



GUN1 involvement in the redox changes occurring during biogenic retrograde signaling

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ABSTRACT

Chloroplast biogenesis requires a tight communication between nucleus and plastids. By retrograde signals, plastids transmit information about their functional and developmental state to adjust nuclear gene expression, accordingly. GENOMES UNCOUPLED 1 (GUN1), a chloroplast-localized protein integrating several developmental and stress-related signals, is one of the main players of retrograde signaling.

Here, we focused on the interplay between GUN1 and redox regulation during biogenic retrograde signaling, by investigating redox parameters in *Arabidopsis* wild type and *gun1* seedlings. Our data highlight that during biogenic retrograde signaling superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) play a different role in response to GUN1. Under physiological conditions, even in the absence of a visible phenotype, *gun1* mutants show low activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX), with an increase in $O_2^{\cdot -}$ accumulation and lipid peroxidation, suggesting that GUN1 indirectly protects chloroplasts from oxidative damage. In wild type seedlings, perturbation of chloroplast development with lincomycin causes H_2O_2 accumulation, in parallel with the decrease of ROS-removal metabolites and enzymes. These redox changes do not take place in *gun1* mutants which, in contrast, enhance SOD, APX and catalase activities. Our results indicate that in response to lincomycin, GUN1 is necessary for the H_2O_2 -dependent oxidation of cellular environment, which might contribute to the redox-dependent plastid-to nucleus communication.

1. Introduction

Plant development, differentiation and appropriate response to environmental fluctuations require a mutual communication between plastids and the nucleus. By anterograde signaling, the nucleus exerts its control over the chloroplasts, while plastids, through retrograde signaling, transmit information about their developmental and functional state to adjust nuclear gene expression (NGE), accordingly [1,2]. Many components and distinctive pathways of retrograde signaling, controlling chloroplast biogenesis (biogenic control) and plastid homeostasis in response to environmental cues (operational control), have been identified in the last decades. These signaling molecules include carotenoid oxidation products [3], intermediates of tetrapyrrole biosynthesis (TPB) [4–6], carbohydrate metabolites [7,8], isoprenoid precursors [9], phosphoadenosines [10] and reactive oxygen species (ROS) [2,11–13].

The role of ROS as oxidants or components of redox signaling mostly

depends on a fine balance between the production and scavenging of these molecules in different organelles [14]. Aerobic metabolism constantly generates ROS in different compartments of plant cells [15, 16]. Chloroplasts represent a significant source of ROS, which comprise production of singlet oxygen at photosystem II and superoxide anion ($O_2^{\cdot -}$) at PSI [17]. The signaling activity and the simultaneous prevention of oxidative damage takes place through the control of ROS levels, which is made possible by enzymatic and non-enzymatic antioxidant systems [16,18]. Major non-enzymatic antioxidants include tocopherols, carotenoids, ascorbate (ASC), and glutathione (GSH) [18]. Amongst the enzymatic systems, superoxide dismutases (SOD) catalyze $O_2^{\cdot -}$ dismutation to hydrogen peroxide (H_2O_2). H_2O_2 is closely controlled by the action of catalases (CAT), ASC peroxidases (APX), class III peroxidases (POD) and thiol-dependent peroxidases (TPX), which include peroxiredoxins [19] and GSH peroxidases (GPX) [20]. APX utilizes ASC to reduce hydrogen peroxide yielding monodehydroascorbate (MDHA), which can be reconverted to ASC by either the action of MDHA

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reductase (MDHAR) or through the non-enzymatic disproportionation to dehydroascorbate (DHA). Subsequently, DHA reductase (DHAR) reduces DHA to ASC utilizing GSH, which is oxidized to glutathione disulfide (GSSG) and regenerated to GSH by NADPH-dependent GSSG reductase (GR) [16].

Redox regulation occurring in cell organelles can also regulate retrograde signaling, greatly influencing plant response to external environment changes [15,21–24]. Cellular redox signaling has been proposed as a crucial integrator of retrograde signals deriving from organelles, which permit communication with the nucleus [25].

One of the main biogenic retrograde signaling pathways involves the plastid-localized GENOMES UNCOUPLED (GUN) proteins, identified in experiments where plastid development was chemically inhibited [26]. In these conditions, *gun* mutants failed to repress nucleus-encoded Photosynthesis-Associated Nuclear Genes (PhANGs). Among the six GUN proteins identified, GUN2-GUN6 are directly involved in TPB, giving rise to intermediate molecules with a possible role in biogenic retrograde signaling [6,27–29]. On the other hand, GUN1 is a nuclear encoded pentatricopeptide repeat (PPR) protein, with a C-terminal Small MutS-Related (SMR) domain, localized in the chloroplast, which takes part in multiple processes to coordinate NGE in response to plastid signals. Since the PPR and SMR proteins are involved in RNA metabolisms and DNA repair and recombination [30,31], GUN1 was initially proposed as a nucleic acid-binding protein acting in either plastid DNA metabolism or repair and involved in plastid gene expression (PGE) [29]. Successive evidence demonstrates that GUN1 interacts with proteins more than with nucleic acids. Co-immunoprecipitation and mass spectrometry studies have detected nearly 300 different GUN1 interacting proteins, involved in several biological processes [32,33], including plastid gene transcription, RNA-editing, translation, protein import and indirectly TPB [34–36].

GUN1 protein level is very low in most plant developmental stages, since Clp protease rapidly degrades the protein after entering the chloroplast [37]. Only during the early stages of chloroplast biogenesis or under stress conditions that perturb plastid protein homeostasis, such as lincomycin (Lin) treatment, GUN1 accumulates to detectable levels [33, 37]. When chloroplast protein synthesis is inhibited by Lin, Clp-protease fails to accumulate, resulting in increased amount of GUN1 protein in the chloroplast [38]. It has been recently proposed that GUN1 functions as a hub by interacting with several protein partners and promoting function by bringing enzymes in proximity with their substrate, or the opposite, inhibiting processes by sequestering specific interactors [35, 39].

Although a growing number of evidence underlines, on one side, the pivotal role of ROS and redox changes and, on the other side, the GUN1 protein in retrograde signaling to our knowledge very few data are present in the literature on the interplay between GUN1 and redox regulation during biogenic retrograde signaling.

Considering these premises, we aimed to study the potential involvement of GUN1 in the control of redox regulation occurring upon activation of GUN1-dependent retrograde signaling. To achieve this goal, we investigated redox parameters in *Arabidopsis* wild type (Col-0) and *gun1* mutant seedlings grown both in presence and absence of Lin. In particular, the levels of ROS, the main oxidative markers, as well antioxidant metabolites and the major ROS removal enzymes were analyzed. Redox changes observed between Col-0 and *gun1* seedlings in response to lincomycin treatment have provided valuable insights into the role of ROS and redox changes in the biogenic retrograde communication.

2. Materials and methods

2.1. Plant materials and growth conditions

The *Arabidopsis* (*Arabidopsis thaliana*, genetic background Col-0) *gun1-102* T-DNA insertion mutant was previously described in Tadini

et al. [32]. Col-0 and *gun1-102* seeds were surface-sterilized and sown out on Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands) supplemented with 2% (w/v) sucrose and 1.5% (w/v) Phyto-Agar (Duchefa). Lincomycin (Lin) was added at the final concentration of 550 μ M. After 2 days incubation at 4 °C in the dark, seedlings were grown for 6 days (80 μ mol m⁻² sec⁻¹ on 16 h/8 h dark/light cycles).

2.2. Determination of ROS and oxidative markers

In situ O₂⁻ and H₂O₂ were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described in Jambunathan (2010) [40] with minor modifications. For anion superoxide visualization, seedlings were vacuum infiltrated (70–100 mbar) for 10 min in NBT-staining solution (50 mM phosphate buffer pH 6.4, 0.1% (w/v) NBT, 10 mM sodium azide). After a further incubation for 15 min with a new NBT-staining solution, seedlings were exposed under cool fluorescent light for 20 min. After the staining, seedlings were bleached by a series of washing steps with 95% ethanol at 45 °C. Superoxide anion was visualized as a blue color produced by NBT precipitation. For H₂O₂ visualization, seedlings were vacuum infiltrated (70–100 mbar) for 5 min with 100 mM phosphate buffer pH 7.4 containing 0.1% 3,3'-diaminobenzidine (DAB) (w/v). The seedlings were incubated under vacuum in the dark for 5–6 h until brown precipitates were observed. Successively, stained seedlings were bleached by a series of wash with 95% ethanol at 45 °C. The staining intensity was quantified on digital images by ImageJ software (<https://imagej.nih.gov/ij/>) as reported in [40]. The relative O₂⁻ and H₂O₂ levels were determined as percentage of NBT- and DAB-stained area of cotyledons, respectively.

The level of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) content determined by the TBA reaction as described by Paradiso et al. [41]. The amount of MDA-TBA complex was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

Protein oxidation was spectrophotometrically determined by measuring the content of carbonyl-groups reacting with dinitrophenylhydrazine (DNPH), according to Romero Puertas et al. [42]. Carbonyl content was calculated using an extinction coefficient of 22 mM⁻¹ cm⁻¹.

For the identification of sulfhydryl groups, proteins were labelled with monobromobimane (mBBR) and separated by sodium dodecyl sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) according to De Gara et al. (2003) [43]. Quantitative densitometric analyses of the main bands in the gels were performed using Quantity One™ software (Biorad).

2.3. Analysis of enzymatic and non-enzymatic antioxidants

For the determination of non-enzymatic antioxidants, *Arabidopsis* seedlings were homogenized with four volumes of cold 5% (w/v) metaphosphoric acid in liquid nitrogen. The homogenates were centrifuged at 20,000 g for 15 min at 4 °C. Supernatants were used to determine contents and redox states of ASC and GSH according to de Pinto et al. [44].

For the determination of enzymatic antioxidants, seedlings were ground in liquid nitrogen and homogenized at 4 °C in a 1:8 (w/v) ratio with the extraction buffer (50 mM Tris-HCl pH 7.5, 0.05% cysteine, 0.1% bovine serum albumin, 1 mM phenylmethanesulfonylfluoride-PMSF). For the determination of APX activity, 1 mM ASC was added to the extraction buffer. Homogenates were centrifuged at 20,000 g for 15 min, and the supernatants were used for spectrophotometric and electrophoretic analyses. Protein concentration was determined according to Bradford [45], using bovine serum albumin as a standard.

Superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) activities were spectrophotometrically measured according to Paradiso et al. [46].

Native PAGE of CAT and SOD were performed according to Villani

et al. [47]. After the electrophoretic run, for CAT determination, gels were incubated for 15 min in 5 mM H_2O_2 . The gels were washed with distilled water and stained with 1% ferrichloride and 1% ferricyanide solution. CAT isoforms appeared as achromatic bands on a dark-blue background. For SOD, gels were incubated in 36 mM sodium phosphate buffer (pH 7.8), containing 28 μM riboflavin and 28 mM N' -Tetramethyl ethylenediamine for 25 min at room temperature. The gels were washed with distilled water and incubated in the dark for 30 min with a gentle shaker in 36 mM sodium phosphate buffer (pH 7.8), containing 2 mM NBT. After illumination under cool fluorescent light for 15 min, the solution was replaced with 36 mM sodium phosphate buffer (pH 7.8) until achromatic bands on a grey background appeared.

Ascorbate peroxidase (APX, EC: 1.11.1.11), dehydroascorbate reductase (DHAR, EC: 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC: 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) activities were determined according to de Pinto et al. [48].

Activity of class III peroxidases (POD, EC: 1.11.1.7) was measured following the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) at 652 nm. The reaction buffer contained 50 mM sodium acetate buffer (pH 5.0), 0.1 mM H_2O_2 , and 0.2 mM TMB. The activity was calculated using an extinction coefficient of $26.9 \text{ mM}^{-1} \text{ cm}^{-1}$.

Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was determined following the NADPH oxidation at 340 nm in a 1 mL reaction mixture composed of 0.1 M Tris-HCl buffer (pH 8.0), 1 mM GSH, 0.2 mM NADPH, 3 U GR and 50 μM H_2O_2 . The activity was calculated using an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.4. Western Blot of SOD, CAT and APX

Total proteins were extracted from seedlings with 10% (w/v) TCA in a 1:10 (w/v) ratio. The homogenates were centrifuged at 20,000 g for 15 min. Pellets were washed with 1 mL of acetone and resuspended in 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% (w/v) SDS, 1 mM PMSF. Proteins were separated by SDS PAGE and then transferred on a polyvinylidene fluoride membrane, as described by Nigro et al. [49]. Filters were then incubated with the following specific antibodies: L-ascorbate peroxidase primary polyclonal antibody (Agrisera Vännäs, Sweden), which recognizes thylakoidal, stromal and cytosolic isoforms; Catalase (peroxisomal marker) primary polyclonal antibody (Agrisera Vännäs, Sweden); Fe-SOD primary polyclonal antibody (Agrisera Vännäs, Sweden). The secondary antibody used was horseradish peroxidase (HRP)-conjugate Anti-Rabbit IgG (Promega, Madison, WI, USA). Filters were revealed by enhanced chemiluminescence using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Cleveland, OH, USA).

2.5. Differential expression analyses

Differential expression data have been retrieved from published datasets on Gene Expression Repository (GEO; <https://www.ncbi.nlm.nih.gov/geo/>; GEO accession GSE5770) [29] and analyzed using GEO2R online tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>).

2.6. Statistical analysis

The data were expressed as the means of five different experiments $\pm$ standard error (SE). One-way analysis of variance (ANOVA) followed by a post-hoc Tukey's comparison test was used to calculate the difference between genotypes and treatments. Differences were considered statistically significant at a p-value < 0.05 . All the statistical analyses were performed by Minitab software (Minitab Inc., State College, PA, USA).

3. Results

3.1. ROS accumulation and oxidative markers differed in wild type and *gun1* seedlings both in presence and absence of Lin

Six-day-old wild type (Col-0) and *gun1* Arabidopsis seedlings grown in the absence of Lin (control conditions, - Lin) did not show any visible phenotypic difference (Fig. 1A). However, under control conditions, the levels of O_2^- , visualized by NBT-staining, were significantly higher in the cotyledons of *gun1* seedlings than in Col-0 (Fig. 1B). On the other hand, H_2O_2 levels, visualized by DAB-staining, did not differ between the two genotypes grown under optimal control conditions (Fig. 1C). In presence of 550 μM Lin, when the proplastid-to-chloroplast transition is completely suppressed (Fig. 1A), O_2^- levels did not change in wild type seedlings but were significantly reduced, i.e. under the limit of detection, in *gun1* cotyledons (Fig. 1B). Conversely, Lin treatment caused a very specific increase in the accumulation of H_2O_2 only in wild type seedlings (Fig. 1C).

Furthermore, under control conditions, the level of lipid peroxidation was higher in *gun1* with respect to wild type seedlings. Interestingly, the Lin treatment did not change lipid peroxidation in Col-0, while the same treatment lowered the level of this oxidative marker in *gun1* seedlings (Fig. 2A). With respect to protein oxidation, the levels of protein carbonyl groups did not show differences between the two genotypes grown under control conditions, while a marked increase occurred in Col-0 seedlings treated with Lin (Fig. 2B). Sulfhydryl groups of proteins, labelled with mBBR and separated by SDS-PAGE, were slightly higher in *gun1* with respect to wild type seedlings grown under control conditions. Treatment with Lin caused a marked oxidation of protein sulfhydryl groups in both genotypes, although more evident in wild type seedlings (Fig. 2C).

3.2. Behavior of ROS scavenging enzymes in wild type and *gun1* seedlings

To clarify the different O_2^- levels found in the two genotypes the activity of SOD was investigated. Under control conditions, total SOD activity in *gun1* was significantly lower than in wild type seedlings. Moreover, Lin treatment did not affect the activity of this enzyme in Col-0, while induced a significant increase in *gun1* seedlings (Fig. 3A). Changes in SOD activity were confirmed by Native PAGE analysis (Fig. 3B), where the SOD isoenzyme activity was lower in *gun1* than in wild type seedlings under control conditions, while both Mn/Fe-SOD and Cu/Zn-SOD activities markedly increased in *gun1* seedlings treated with Lin. A similar behavior could be observed by monitoring the changes in the amount of Fe-SOD protein by immunoblot and densitometric analysis (Fig. 3C).

To explain the different H_2O_2 accumulation in the two genotypes the behavior of the two enzymes directly involved in the removal of this ROS, namely CAT and APX, was investigated (Figs. 4, 5). In control conditions, CAT activity did not differ significantly among the two genotypes (Col-0 and *gun1*), as shown by spectrophotometric analysis and Native PAGE. On the contrary, the presence of Lin in the growth medium induced a significant reduction of CAT activity only in Col-0 seedlings (Figs. 4A, 4B). Interestingly, the protein level of CAT2, analyzed by western blotting, was much higher in *gun1* seedlings under both control and Lin treatment conditions (Fig. 4C).

Regarding APX enzyme, its activity was lower in *gun1* seedlings under control conditions; however, the presence of Lin determined a reduction of the enzyme activity in Col-0 and an enhancement in *gun1* mutants (Fig. 5A). Western blotting showed that the various APX isoforms behaved differently. Indeed, Lin inhibited the accumulation of thylakoidal APX in both the genotypes, as thylakoid formation itself is inhibited, and caused a clear increase in the cytosolic isoforms only in *gun1* seedlings, especially upon Lin treatment (Fig. 5B, C).

The expression analysis of genes coding for different isoforms of SOD, CAT and APX, taken from published microarray experiments as in

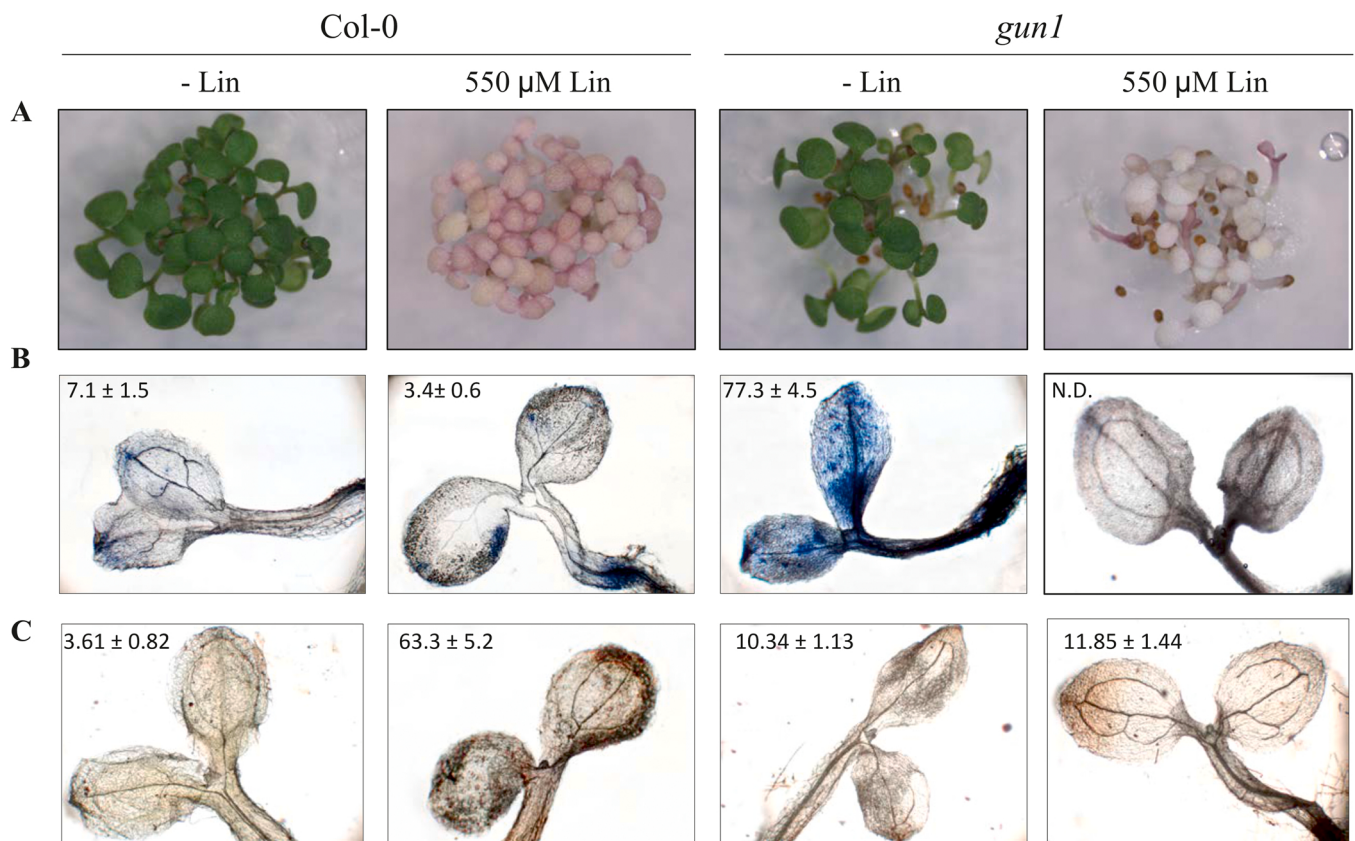


Fig. 1. Accumulation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in Arabidopsis wild type (Col-0) and *gun1* seedlings, grown in the absence or presence of 550 µM lincomycin (Lin). Representative images of (A) phenotypes, (B) O_2^- accumulation, visualized by nitroblue tetrazolium (NBT)-staining and (C) H_2O_2 accumulation, visualized by diaminobenzidine (DAB)-staining. The experiments of O_2^- and H_2O_2 detection were repeated three times showing reproducible results. In (B) and (C) the percentage area ± standard errors of 60 cotyledons (20 for each experiment) stained with NBT and DAB, respectively, is reported inside the images.

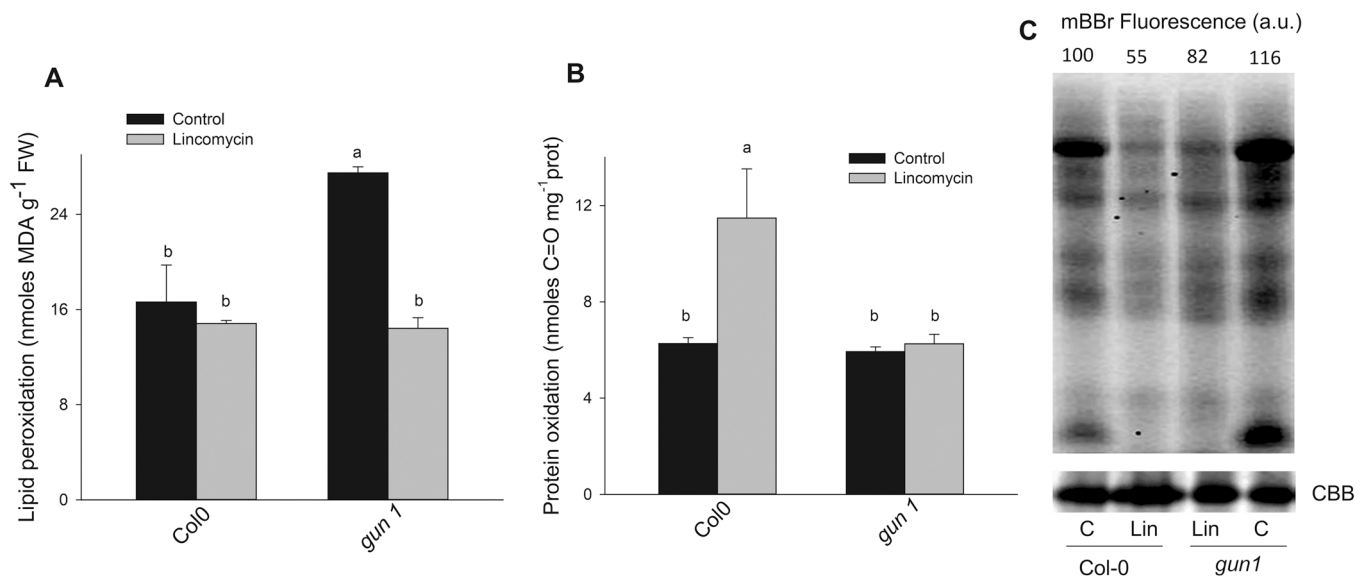


Fig. 2. Oxidative markers in Arabidopsis wild type (Col-0) and *gun1* seedlings after six days of growth in the absence (control) or presence of lincomycin (Lin). (A) Lipid peroxidation, measured as malondialdehyde content and (B) protein oxidation measured as total protein carbonyl groups. The values are the means ± standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$). (C) Representative image from three independent experiments of the levels of sulphhydryl protein groups. Proteins were labelled with monobromobimane (mBBr) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Each well was loaded with 100 µg of proteins. To control for loading, gel was stained with Coomassie Brilliant Blue (CBB); quantification of signals (by Quantity One) relative to Col-0 without Lin (100%) is provided above the panel.

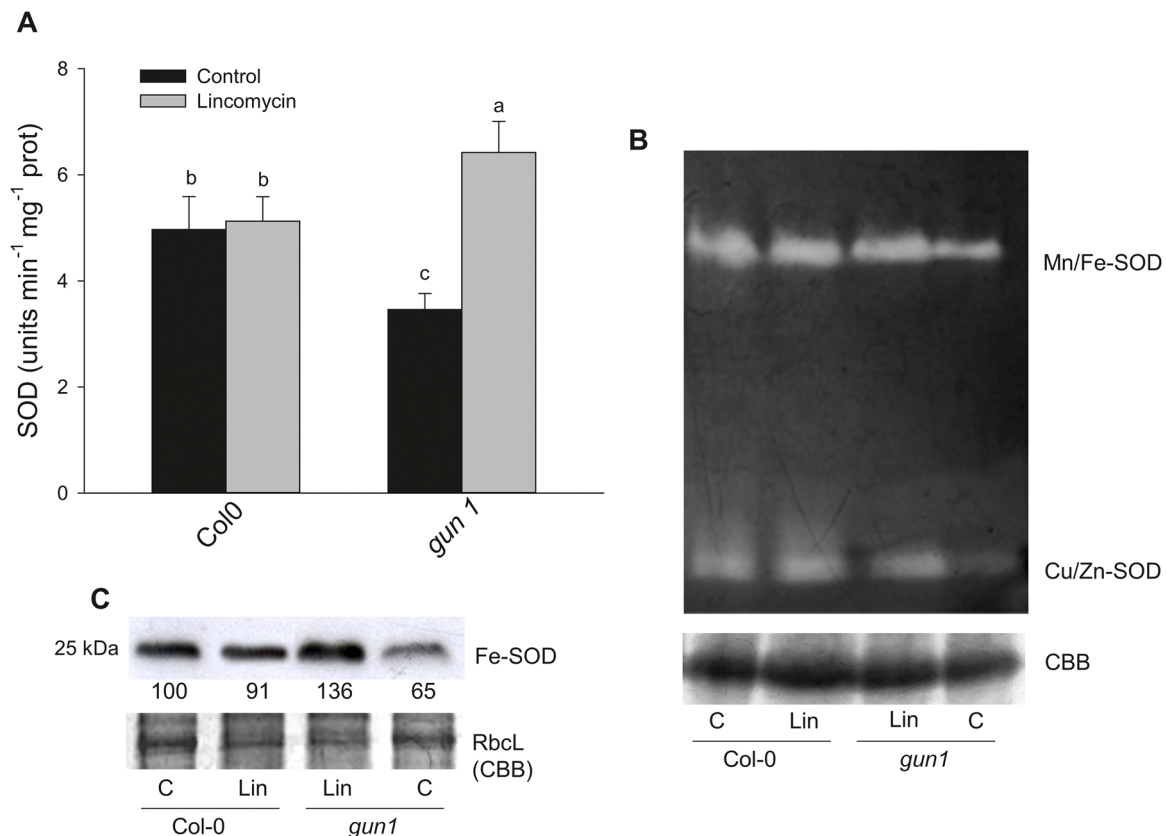


Fig. 3. Superoxide dismutase (SOD) behaviour in wild type (Col-0) and *gun1* seedlings after six days of growth in the absence (control) or presence of lincomycin (Lin). (A) Total SOD activity of *Arabidopsis* seedlings (Col-0 and *gun1*), grown in the absence or presence of Lin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$). (B) Representative image from three independent experiments of Native Polyacrylamide Gel Electrophoresis of SOD. Each well was loaded with 150 μ g of proteins. To control for loading, gel was stained with Coomassie Brilliant Blue (CBB). (C) Representative image from three independent experiments of Fe-SOD immunoblotting; each well was loaded with 30 μ g of proteins. RbcL band, stained with Coomassie Brilliant Blue (CBB), was used as a loading control between genotypes. The decrease in RbcL in the "Lin" lanes confirmed the action of the treatment. Quantification of signals (by ImageJ) relative to the Col-0 without Lin (100%) is provided below the panel.

Koussevitzky, et al. (2007) [29], showed that genes encoding the plastid-located FSD1, CSD2, tAPX and APX4, as well the gene coding for CAT2, were downregulated by Lin in Col-0 plants. On the other hand, Lin treatment induced the expression of APX2, and CDS3 in *gun1* mutants. Moreover, in Lin-treated plants many of the genes coding for the different SOD (FSD1, FSD2, FSD3, CSD3) and APX (APX2, APX3, APX4, APX6, TAPX) isoforms were upregulated in *gun1* compared to Col-0 (Table S1).

With respect to GPX, its activity did not differ significantly in the two genotypes grown under control conditions, while it appeared to be significantly higher in Lin-treated Col-0 seedlings (Fig. 6A). Lin treatment in Col-0 seedlings caused a repression of genes encoding the chloroplastic GPXs, namely GPX1 and GPX7, and an induction of GPX3 and GPX8 (Table S1) [29].

Similarly, POD activities did not change in the two genotypes under control conditions and increased only in wild type seedlings grown in presence of Lin (Fig. 6B).

3.3. Hydrophilic antioxidants decreased and were more oxidized only in wild type seedlings grown in presence of Lin

The different redox environment of wild type and *gun1* mutants grown in presence of Lin was confirmed through the analyses of the two major hydrophilic antioxidants, ASC and GSH (Fig. 7). Under control conditions, total contents, and redox state of the two antioxidants did not vary significantly between Col-0 and *gun1*. However, Lin triggered a reduction in total ascorbate content in both the genotypes, although this

decrease was significantly greater in Col-0 than in *gun1* seedlings (Fig. 7A). On the other hand, total glutathione levels were lowered only in Col-0 grown in presence of Lin (Fig. 7C). Furthermore, the presence of Lin affected the oxidation of both antioxidants only in wild type seedlings (Figs. 7B, 7C).

To clarify the different redox state of ascorbate and glutathione in the two genotypes, the activity of the enzymes involved in the reduction of the oxidized forms of the two metabolites was determined. The activities of the three enzymes, namely MDHAR, DHAR and GR did not show significant differences between the two genotypes grown under control conditions (Fig. 8). Lin treatment was responsible, instead, for the increase in the activities of MDHAR and GR in *gun1* seedlings (Fig. 8A and C). On the contrary, Lin led to an induction of DHAR activity only in Col-0 seedlings (Fig. 8B).

4. Discussion

GUN1-dependent signaling has been proposed as one of the main retrograde signaling pathways active during plastid biogenesis. Our results indicate that during the GUN1-dependent biogenic retrograde communication O_2^- and H_2O_2 might contribute differently to the chloroplast-to-nucleus retrograde communication.

Under optimal physiological conditions, except for a small percentage of chlorophyll-deficient variegated cotyledons [50], *gun1* mutants are mostly phenotypically indistinguishable from wild type (Fig. 1A). However, *gun1* mutants, grown in control conditions, accumulate a higher O_2^- amount and a greater level of lipid peroxidation than Col-0

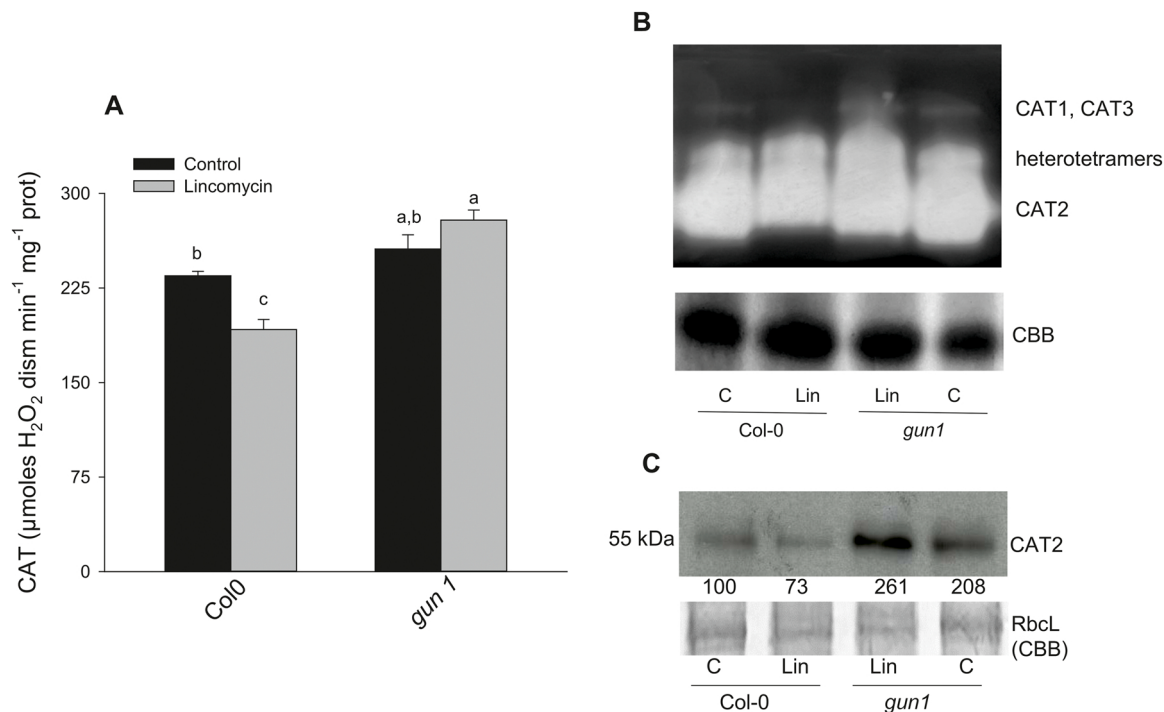


Fig. 4. Catalase (CAT) behaviour in response to lincomycin (Lin) in wild type (Col-0) and *gun1* seedlings. **(A)** Total CAT activity of Arabidopsis seedlings (Col-0 and *gun1*), grown in the absence (control) or presence of Lin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$). **(B)** Representative image from three independent experiments of Native polyacrylamide gel electrophoresis of CAT. Each well was loaded with 50 μ g of proteins. To control for loading, gel was stained with Coomassie Brilliant Blue (CBB). **(C)** Representative image of western blotting of CAT2. Each well was loaded with 10 μ g of proteins; RbcL band, stained with Coomassie Brilliant Blue (CBB), was used as a loading control between genotypes. The decrease in RbcL in the "Lin" lanes confirmed the action of the treatment. Quantification of signals (by ImageJ) relative to the Col-0 without Lin (100%) is provided below the panel.

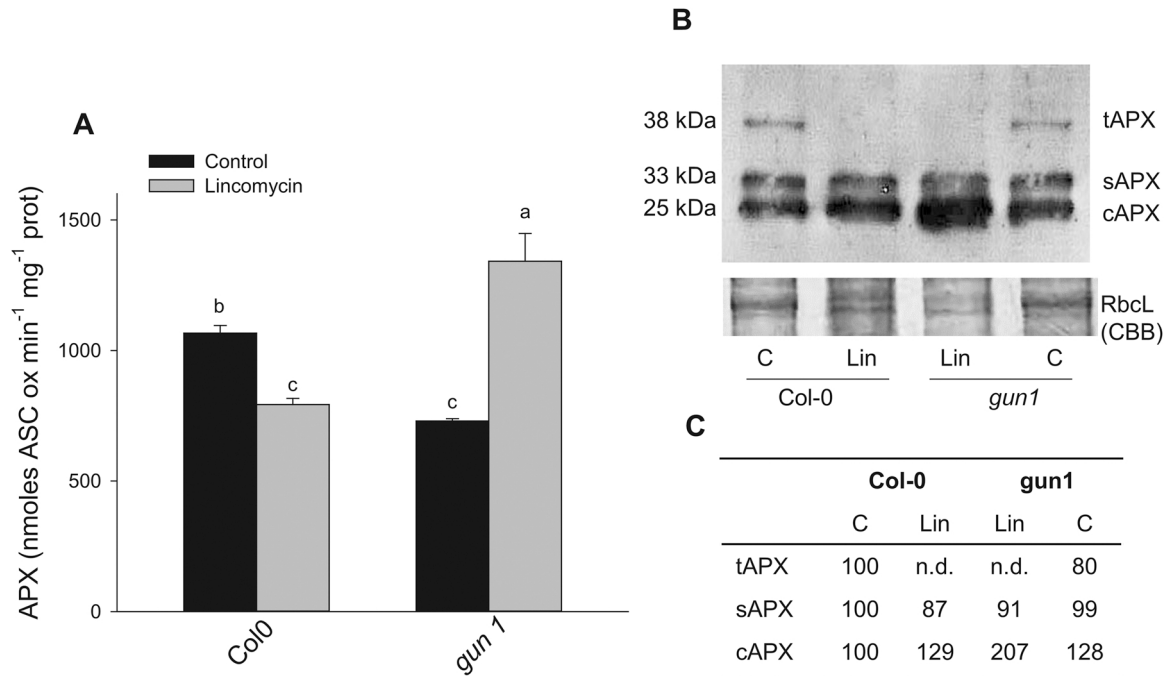


Fig. 5. Ascorbate peroxidase (APX) in wild type (Col-0) and *gun1* seedlings after six days of growth in the absence (control) or presence of lincomycin (Lin). **(A)** Total APX activity of Arabidopsis seedlings (Col-0 and *gun1*), grown in presence or absence of Lin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$). **(B)** Representative image of western blotting of CAT2; each well was loaded with 10 μ g of proteins. RbcL band, stained with Coomassie Brilliant Blue (CBB), was used as a loading control between genotypes. The decrease in RbcL in the "Lin" lanes confirmed the action of the treatment. tAPX, sAPX and cAPX are thylacoidal, stromal and cytosolic APX, respectively. **(C)** Quantification of tAPX, sAPX and cAPX immunoblot signals (by ImageJ) relative to Col-0 without Lin (100%).

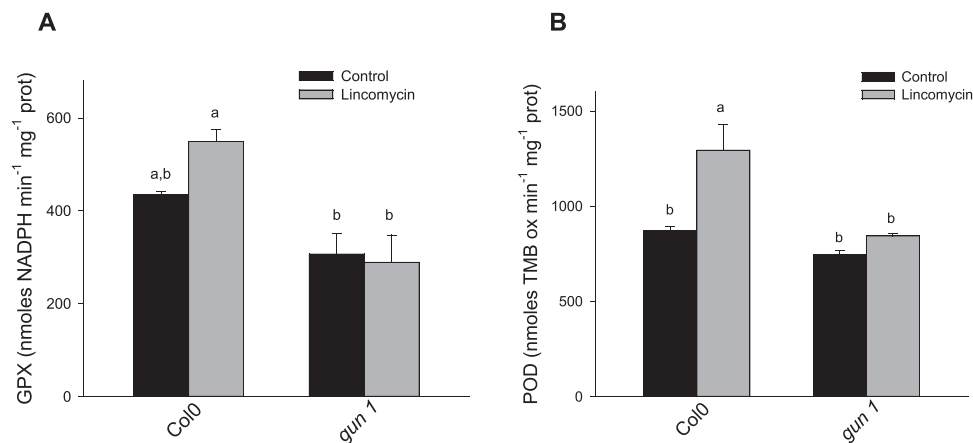


Fig. 6. Lincomycin increases glutathione peroxidase (GPX) and class III peroxidases (POD) activities only in wild type (Col-0) *Arabidopsis* seedlings. (A) GPX and (B) POD activity of *Arabidopsis* seedlings (Col-0 and *gun1*), grown in the absence (control) or presence of lincomycin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$).

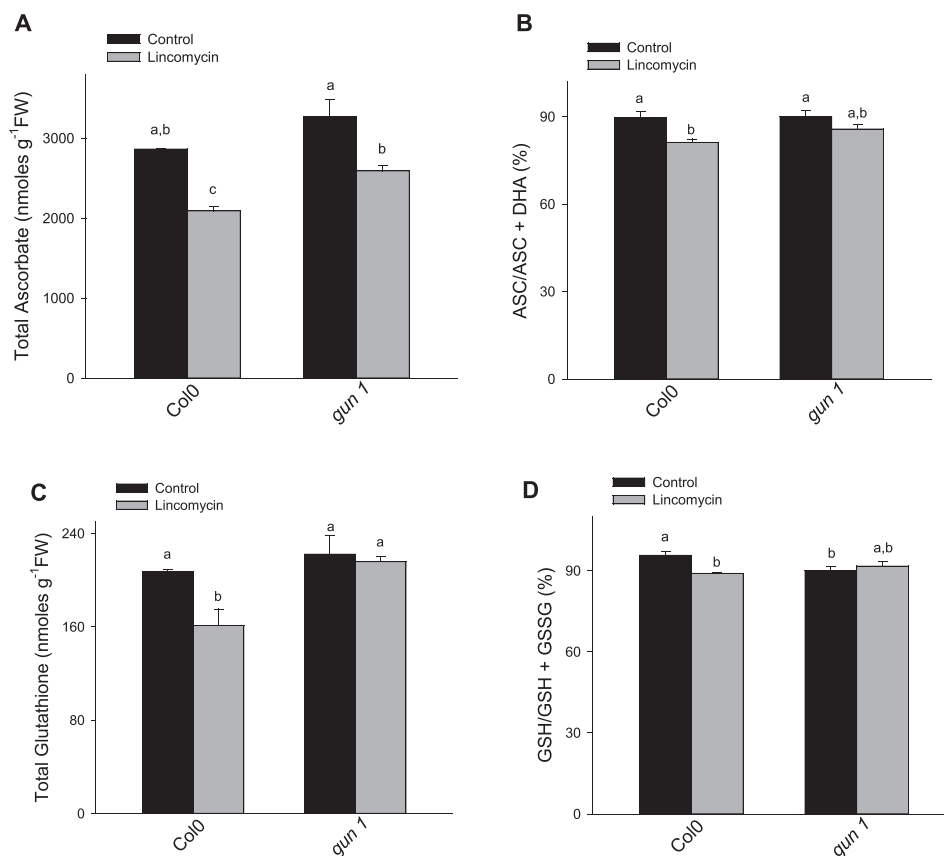


Fig. 7. Lincomycin induces a decrease of total content and redox state of ascorbate (ASC) and glutathione (GSH) only in wild type (Col-0) *Arabidopsis* seedlings. (A) Total content (ASC + dehydroascorbate-DHA) and (B) redox state (ASC/ASC + DHA) of ascorbate and (C) total content (GSH + glutathione disulfide -GSSG) and (D) redox state (GSH/GSH+GSSG) of glutathione in *Arabidopsis* seedlings (Col-0 and *gun1*), grown in the absence (control) or presence of lincomycin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$).

seedlings (Figs. 1B, 2A). O_2^- accumulation in *gun1* mutants may contribute to cellular injuries oxidizing the iron-sulfur centers of proteins; moreover, O_2^- can lead to the formation of hydroxyl radicals, which promptly react with lipids, causing peroxidation [51,52]. The accumulation of O_2^- in *gun1* occurs in parallel with a significant decline in the activities of SOD and APX, two main players of ROS removal (Figs. 3, 5). SOD, catalyzing the dismutation of O_2^- to H_2O_2 , represents the primary line of resistance against ROS [53]. In different plant species, high activities of SOD contribute to improve resistance to high light intensities [54,55]. Moreover, in *Arabidopsis* chloroplastic APX has been shown to play a significant role for photoprotection [55,56]. Thus, the more sensitive phenotype to photo-oxidative stress observed in *gun1*

mutants compared with Col-0 plants [50,57], could be related to the high O_2^- accumulation and the low activity of SOD and APX (Figs. 1, 3, 5), suggesting that *gun1* plastids are more inclined to suffer ROS-mediated damage. Accordingly, the modest percentage of *gun1* cotyledons that fail the greening process [50] could be related to the photo-protective functions provided by GUN1 during chloroplast biogenesis. Our data support the idea that GUN1 optimizes chloroplast biogenesis minimizing the consequences of failures in developing chloroplasts, mainly preventing, or at least reducing, photo-oxidative damage [58,59]. The higher O_2^- level in *gun1* control conditions could also explain the enhanced sensitivity to low concentrations of Lin and norflurazon, when plastid functions are only mild impaired [59,60].

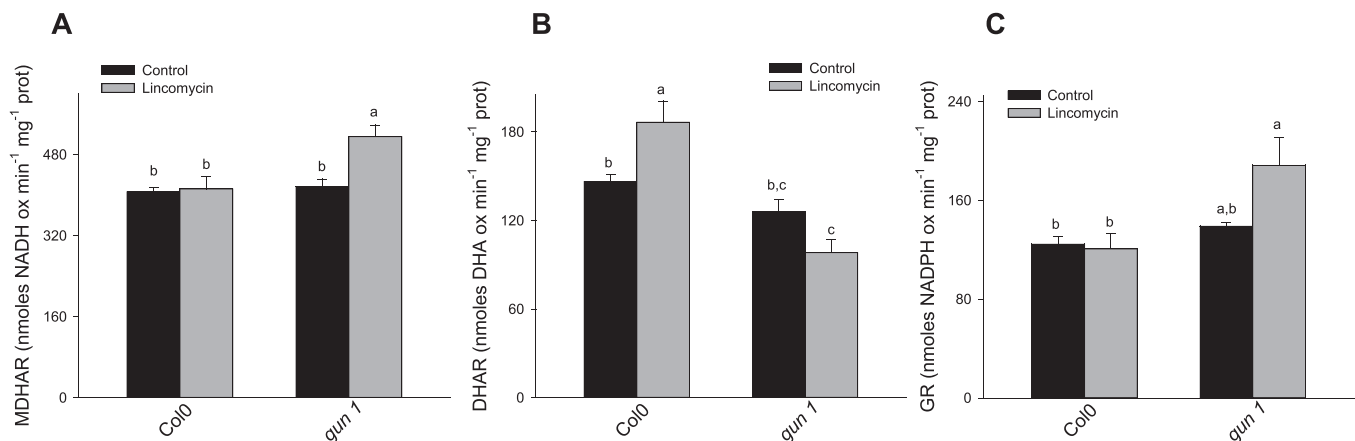


Fig. 8. Effects of lincomycin on ascorbate-glutathione recycling enzymes of Col-0 and *gun1* Arabidopsis seedlings. (A) Monodehydroascorbate reductase (MDHAR), (B) dehydroascorbate reductase (DHAR) and (C) glutathione reductase (GR) of *Arabidopsis* seedlings (Col-0 and *gun1*) grown in the absence (control) or presence of lincomycin. The values are the means $\pm$ standard errors of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($P < 0.05$).

The block of translation in the chloroplast of wild type *Arabidopsis* seedlings by 550 μ M Lin suppresses chloroplast development and impacts retrograde signaling, causing a strong downregulation of PhANGs [61]. Our results show that Lin treatment of *Arabidopsis* Col-0 seedlings causes an increase in the level of H_2O_2 (Fig. 1C). Due to its quite long half-life, its capability to move across the plasma membrane and to oxidize proteins, among ROS, H_2O_2 is considered the crucial signaling molecule [62]. Hence, H_2O_2 can trigger retrograde signaling from chloroplasts to the nucleus reprogramming NGE [63,64]. Intracellular H_2O_2 levels can influence cellular redox regulation leading to the oxidation of protein thiols [65]. By using roGFP2, an *in vivo* reporter of redox changes, it has been shown that the treatment of *Arabidopsis* plants with Lin increases the oxidation of cytosol and nuclei, suggesting that this oxidation can work as a redox signal that permits communication between chloroplasts and the nucleus [25]. Consistently, our results indicate that high H_2O_2 levels in *Arabidopsis* Lin-treated Col-0 seedlings correlate with a higher protein oxidation, measured as increase in carbonylated proteins as well as oxidation of protein sulfhydryl groups (Fig. 3B, C). The effect of Lin in increasing the oxidation of cellular environment of wild type seedlings has been confirmed by the decrease of redox state of the two major hydrophilic antioxidants, ASC and GSH (Fig. 7B, D). Crosstalk between redox pools of different cellular compartments, possibly transferred by a redox shift in cellular components, has also been considered critical for controlling NGE [66,67]. Thus, our results indicate that the H_2O_2 -dependent oxidation of cellular environment caused by Lin treatment could act as a redox signaling communicating to the nucleus the impairment of chloroplast development.

It has been previously proposed that plastid redox state and GUN1-dependent signaling can be interconnected [32,64]. Our data show that contrary to what happens in wild type plants, *gun1* mutants grown in presence of Lin do not accumulate H_2O_2 and do not have an oxidized cellular environment, as shown by the unchanged levels of oxidized proteins and the maintenance of redox state of ASC and GSH pools (Figs. 1C, 2B, C, 7B, D). The preservation of the reduced forms of ASC and GSH in Lin-treated *gun1* mutants is justified by the increase in the activities of MDAR and GR (Fig. 8). Thus, the redox-dependent communication from plastids to nucleus occurring in wild type seedlings fails in *gun1* mutants. The data emphasize the idea of an involvement of GUN1 in the control of the H_2O_2 -dependent redox changes occurring during biogenic retrograde signaling.

Consistently, also the decrease in the content of GSH and ASC occurring in Lin-treated Col-0 seedlings is GUN1-dependent (Fig. 7). Our data are in accordance with literature data showing that GUN1 is

required for the PGE-dependent suppression of ASC biosynthesis; indeed, Lin markedly decreases the transcript levels of many genes involved in ASC biosynthesis in the wild-type plants but does not significantly affect the expression of these genes in the *gun1* mutants [68]. A lowered ASC synthesis and therefore a lowered ROS buffering capacity in Lin-treated seedlings might be part of the cause for the oxidized environment. It is interesting to point out that cellular ASC homeostasis may affect NGE, particularly the expression of defense genes [69,70].

In wild type plants, Lin also induces a decrease in the activities of CAT and APX, which may contribute to the overaccumulation of H_2O_2 (Figs. 4, 5). Western blotting analysis (Figs. 5B, 5C), as well transcriptomic data (Table S1), highlight that the decrease in APX activity is particularly due to the thylakoidal isoform. Remarkably, it has been shown that when the expression of tAPX was silenced in leaves, levels of oxidized protein in chloroplasts increased in the absence of stress [12]. Moreover, in Lin-treated Col-0 seedlings, also the chloroplastic GPX, namely GPX1 and GPX7, are downregulated (Table S1), demonstrating a severe impairment of the chloroplastic H_2O_2 -removal enzymes. On the other hand, the non-chloroplastic GPX3 and GPX8 increase, explaining the higher GPX activity (Fig. 6A) and suggesting their involvement in maintaining the thiol/disulfide balance [20]. Finally, the increase in POD activity observed in Lin-treated Col-0 plants (Fig. 6B) highlight the different behavior of class III peroxidases, which are closely connected with ROS dynamics, working in both H_2O_2 detoxification and production, mainly in the apoplast and vacuole [71]. The rise in POD activity may indicate a contribution of these enzymes to H_2O_2 accumulation caused by Lin. In Lin-treated Col-0 seedlings, the differential responses of CAT and APX on one side and POD on the other indicate that perturbation of chloroplast development triggers specific GUN1-dependent redox processing and signaling pathways.

The behavior of *gun1* mutants grown in presence of Lin is very different from that of wild type plants, which show a significant increase in SOD, CAT and cytosolic APX (Figs. 3–5). It has been recently reported that in Lin-treated *gun1* mutants, an altered chloroplast protein import causes an overaccumulation of unimported precursor with a subsequent cytosolic proteotoxic stress [33,59]. Thus, it is plausible to assume that the increase in the ROS removal enzymes, observed in *gun1* mutants, may represent an indirect response to the cytosolic proteotoxic stress, resembling what occurs in response to heat stress [46]. The increase in SOD activity can explain the decrease in O_2^- accumulation observed in the *gun1* mutants after the Lin treatment. Moreover, in Lin-treated *gun1* mutants, the induction of APX2 and the failure in CAT2 downregulation (Table S1), followed by the increase in the activity of both the enzymes

(Figs. 4, 5), significantly contribute to the maintenance of low H_2O_2 levels, inhibiting the oxidative signaling to the nucleus.

5. Conclusions

This study revealed that during the GUN1-dependent biogenic retrograde signaling O_2^- and H_2O_2 might play a different role. During plastid biogenesis, occurring under optimal physiological conditions, GUN1 appears to influence the O_2^- accumulation, through the regulation of SOD and APX enzyme activities, playing a role in protecting the organelles from potential oxidative damage. On the other hand, in response to Lin treatment, GUN1 mediates the formation of an H_2O_2 -dependent oxidized environment, which can represent a redox signal communicating to the nucleus the perturbation of chloroplast development [25].

Further investigation will be aimed at understanding whether the oxidation of the cellular environment is a common event when PGE is altered and to explore the molecular mechanisms through which GUN1 mediates cellular oxidation.

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Author contributions

S.F., P.P., L.T., MCDP. conceived and designed research. S.F., C.L. N. J. F.V. performed the experiments. M.C.dP. advised on the experiments. All authors contributed drafting the manuscript. S.F., M.C.dP. coordinated the study and took care of the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111265.

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