) and to induce peroxidation of low-density lipoproteins (Yoshida et al. 2004) and of phosphatidylcholine liposomes (Mendez-Sanchez et al. 2009). It is also used in ORAC (oxygen radical absorbance capacity) assays to evaluate the antioxidant capacity of different compounds to protect target molecules (e.g., phycoerythrine, fluoresceine, pyrogallol red) against peroxy radicals (Prior et al. 2005). In contrast to these numerous examples, the effects of AAPH on plants are rare. For example, AAPH stimulated the ROS defense system in plant tissue cultures while repressing a *cab* gene encoding the chlorophyll a/b-binding protein of the light-harvesting antenna complex (Ohlsson et al. 1995; Henkow et al. 1996).

Based on the above considerations, we aimed in this study to demonstrate the uncoupling effect of lipid peroxidation in spinach thylakoids exposed to peroxy radicals generated by different concentrations of AAPH, by comparing 1) the relative susceptibilities of photosynthetic electron transport and ΔpH to lipid peroxidation, and 2) the effects of AAPH on O_2 uptake measured in presence and in absence of the artificial uncoupler NH_4Cl . We observed that the extent of lipid peroxidation was closely related to the inhibition of the light-induced ΔpH , which occurred at much lower concentrations than those required to inhibit significantly the photosynthetic electron transport. The uncoupling effect of AAPH, indicated by the presence of significant electron transport in absence of ΔpH , was confirmed by the stimulation at moderate AAPH concentrations of the O_2 uptake measured in thylakoids resuspended in absence, but not in presence, of the artificial uncoupler NH_4Cl .

Materials and Methods

Thylakoid isolation and treatments

Thylakoids were isolated from fresh market spinach as in Whitmarsh and Ort (1984). Thylakoids were kept on ice in darkness and were used within 6-8 hours after their isolation. $\text{Chl}a+b$ concentration was estimated in 80% acetone according to Porra et al. (1989). For the treatments, the thylakoids were resuspended at a concentration of $10 \mu\text{g Chl}a+b \text{ mL}^{-1}$ in an assay medium containing 200 mM sorbitol, 25 mM tricine- NaOH (pH 8.0), 5 mM MgCl_2 , and 0.5 mg mL^{-1} BSA (fatty acid free). Thylakoids were incubated at 33°C for 20 min in presence of different AAPH concentrations ranging from 0 to 200 mM.

Quantum yields of photosystem II photochemistry

The chlorophyll-*a* fluorescence (ChlF) was measured with a XE-PAM fluorimeter (Walz, Germany) to estimate the maximum (F_v/F_m) and effective ($\Delta F/F_m'$) quantum yields of photosystem II photochemistry according to Klughammer and Schreiber (2008). Two mL of AAPH-treated thylakoids were placed in a quartz cuvette, and methyl viologen (MV) (0.3 mM) was added as an electron acceptor. Blue modulated (2 Hz) and non-actinic excitation was obtained by placing BG18, KV418, and neutral density filters in front of the Xe-flash lamp connected to the cuvette via a quartz rod. ChlF was collected via a plastic rod by a photodetector positioned at 90° from the Xe-flash lamp, and protected with a RG715 Schott filter. The saturating light pulse and the actinic light ($90 \mu\text{moles photons m}^{-2} \text{ s}^{-1}$) were directed by an optic fiber placed just below the cuvette.

Estimation of light-induced pH gradient

Light-induced pH gradient (ΔpH) in spinach thylakoids was estimated by the quenching of the 9-aminoacridine fluorescence (Q9-AA) (Schuldiner et al. 1972). This fluorescence was measured

with the Xe-PAM fluorometer in the same configuration as for the ChlF measurements, except the presence of DUG11 + UG11 UV filters in front of the Xe-flash lamp, and the blue-green KV418 and BG18 filters in front of the photodetector. Two mL of treated thylakoids were placed in a quartz cuvette in presence of MV (0.3 mM). After the recording of a baseline (F0), 1 μ M 9-aminoacrine was added to the cuvette and the unquenched signal of 9-aminoacrine fluorescence (F1) was measured in darkness. Then, the actinic light (90 μ mole photons $\text{m}^{-2} \text{s}^{-1}$) was turned on for 30 sec, sufficient to induce a quenched fluorescence level (F2). The Q9-AA related to Δ pH was calculated as $\text{Q9-AA} = 100 \times (\text{F1-F2})/(\text{F1-F0})$.

Estimation of lipid peroxidation with the TBARS assay

The thiobarbituric acid reactive substances (TBARS) assay is the most common estimation of lipid peroxidation in simple biological systems (Hodges et al. 1999). It is based on the absorbance at 532 nm of the complex formed between thiobarbituric acid and mainly malondialdehyde, one of the secondary products of lipid peroxidation.

At the end of the 20-min light treatment, 0.1 mL of trichloroacetic acid (TCA) was added to 5.0 mL of a suspension of AAPH-treated thylakoids to stop the peroxidation reaction. Then, 2 mL of the thylakoid suspension were transferred to a test tube containing 2 mL TCA 30%, 0.2 mL HCl 5 M, and 2 mL of thiobarbituric acid (TBA) 0.75%. For each sample, the mixture was heated in a dry bath at 90°C for 45 min, and then the samples were centrifuged for 10 min at 10 000 g and 4°C. The absorbance at 532 nm of the supernatant was measured, from which the non-specific absorbance at 600 nm was subtracted. The concentrations of MDA equivalents were calculated using an extinction coefficient of 155 mM^{-1} (Hodges et al. 1999).

Rates of photosynthetic electron transport measured by O₂ uptake

To complement the estimations of photochemical efficiency of photosystem II by ChlF, we also determined the rates of photosynthetic electron transport by O₂ uptake measured with a Clark electrode connected to an Oxylab system (Hansatech, England). Immediately after the AAPH treatment, thylakoids were placed in the reaction vessel of a DW2/2 electrode chamber connected to a thermoregulated circulating water bath adjusted at 25°C. MV (0.3 mM) was added to the thylakoids maintained in darkness to record a drifting signal when present. Then, the thylakoids were illuminated with a saturating white light and the rate of O₂ uptake was measured. After about 30 seconds, 1 mM NH₄Cl was added to the thylakoids and the rate of O₂ uptake by uncoupled thylakoids was measured.

Statistical Analysis

Data are the means $\pm$ SEM calculated from five measurements made from five different thylakoid preparations, except for O₂ evolution where n= 3.

Results

In most experiments with AAPH such as ORAC assays, peroxy radicals are generated at 37° C, a temperature too high to ensure the stability of the thylakoids. Preliminary experiments were needed to determine the temperature and the duration of the treatments that optimize the production of peroxy radicals by AAPH while maintaining the integrity of control thylakoids (without AAPH). To this end, we monitored the time-dependent bleaching of pyrogallol red (López-Alarcón and Lissi 2005) induced by the peroxy radicals generated by AAPH at different temperatures. The absorbance of pyrogallol red linearly decreased at a rate that was directly temperature-dependent between 29° and 37° C (Fig. S1). On the other hand, we observed that the quenching of the 9-aminoacridine fluorescence (Q9-AA) in thylakoids without AAPH was stable over 30 min at temperatures from 29° and 33° C, but markedly decreased after 10 min at 35° C (Fig. S2). Therefore, a temperature of 33° C and a duration of 20 min were used for the AAPH treatments. The production rate of peroxy radicals at 33° C was 66% relative to the rate at 37° C (Fig. S1B).

We then verified the effectiveness of the peroxy radicals generated by AAPH to induce lipid peroxidation in spinach thylakoids under the selected experimental conditions (33° C x 20 min). We detected the presence of TBA-MDA complexes (TBARS) at sub-millimolar AAPH concentrations, and their values increased exponentially with higher concentrations to reach a maximum near 100 mM AAPH (Fig. 1).

The consequences of lipid peroxidation on the main functions of spinach thylakoids were assessed first by measuring the effects of different AAPH concentrations on the ΔpH estimated by the Q9-AA (Fig.2). Inhibition of Q9-AA was already apparent at 0.1 mM AAPH. Q9-AA values decreased exponentially with increasing AAPH concentrations, and it was abolished between 10 and 20 mM AAPH. The AAPH concentration required to inhibit by 50% the initial values (IC_{50}) of Q9-AA was estimated at 1.5 mM. Interestingly, the AAPH-induced inhibition of Q9-AA is closely correlated ($R^2 = 0.981$) to the presence of TBARS in spinach thylakoids treated with different AAPH concentrations (from 0 to 10 mM) (Fig.3).

Lipid peroxidation could inhibit the formation of light-induced ΔpH either as a consequence of an increase in membrane leakiness to H^+ , or because of an inhibition of the photosynthetic electron transport. The most likely of these two possibilities was determined by measuring the maximum (F_v/F_m) and effective ($\Delta F/F_m'$) quantum yields of PSII photochemistry (Fig. 4). Both quantum yields remained unaffected up to 2 mM AAPH, but their values decreased exponentially at higher concentrations. The IC_{50} values were estimated at 120 mM for F_v/F_m , and 40 mM for $\Delta F/F_m'$.

The much lower IC_{50} found for light-induced ΔpH than for photosynthetic electron transport (1.5 vs 40 mM) indicates an uncoupling effect of AAPH in spinach thylakoids. This possibility was supported by measuring the rates of MV-supported O_2 uptake in presence and in absence of the artificial uncoupler NH_4Cl . At 0 mM AAPH, the uncoupling effect of NH_4Cl can be seen by its large stimulation (130 %) of the rate of O_2 uptake compared to the rate measured without NH_4Cl (Fig. 5). As the concentration of AAPH increased, the rates of O_2 uptake decreased in presence of NH_4Cl , similarly to the decline of $\Delta F/F_m'$. In fact, the declines of the O_2 uptake rates measured with NH_4Cl and of $\Delta F/F_m'$ are highly correlated ($R^2 = 0.976$) in thylakoids treated with different

AAPH concentrations, (Fig. 6). A different response to AAPH was observed when O_2 uptake rates were measured without NH_4Cl . The rates of O_2 uptake increased at low AAPH concentrations, to reach a maximum at 10-20 mM, to a value 62% higher than the control value (no AAPH and no NH_4Cl). At 10-20 mM AAPH concentrations, the rates of O_2 uptake in presence and in absence of NH_4Cl became similar. Interestingly, 10-20 mM correspond to the concentrations where AAPH completely inhibited Q9-AA. At higher AAPH concentrations, the rates of O_2 uptake measured in presence and in absence of NH_4Cl declined similarly.

Discussion

The production of NADPH and ATP by the light reactions in the thylakoid membranes requires the efficient coupling between the electron transport chain and the transfer of protons into the luminal spaces (ΔpH). Our results indicate that a slight lipid peroxidation in thylakoids induced by AAPH-generated peroxy radicals can alter membrane integrity that ensures this coupling. Indeed, we observed a close relation between the extent of lipid peroxidation and the inhibition of ΔpH at low to moderate AAPH concentrations which still allowed substantial electron transport. This uncoupling effect of lipid peroxidation is supported by the 62% stimulation of MV-mediated O_2 uptake by thylakoids treated with moderate AAPH concentration (10-20 mM).

An uncoupling effect of lipid peroxidation in spinach thylakoids is consistent with the reversible uncoupling of oxidative phosphorylation concomitant with the variations of TBARS concentrations observed *in vivo* in mitochondria isolated from rats after they received intraperitoneal injection of AAPH (Kanno et al. 1994). These authors observed that the extents of lipid peroxidation and uncoupling were maximum one hour after the injection and that the *in vivo* mitochondria gradually recovered to normal levels 14 hours later. This recovery is coherent with the transient photo-oxidative responses frequently occurring during plant acclimation to stresses (Muñoz et al. 2021).

It could be argued that the observed inhibitions of ΔpH and electron transport could be the results of a direct effect of the AAPH compound altering the thylakoid structure instead of the consequence of lipid peroxidation initiated by the peroxy radicals. Indeed, Isaakidou and Forti (1977) reported an uncoupling of electron transport from photophosphorylation in spinach thylakoids incubated in the dark with xanthine oxidase, a superoxide-producing enzyme. In that case, the uncoupling was observed in absence of xanthine, the enzyme substrate, and thus without the generation of O_2^- . In our study, a direct effect of AAPH on membrane permeability to protons is unlikely considering 1) the hydrophilic nature of AAPH and 2) the strong correlation observed between the extents of lipid peroxidation (TBARS) and the inhibition of ΔpH with increasing AAPH concentrations. However, we cannot rule out that the inhibitions of electron transport observed at the highest concentration used in our study was the result of both membrane peroxidation and due to a direct effect of AAPH on the components of the electron transport chain.

Numerous studies have used AAPH as a model reagent to study the consequences of oxidative damages on whole organisms, cell cultures, erythrocytes or artificial membranes. The AAPH effects on biomembranes have been more extensively studied in erythrocytes, where radical chain oxidation of membrane components eventually causes hemolysis (Takahashi and Niki 1998).

At an early stage of the peroxidation process in erythrocytes, there is a leakage of K^+ , then Ca^{2+} . Further peroxidation causes the accumulation of secondary peroxidation products, concurrently with the oxidation and redistribution of the band 3 protein. These reactions result in the formation of hemolytic holes responsible for the leakage of hemoglobin and other proteins (Sato et al. 1992; Sato et al. 1995). Interestingly, the IC_{50} for the AAPH-induced hemolysis of erythrocytes after 1 hour at 37° C (about 75 mM) is much higher than the IC_{50} of Q9-AA inhibition (1.5 mM) that we observed after a milder incubation of 20 minutes at 33° C. A lower IC_{50} and then a larger difference between thylakoids and mitochondria may have been observed since the incubation at 33° C for 20 min, even if thylakoid integrity was ensured during this period (Fig. 2), may have increased the thylakoid fluidity, consequently increasing their susceptibility to peroxy radicals. Still, this large difference of EC_{50} indicates that minor alteration of the thylakoid membranes is sufficient to increase its permeability to protons, whereas more extensive oxidation of membrane components (both lipids and proteins) is necessary to form hemolytic holes in erythrocyte membranes. The large difference of IC_{50} between thylakoids and erythrocytes exposed to AAPH might also originate from their different lipid composition: thylakoids possess distinctive polar head groups, notably the dominant di- and monogalactosyldiacylglycerol (DGDG, and MGDG) associated with a large proportion of polyunsaturated fatty acids (Sakurai et al. 2006; Van Eerden et al. 2015) compared to the typical phosphoglycerides (ethanolamine, serine and choline) with less unsaturated fatty acids present in the erythrocyte membrane (Krachler et al. 2008). Besides the main lipid composition, the presence of α -tocopherol (Vit E), an efficient quencher of peroxidation, was shown to significantly delay the AAPH-induced hemolysis of erythrocytes (Mino et al. 1988). Interestingly, α -tocopherol contributes to plant stress tolerance since its levels in thylakoids can change significantly during plant development and in response to environmental stress (Munné-Bosch 2005).

Besides the effects of AAPH on rat mitochondria, other observations from the literature indirectly support an uncoupling effect of lipid peroxidation in chloroplasts. Indeed, there are several examples of inhibition of ATP synthesis in plants exposed to various environmental stresses (Ortiz-Lopez et al. 1991; Tezara et al. 1999; Lawlor and Cornic 2002; Allakhverdiev et al. 2008). Although inhibition of ATP synthesis can also result from the inactivation of the F-ATPase and from the inhibition of the photosynthetic electron transport, only the uncoupling of thylakoids can cause an inhibition of non-photochemical quenching (qN) accompanied by an overreduction of the photosynthetic electron transport chain (i.e., a decrease of photochemical quenching qP). Such simultaneous decreases of both qN and qP have been reported in a variety of environmental stresses: low temperatures (Strand and Öquist 1988), ozone (Calatayud et al. 2002), copper (Pádua et al. 2010), acid rain (Qiu and Liu 2002), and methyl viologen (Samson et al. 2020). To further support our conclusions, it would have been interesting to directly measure the inhibition of qN formation in thylakoids treated with low to moderate AAPH concentrations. However, activation of qN requires prolonged (10-20 min) of illumination not feasible in our conditions, or the accumulation of zeaxanthin in thylakoids just before their isolation by the pre-illumination of the leaves, ideally in CO_2 -free atmosphere for 50 min in the presence of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Noctor et al. 1991).

Loss of qN and overreduction of the plastoquinone (PQ) pool caused by increased H^+ -leakiness of thylakoid membranes promote photoinhibition and ROS production. Fortunately,

reduction of the photosynthetic electron chain, mainly the PQ pool and the ferredoxin/thioredoxin system, can activate signaling pathways that lead to appropriate acclimation in response to photooxidative stress (Yang et al. 2001; Havaux 2020). For instance, it was shown in *Lemna minor* that a more reduced PQ pool caused by inhibitors of PQH₂ oxidation by the cytochrome b₆f complex stimulate the synthesis of polyphenols, more specifically flavonoids (Akhtar et al. 2010). These secondary metabolites accumulate in plants exposed to diverse environmental stresses such as heavy metals, ozone, herbivory, drought, nutrient limitations, etc. (Dixon and Paiva 1995). Also, exogenous salicylic acid increases polyphenol contents in plants (Chitra et al. 2008), and improves plant resistance against biotic (Chitra et al. 2008) and abiotic (Hayat et al. 2010) stresses. Interestingly, salicylic acid was shown to act as an uncoupler in mitochondria (Norman et al. 2004). Like the dual role of ROS as both damaging and signaling molecules (Noctor et al. 2018), the uncoupling effect of lipid peroxidation could inhibit ATP synthesis but on the other hand, it could activate via a more reduced PQ pool a complex network of pathways, leading to the acclimation of the photosynthetic system to the stress conditions.

In short, our results demonstrate that moderate lipid peroxidation induced by peroxyl radicals caused the uncoupling of spinach thylakoids. We suggest that the increased H⁺-leakiness of thylakoid membranes could be one of the primary effects of mild oxidative stress in chloroplasts.

Credit authorship contribution statement

O La Haye Yergeau: Realization of most of the experiments, data analysis, reading and approval the manuscript.

G Samson: Conception of the experiments, realization of some experiments (TBARS and O₂ uptake), supervision, data analysis, writing the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Figure legends

Figure 1 Concentrations of thiobarbituric acid reactive substances (TBARS) as markers of lipid peroxidation in spinach thylakoids exposed to peroxy radicals generated by different concentrations of AAPH for 20 min at 33° C. Symbols represent the means ($n=5$) and the error bars, the standard errors of the mean.

Figure 2 Decrease of the light-induced quenching of the 9-aminoacridine fluorescence Q9-AA in spinach thylakoids incubated in presence of different concentrations of AAPH. Symbols represent the means ($n=5$) and the error bars, the standard errors of the mean.

Figure 3 Correlation between the values of the light-induced quenching of the 9-aminoacridine fluorescence Q9-AA and the TBARS concentrations in spinach thylakoids incubated in presence of different concentrations of AAPH.

Figure 4 Decreases of the maximum (F_v/F_m ●) and effective ($\Delta F/F_m'$ ○) quantum yields of Photosystem II photochemistry measured from spinach thylakoids exposed to peroxy radicals generated by different concentrations of AAPH. Symbols represent the means ($n=5$) and the error bars, the standard errors of the mean.

Figure 5 Rates of methyl viologen-supported O_2 uptake by spinach thylakoids exposed to peroxy radicals generated by different concentrations of AAPH. O_2 uptake was measured in absence (○) and in presence (●) of the uncoupler NH_4Cl (1 mM).

Figure 6 Correlation between the effective quantum yield of Photosystem II photochemistry $\Delta F/F_m'$ and the rates of methyl viologen-supported O_2 uptake measured in presence of NH_4Cl from spinach thylakoids exposed to peroxy radicals generated by different concentrations of AAPH. Symbols represent the means ($n=3$) and the error bars, the standard errors of the mean.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6