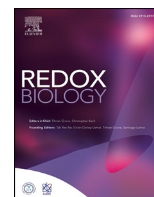
Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox



Plastid 2-Cys peroxiredoxins are essential for embryogenesis in Arabidopsis

Antonia M. Gallardo-Martínez, Julia Jiménez-López, María Luisa Hernández, Juan Manuel Pérez-Ruiz, Francisco Javier Cejudo *

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla and CSIC, Avda. Américo Vespucio, 49, 41092, Sevilla, Spain

ARTICLE INFO

Keywords:
Arabidopsis
Embryogenesis
NTRC
Peroxiredoxin
Redox regulation
Seed development

ABSTRACT

The redox couple formed by NADPH-dependent thioredoxin reductase C (NTRC) and 2-Cys peroxiredoxins (Prxs) allows fine-tuning chloroplast performance in response to light intensity changes. Accordingly, the Arabidopsis *2cpab* mutant lacking 2-Cys Prxs shows growth inhibition and sensitivity to light stress. However, this mutant also shows defective post-germinative growth, suggesting a relevant role of plastid redox systems in seed development, which is so far unknown. To address this issue, we first analyzed the pattern of expression of NTRC and 2-Cys Prxs in developing seeds. Transgenic lines expressing GFP fusions of these proteins showed their expression in developing embryos, which was low at the globular stage and increased at heart and torpedo stages, coincident with embryo chloroplast differentiation, and confirmed the plastid localization of these enzymes. The *2cpab* mutant produced white and abortive seeds, which contained lower and altered composition of fatty acids, thus showing the relevance of 2-Cys Prxs in embryogenesis. Most embryos of white and abortive seeds of the *2cpab* mutant were arrested at heart and torpedo stages of embryogenesis suggesting an essential function of 2-Cys Prxs in embryo chloroplast differentiation. This phenotype was not recovered by a mutant version of 2-Cys Prx A replacing the peroxidatic Cys by Ser. Neither the lack nor the overexpression of NTRC had any effect on seed development indicating that the function of 2-Cys Prxs at these early stages of development is independent of NTRC, in clear contrast with the operation of these regulatory redox systems in leaves chloroplasts.

1. Introduction

Seed development in Arabidopsis is initiated by a double fertilization event that generates a diploid zygote and a triploid endosperm [1]. Then, the formation of the mature seed progresses following a process in which the phases of embryogenesis, maturation, and desiccation can be distinguished [2,3]. Embryogenesis, the process of formation of the embryo, follows a simple pattern progressing through well-defined stages termed globular, heart, torpedo, and walking stick to generate the mature embryo [3,4]. Even though the light intensity that reaches the embryo in a developing seed is limited by filtration through the silique and the pericarp, it is well established that developing embryos perform photosynthesis [5,8], which takes place in specialized organelles, the chloroplasts, the formation of which is initiated at the globular stage and completed during heart and torpedo stages of embryogenesis [9]. Then, at the end of the seed maturation phase, chloroplasts de-differentiate to form non-photosynthetic plastids, termed eoplasts, present in the dry seed [9]. Different functions have been proposed for photosynthesis during embryogenesis. First, it allows refixation of CO_2 produced by respiration to yield storage compounds, starch, proteins, and lipids, which support cotyledon development in post-germinating seeds [6]. In addition, it has been suggested that the generation of oxygen by photosynthesis may alleviate hypoxic conditions within the developing seed [10,11]. Moreover, the analyses of photosynthetic parameters in Arabidopsis developing seeds showed that seed vigor is highly affected by embryonic photosynthesis [5,7].

By performing photosynthesis, chloroplasts act as factories for the synthesis of metabolic intermediates hence being essential for plant growth and development. However, chloroplasts have also an important signaling function acting as sensors of environmental cues, which is essential for plant acclimation to the environment. Among these factors, photosynthesis is continuously fine-tuned in response to light intensity.

Abbreviations: Fdx, ferredoxin; FTR, ferredoxin-dependent thioredoxin reductase; FBPase, fructose bisphosphate phosphatase; NTRC, NADPH-thioredoxin reductase; Prx, peroxiredoxin; Trx, thioredoxin.

* Corresponding author.
E-mail addresses: antonia.gallardo@ibvf.csic.es (A.M. Gallardo-Martínez), julia.jimenez@ibvf.csic.es (J. Jiménez-López), mhjimenez@us.es (M.L. Hernández), jperez4@us.es (J.M. Pérez-Ruiz), fjcejudo@us.es (F.J. Cejudo).

https://doi.org/10.1016/j.redox.2023.102645
Received 29 December 2022; Received in revised form 8 February 2023; Accepted 23 February 2023
Available online 1 March 2023
2213-2317/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

It is known for more than fifty years that redox regulation, based on dithiol-disulfide exchange, plays a key role in the rapid adaptation of chloroplast photosynthetic metabolism to light [12,13]. This regulatory mechanism relies on the disulfide reductase activity of thioredoxins (Trxs) of which plant chloroplasts are equipped with a complex set of more than 20 isoforms [14–17], which use reducing equivalents from photosynthetically reduced ferredoxin (Fdx) via a Fdx-dependent Trx reductase (FTR) [18]. Thus, Fdx, FTR and Trxs constitute a redox system that links redox regulation to light [14,17,19,20]. Since more than 400 putative targets of chloroplast Trxs have been identified [21], virtually any process that takes place in the organelle has at least one redox regulated step [22–24], emphasizing the essential role of redox regulation for chloroplast performance.

In addition to the Fdx-FTR-Trxs redox system, chloroplasts are equipped with an enzyme termed NTRC, which contains NADPH-dependent Trx reductase (NTR) and Trx domains in a single polypeptide [25]. NTRC shows high affinity for NADPH [26] thus allowing the use of this pyridine nucleotide in chloroplast redox homeostasis. Based on the finding that the thiol-dependent hydrogen peroxide scavenging enzyme 2-Cys peroxiredoxin (Prx) is efficiently reduced by NTRC [27–29], it was initially proposed an antioxidant role for this enzyme. However, the Arabidopsis *ntrc* mutant, which is a knockout for NTRC, shows a severe growth retard phenotype, pale green leaves and altered chloroplast structure [25,29,30]. The strong effect of NTRC on chloroplast performance is due to the participation of the enzyme in different redox-regulated processes such as the efficiency of light use [31–33], and the biosynthesis of starch [34,35] and chlorophyll [36,37]. Therefore, though the number of putative NTRC targets so far identified [38,39] is much lower than those of Trxs [21], the analyses of the *ntrc* mutant suggest a very relevant role of this enzyme in chloroplast redox regulation.

More recently, it was found that decreased contents of 2-Cys Prxs suppress the phenotype of the *ntrc* mutant, suggesting that the effects of NTRC on chloroplast performance are exerted via the redox regulation of 2-Cys Prxs [40]. These results uncovered the close functional relationship between NTRC and 2-Cys Prxs indicating the concerted action of thiol-dependent antioxidant and regulatory systems in maintaining chloroplast redox homeostasis [19]. An important implication of this model is the possibility that hydrogen peroxide might act as sink of reducing equivalents allowing the oxidation of chloroplast enzymes. The delayed rate of enzyme oxidation in the dark in mutant plants devoid of 2-Cys Prxs [41–43] confirmed the participation of these enzymes in protein oxidation and showed the relevance of the oxidizing component of redox regulation for chloroplast performance in response to changes in light intensity and darkness [44,45].

In line with the proposed central function of 2-Cys Prxs in chloroplast redox regulation, the Arabidopsis double mutant *2cpab* lacking the two 2-Cys Prxs, A and B, localized in the organelle shows growth retard and sensitivity to high light intensity [46]. Strikingly, the most remarkable phenotypic features of this mutant were observed at early stages of development since it shows aberrant cotyledon phenotypes [47], hence suggesting an important function of 2-Cys Prxs in seed development and post-germinative growth. However, most of the research on plastid redox regulation has been performed on adult leaves, hence on mature chloroplasts and, consequently, their function during seed development remains unknown. In this work, we have addressed this issue by analyzing the roles of NTRC and 2-Cys Prxs in developing seeds. The analysis of the pattern of expression of NTRC and 2-Cys Prxs during seed development in addition to the seed phenotype of Arabidopsis mutants and transgenic lines with altered plastid redox balance reveals an essential role of 2-Cys Prxs, which is independent of NTRC, at early stages of plant development. The different effects of NTRC and 2-Cys Prxs in plastids of developing seeds and fully differentiated chloroplasts of adult leaves will be discussed.

2. Materials and methods

2.1. Biological material and growth conditions

Wild type (WT) *Arabidopsis thaliana* (ecotype Columbia), mutant plants and transgenic lines were routinely grown in soil in growth chambers under long-day (16-h light/8-h darkness), short-day (8-h light/16-h darkness) or continuous light at 22 °C and 20 °C during light and dark periods, respectively, and light intensity of 125 $\mu E\ m^{-2}\ s^{-1}$. The Arabidopsis mutants *ntrc* [25], *Δ2cp* [48], *2cpab* [41] and the transgenic NTRC-OE line [41] were previously described.

2.2. Generation of Arabidopsis transgenic lines expressing NTRC and 2-Cys Prxs A and B fused to GFP

To analyze the pattern of expression of the *NTRC*, *2-Cys PRX A* and *2-Cys PRX B* genes in developing seeds and the localization of the corresponding proteins, genes expressing C-terminal GFP fusion proteins were constructed according to the scheme shown in Fig. S1A. For the NTRC::GFP fusion a fragment of 4249 bp containing the Arabidopsis *NTRC* gene including its putative promoter (1572 bp upstream the translation start codon) and excluding the translation stop codon, was amplified from genomic DNA with iProof High-Fidelity DNA Polymerase (Bio-Rad) using the oligonucleotides indicated in Table S1, which added attB recombination sites at the 5′and 3′ends. For 2-Cys Prx A::GFP and 2-Cys Prx B::GFP fusions, fragments of 3331 bp and 3533 bp containing *2-Cys PRX A* and *2-Cys PRX B* genes, respectively, including their putative promoters (1585 bp and 1704 bp upstream their respective translation start codons) and excluding the translation stop codons, were amplified from genomic Arabidopsis DNA as described above. These genomic fragments were cloned into the Gateway vector pDONR207 (Invitrogen), verified by sequencing, and recombined into the pGWB604 destination plasmid according to the manufacturer's instructions. The P_{NTRC}-NTRC::GFP and the P_{2CPA}-2-Cys Prx A::GFP and P_{2CPB}-2-Cys Prx B::GFP constructs were introduced into the *ntrc* and the *2cpab* mutants, respectively, via the *Agrobacterium tumefaciens* (GV3101) floral dipping method [49].

2.3. Generation of Arabidopsis transgenic lines expressing the WT and 2CPA-C$_P$-S mutant versions of 2-Cys Prx a in the 2cpab mutant background

The 2CPA-C_P-S mutant version of 2-Cys Prx A, in which the peroxidatic Cys (C_P) was replaced by Ser (C119S: codon change TGC to AGC), was produced by site-directed mutagenesis using the pDONR-207/P_{2CPA}-2CPA plasmid and oligonucleotides 2CPAg_C119S_for and 2CPAg_C119S_rev (Table S1). Plasmids obtained above, pDONR207/P_{2CPA}-2CPA and pDONR207/P_{2CPA}-2CPA-C_P-S, were recombined into the pGWB616 vector to generate the P_{2CPA}-2CPA-cmyc and P_{2CPA}-2CPA-C_P-S-cmyc constructs, which were introduced into the *2cpab* mutant as described above.

2.4. Analysis of seed and embryo development

Seed development was analyzed in siliques of the different Arabidopsis lines under study, which were harvested at 10–11 days post anthesis (dpa), opened under a stereomicroscope (SZ40, Olympus) and photographed. For embryo observation, ovules were dissected from siliques and incubated overnight at room temperature in Hoyer's solution [chloral hydrate:glycerol:water, 8:1:3 (w/v/v)] in darkness [50]. After ovule clearing, embryo images were obtained by differential interference contrast (DIC) microscopy (BX60, Olympus) and photographs were taken with a DFC 300 FX camera (Leica).

2.4.1. Confocal microscopy and scanning electron microscopy

The pattern of expression of *NTRC* and 2-Cys *PRXs A* and *B* genes was analyzed by confocal microscopy (FLUOVIEW FV3000, Olympus) of

embryos dissected from the Arabidopsis transgenic lines expressing the corresponding GFP fusion proteins. Embryos were removed from ovules harvested at different stages and placed onto glass slides with 6% (v/v) glycerol solution [50]. GFP fluorescence was observed by excitation at 488-nm and 640-nm, and emission at 500-520-nm was detected. For chlorophyll autofluorescence, samples were excited at 488 nm and emission between 650-nm and 750-nm was detected. Seed morphology was obtained by scanning electron microscopy (SEM) with a HITACHI S-4800 SEM-FEG microscope operated at 2 kV. Previously, the samples were coated with gold.

2.5. Protein extraction, alkylation assays and Western blot analysis

Plant material, either isolated embryos from developing seeds or leaves from adult plants, was ground under liquid nitrogen to a fine powder. Extraction buffer (50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% (v/v) Nonidet P-40) was immediately added, mixed on a vortex, and centrifuged at 16,100 *g* at 4 °C for 20 min. Protein was quantified using the Bradford reagent (Bio-Rad). Alkylation assays were performed as previously described [40] using 60 mM iodoacetamide. Protein samples were subjected to SDS-PAGE under reducing (NTRC, 2-Cys Prxs and GFP) or non-reducing (FBPase and 2-Cys Prxs) conditions using acrylamide gel concentration of 9.5% (FBPase), or 15% (NTRC, 2-Cys Prxs and GFP). Resolved proteins were transferred to nitrocellulose membranes and probed with the indicated antibodies. Specific antibodies for NTRC [25] and 2-Cys Prxs [29] were previously raised in our laboratory. The anti-FBPase was kindly provided by Dr. Sahrawy (Estación Experimental del Zaidín, Granada, Spain). The anti-GFP antibody was purchased from Agrisera.

2.6. Total fatty acids extraction and analysis

Total fatty acid content and composition from Arabidopsis dry seeds were determined using the whole seed acid-catalyzed transmethylation protocol [51]. The fatty acid methyl esters were analyzed by gas chromatography using a GC-MS-QP2010 Plus equipment (Shimadzu, Kyoto, Japan) fitted with a Suprawax 280 capillary column (10-m length, 0.1-mm i.d. and 0.1-μm film thickness) and one quadrupole mass detector. Helium was used as the carrier gas at a flow rate of 16.3 ml min^{-1}. Ion source was held at a temperature of 200 °C, and an interphase temperature of 280 °C. Heptadecanoic acid was used as internal standard for fatty acids content calculation.

3. Results

3.1. NTRC and 2-Cys Prxs show different expression patterns during seed development

The finding that the Arabidopsis mutant *2cpab*, which is devoid of 2-Cys Prxs A and B, shows defective post-germinative growth [47] suggests a relevant function of plastid redox systems during seed development. It is well established the functional relationship of 2-Cys Prxs and NTRC in the redox balance of leaves chloroplasts, however, little is known about the contribution of these enzymes at early stages of plant development. Thus, to address this issue, we first sought to determine the pattern of expression of NTRC and 2-Cys Prxs throughout embryogenesis and seed development. Western blot analysis of protein extracts from seeds harvested at early (4 dpa), medium (6 dpa), and late (9 dpa) stages of development revealed the presence of NTRC and 2-Cys Prxs in embryos and showed a slight increase of the contents of both enzymes during embryogenesis progression, though at levels significantly lower than in leaves (Fig. 1A and B). To further analyze the pattern of expression of the *NTRC* and *2-Cys PRX A* and *B* genes and the localization of the corresponding proteins during embryogenesis, their respective coding sequences, C-terminally fused to GFP, were expressed under the control of their endogenous promoters in the *ntrc* and *2cpab* mutant

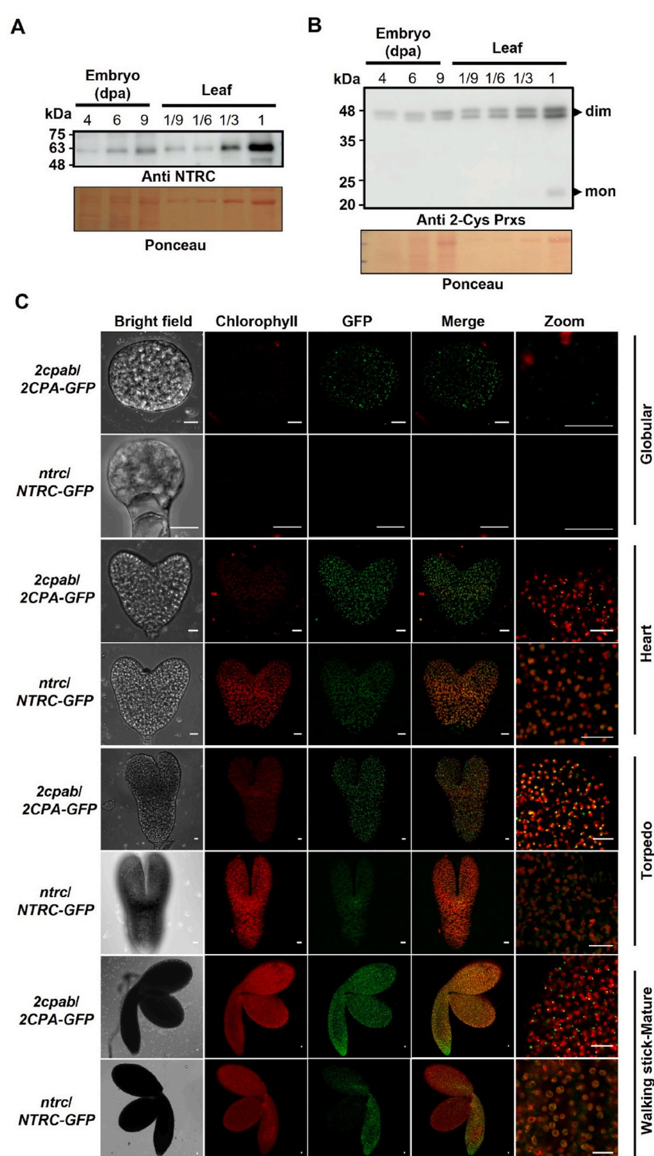


Fig. 1. Expression pattern of NTRC and 2-Cys Prx A in Arabidopsis embryos. Western blot analysis of the levels of NTRC (A) and 2-Cys Prxs (B) in extracts from embryos harvested at 4, 6 and 9 dpa, as indicated, and from rosette leaves from WT plants grown under long-day conditions for 25 days. Embryo protein extracts, 13 μg, and aliquots of 1.44 μg (1/9), 2.17 μg (1/6), 4.33 μg (1/3) and 13 μg (1) of leaf extracts, as indicated, were subjected to SDS–PAGE under reducing (NTRC) or non-reducing (2-Cys Prxs) conditions, transferred to nitrocellulose filters, and probed with anti-NTRC or anti-2-Cys Prxs antibodies. Molecular mass markers (kDa) are indicated at the left and even loading was monitored by Ponceau staining. Monomeric (mon) and dimeric (dim) forms of 2-Cys Prxs are indicated at the right. (C) Expression pattern analysis of 2-Cys Prx A and NTRC in embryos from *2cpab/2CPA-GFP* and *ntrc/NTRC-GFP* plants at different stages of embryogenesis, as indicated. From left to right, panels show representative confocal images corresponding to bright field, chlorophyll red autofluorescence, GFP fluorescence, merged of green and red channels and a higher magnification view of the overlay. For *ntrc/NTRC-GFP* panels, the intensity of the green channel was increased 5-fold. For zoom images, the intensity of the red channel was increased 3-fold. Scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

backgrounds, respectively (Figs. S1A–C). Among the transformants, transgenic plants expressing GFP fusions to NTRC (Fig. S1D) or 2-Cys Prxs A and B (Fig. S1E) in the *ntrc* or *2cpab* genetic backgrounds, respectively, here termed *ntrc/NTRC-GFP* (Fig. S1B), *2cpab/2CPA-GFP*

and *2cpab/2CPB-GFP* (Fig. S1C), were selected for confocal microscopy analyses. The 2-Cys Prx A (Fig. 1C) and 2-Cys Prx B (Fig. S2) genes showed a similar pattern of expression throughout embryogenesis. The expression of both genes was detected at low level at globular stage, the GFP signal being enhanced at later stages embryogenesis (Fig. 1C, Fig. S2), in agreement with the Western blot data. The pattern of expression of the *NTRC* gene was slightly different as no signal was detected at the globular stage and GFP fluorescence remained lower than that of 2-Cys Prxs at later stages (Fig. 1C). The signal of the three GFP fusion proteins was coincident with chlorophyll autofluorescence (Fig. 1C; Fig. S2), indicating their plastid localization. However, relevant differences of the pattern of localization of the three proteins were observed. While the magnified images showed the uniformly distributed signal of NTRC, probably reflecting the stromal localization of the enzyme, 2-Cys Prxs A and B were detected as speckles (Fig. 1C; Fig. S2), suggesting that these proteins may form aggregates at specific sites of the embryo plastid.

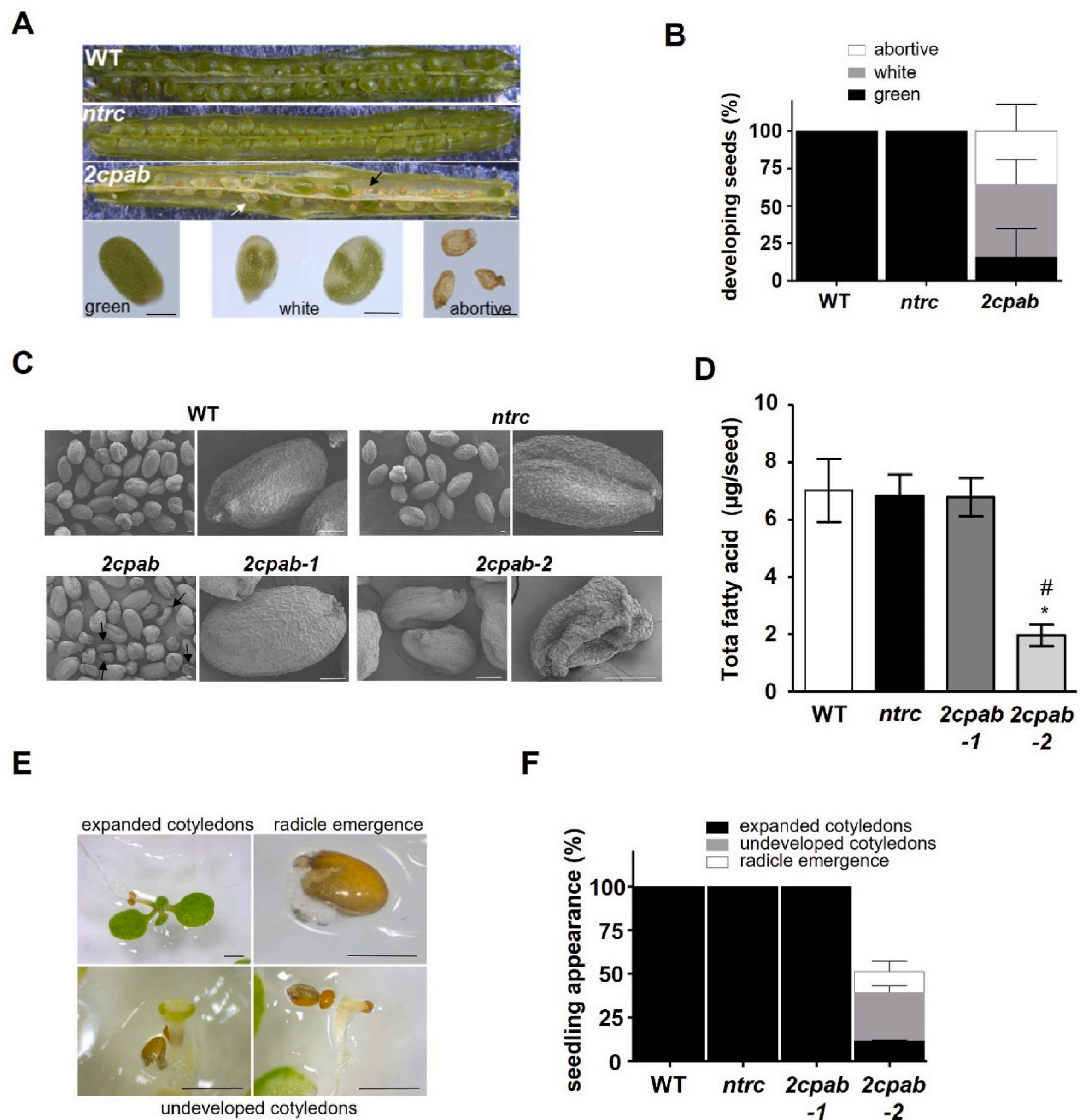


Fig. 2. Comparative analysis of seed development in WT and *ntrc* and *2cpab* mutants. (A) Siliques of the wild type and the *ntrc* and *2cpab* mutants were harvested at 10 dpa from plants grown under long day photoperiod, and enlarged images of representative green, white and aberrant seeds. Selected white and abortive seeds in the *2cpab* silique are indicated by white and black arrows, respectively. Scale bars: 200 µm. (B) Percentages of green, white, and abortive seeds in the indicated lines, represented as mean values ± SEM from four replicates (siliques from different plants). (C) Scanning electron microscope images of mature seeds of the indicated genotypes. Enlarged images of normal (WT, *ntrc* and *2cpab-1*) and abnormal (*2cpab-2*) seeds. Scale bars: 1000 µm. (D) Total fatty acid content from WT, *ntrc*, *2cpab-1* and *2cpab-2* mature dry seeds, shown as the mean ± SD of five independent replicates of 20 seeds. Asterisk indicates statistical significance compared with the *2cpab-1* and hash indicates statistical significance compared to WT according to one-way ANOVA with a Bonferroni post-tests ($p < 0.05$). (E) Seedling phenotypes yielded by the *2cpab-2* mutant seeds after 8 days on MS synthetic medium under long day photoperiod. Scale bars: 10 µm. (F) Among the germinated seeds, the percentages of seedlings displaying developed or undeveloped cotyledons and the emergence of the radicle is represented as mean values ± SEM from four replicates of at least 20 seeds.

3.2. 2-Cys Prxs, but not NTRC, contribute to seed development

Once established the pattern of expression of NTRC and 2-Cys Prxs A and B in developing seeds, we sought to determine their impact on seed development by analyzing this developmental process in the *ntrc* and the *2cpab* mutants. When grown under long-day photoperiod, siliques of the *ntrc* mutant harvested at 10 dpa contained exclusively green seeds, hence being indistinguishable from those of the WT (Fig. 2A and B). In contrast, only a minor proportion (~15%) of the developing seeds in siliques of the *2cpab* mutant were green, most of them presenting abnormal morphologies ranging from white to brown/wrinkled (labeled as abortive), at a frequency of ~50% and ~35%, respectively (Fig. 2A and B). The light regime during plant growth affected the seed phenotype of the *2cpab* mutant as shown by the higher contents of white seeds (~70%) and lower contents of abortive seeds (~15%) in plants grown under continuous light, whereas short day conditions caused the opposite effect lowering the contents of white seeds (~40%) and increasing that of abortive seeds (~50%) (Fig. S3). The contents of green seeds in siliques of the *2cpab* mutant remained invariable under any of these growth conditions (Fig. S3). In contrast with the severe seed development phenotype of the *2cpab* mutant, the *ntrc* mutant showed exclusively green seeds under any of the light conditions tested (Fig. 2A and B; Fig. S3). The different contribution of 2-Cys Prxs and NTRC to seed development was also reflected in the morphology of the mature dry seeds. Scanning microscopy analysis showed that seeds of the *ntrc* mutant displayed WT appearance whereas *2cpab* plants produced both WT-like seeds, labeled as *2cpab-1*, as well as seeds displaying heterogenous sizes and morphologies, referred as *2cpab-2* (Fig. 2C).

Green oilseeds accumulate lipids as energy reserves in the form of triacylglycerol (TAG) and embryo photosynthesis has been proposed to provide reducing equivalents and ATP for fatty acid synthesis [8]. Thus, to further test the effect of impaired plastid redox regulation during seed development, the fatty acid content in dry seeds of the *ntrc* and *2cpab* mutants was analyzed. In agreement with the lack of effect of NTRC on seed development, the total fatty acid content of *ntrc* mutant and WT seeds was indistinguishable (Fig. 2D). Notably, the fatty acid content of the *2cpab-1* seeds showing a WT-like appearance were like that of the *ntrc* mutant and the WT, in clear contrast with the markedly lower content of the *2cpab-2* abnormal seeds (Fig. 2D). Moreover, fatty acid composition in seeds of the different lines under analysis showed only minor differences among WT, *ntrc* and *2cpab-1* seeds, whereas abnormal *2cpab-2* seeds showed a remarkable accumulation of 16:0 and 18:0 fatty acids and lower contents of 18:1, 18:2 and, particularly, 18:3 and 20:1 fatty acids (Fig. S4). Such effects on fatty acid content and composition prompted us to investigate the germination capacity, scored as the emergence of the radicle, and early seedling development of the different lines under study. Like the WT and the *ntrc* mutant seeds, the *2cpab-1* seeds germinated normally on synthetic medium, yielding green seedlings with fully expanded cotyledons after 8 days of imbibition (Fig. 2E and F). On the contrary, abnormal *2cpab-2* seeds displayed impaired germination since only ~50% of the seeds had germinated after 5 days of imbibition, most of them yielding non-viable seedlings with undeveloped cotyledons (Fig. 2E and F), though a minor proportion of these seeds yielded normal seedlings. Thus, the lack of 2-Cys Prxs severely affects seed development and post-germinative growth. Moreover, the almost WT seed phenotype of the *ntrc* mutant reveals the different function of 2-Cys Prxs and NTRC in developing seeds and leaves.

3.3. The lack of 2-Cys Prxs causes embryogenesis delay and seed development arrest

To get more insight into the effect of the lack of 2-Cys Prxs on seed development, a comparative study of the progression of embryogenesis in WT and *2cpab* seeds was performed. At 4 dpa, developing seeds of the *2cpab* mutant contained more embryos at globular stage (~95%) than the WT (~75%), in which ~25% of embryos were already at the transition stage (Fig. 3A), hence indicating a slight delay of embryogenesis caused by the lack of 2-Cys Prxs since early stages. Such delay became aggravated at later stages as shown by the low frequency (~20%) of embryos in developing seeds of the *2cpab* mutant that had reached the torpedo stage at 6 dpa, as compared with the ~80% observed in the WT (Fig. 3A). Similarly, by 8 dpa, all the embryos of the WT seeds had reached the mature stage whereas most of the embryos of the *2cpab* mutant were still at earlier developmental stages, torpedo (~53%) and heart (~23%), only ~24% of them reaching the mature stage (Fig. 3A).

To further investigate whether delayed embryogenesis ultimately leads to seed abortion in embryos devoid of 2-Cys Prxs, siliques of the WT and the *2cpab* mutant harvested at 11 dpa were dissected and the embryo stage was scored in green, white, and abortive seeds (Fig. 3B). At this time, all embryos from green seeds had progressed to the mature stage, regardless of the genetic background. On the contrary, most of the embryos in white seeds were at earlier stages of development, torpedo

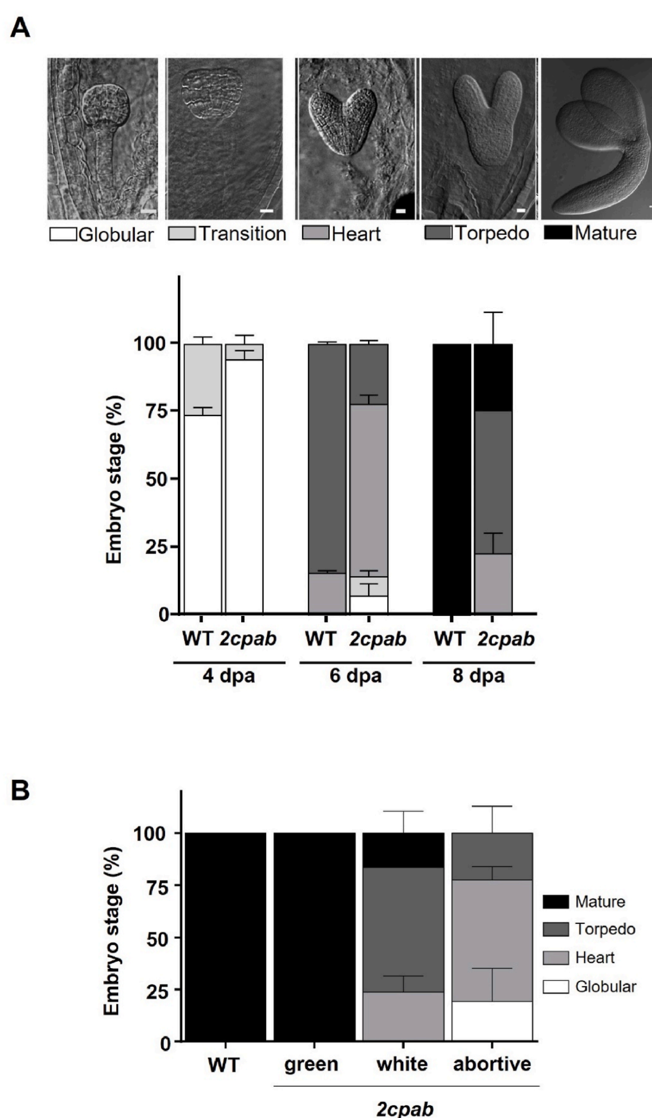


Fig. 3. Comparative analysis of embryo development in WT and *2cpab* mutant. (A) Enlarged images of representative embryos at globular, transition, heart, torpedo, and mature stages. Percentages of embryos at the indicated stages in seeds from WT and *2cpab* plants harvested at 4, 6 and 8 dpa. (B) Percentages of embryos at the indicated developmental stages in WT and green, white, and abortive *2cpab* developing seeds harvested at 10 dpa. Values are mean ± SEM from four replicates (siliques from different plants).

(~60%) and heart (~24%), the remaining (~16%) reaching the mature stage. On the contrary, none of the embryos in abortive seeds of the *2cpab* mutant had reached the mature stage, however, embryos progressed beyond the globular stage, most of them being arrested at the heart stage (~59%), which is consistent with the seedling phenotypes observed upon germination (Fig. 2E and F). Taken together, these findings reveal the prominent role of 2-Cys Prxs during embryogenesis, which becomes more essential at the heart and torpedo stages. Developmental arrest at these stages in the seeds devoid of 2-Cys Prxs may ultimately lead to abortion.

3.4. The function of 2-Cys Prxs on seed development is independent of NTRC-mediated redox regulation

The different progression of embryogenesis in the *2cpab* and the *ntrc* mutants may indicate that plastid oxidizing signals, mediated by 2-Cys Prxs, play a more relevant role than NTRC-dependent reducing signals in this developmental process. Thus, to clarify the molecular basis of the effects of 2-Cys Prxs at this early stage of plant development, we analyzed seed phenotypes and the progression of embryogenesis in Arabidopsis lines in which the redox balance of plastid embryos was altered in different ways. First, we analyzed the seed and embryo phenotypes of an Arabidopsis line over expressing NTRC under the control of the cauliflower mosaic virus (CaMV) 35S promoter (NTRC-OE), which was previously reported [41]. Western blot analysis showed highly increased levels of NTRC in embryos of the NTRC-OE line, as compared with the WT, though the contents of the enzyme tended to decline as embryogenesis progressed (Fig. 4A). As expected, the higher contents of NTRC in embryos of the NTRC-OE line provoked the over reduction of 2-Cys Prxs as shown by the increased contents of the monomeric, hence reduced form of the enzyme in comparison with the WT (Fig. 4B). However, the contents of green seeds in siliques harvested at 10 dpa (Fig. 4C) and the number of embryos that reached maturity at this timepoint (Fig. 4D) were like those of the WT and the *ntrc* mutant, here included for comparison. Thus, these results further confirm the lack of effect of NTRC at this developmental stage.

As a second approach to test the relevance of the redox activity of 2-Cys Prxs for embryogenesis, we generated Arabidopsis transgenic plants expressing a mutated version of 2-Cys Prx A in which the peroxidatic Cys residue (C_P) at the active site of the enzyme was replaced by Ser. To this end, the mutant 2CPA-C_P-S and WT versions of 2-Cys Prx A (2CPA) were fused to a c-myc tag and expressed in the *2cpab* mutant background under the control of the promoter of the endogenous Arabidopsis *2-Cys PRX A* gene (Fig. S5A). The two myc-tagged versions of 2-Cys Prx A showed similar levels of expression in the transgenic plants (Fig. S5B), however, in contrast with the WT version of 2-Cys Prx A, the expression of the mutated 2CPA-C_P-S enzyme did not complement neither the growth inhibition phenotype (Figs. S5A and C) nor the light-dependent reduction of FBPase (Figs. S5D and E) in the *2cpab* mutant, hence confirming the lack of functionality of the mutated enzyme. Remarkably, most of the seeds of the Arabidopsis line expressing the 2CPA-C_P-S enzyme were either white (~52%) or abortive (~38%), whereas the expression of the WT version of 2-Cys Prx A almost completely restored the WT seed phenotype of the *2cpab* mutant (Fig. 5A), indicating that the peroxidatic cysteine is essential for the function of the enzyme in embryo plastids. Moreover, embryo dissection of these seeds revealed embryogenesis arrest predominantly at heart and torpedo stages (Fig. 5B), like the *2cpab* mutant, further confirming the inability of the mutant version of 2-Cys Prx A to restore the impaired embryogenesis caused by the lack of 2-Cys Prxs. It should be noted that the levels of 2-Cys Prxs in transgenic lines, corresponding to the 2-Cys Prx A isoform, is markedly lower than in WT plants (Fig. S5B), suggesting that decreased levels of these enzymes are sufficient to restore the *2cpab* mutant phenotype. To further explore this possibility, seed development and embryogenesis were also tested in the *Δ2cp* mutant, which is a severe knock down containing no 2-Cys Prx B and very low amounts of 2-Cys Prx A [48]. Siliques of this mutant harvested at 10 dpa showed almost WT levels of green seeds (Fig. 5A), which contained mature embryos (Fig. 5B), hence indicating that decreased contents of 2-Cys Prxs are sufficient for normal embryogenesis and seed development. Taken together, these data further confirm the negligible effect of NTRC on embryogenesis and seed development and establish that developmental defects caused by the absence of 2-Cys Prxs extend beyond redox signaling mediated by NTRC.

4. Discussion

To date, most studies of chloroplast redox regulation have been performed on leaves from adult plants, i.e., in fully developed chloroplasts, hence, little is known about the biological significance of this regulatory mechanism at early stages of development. Therefore, the central objective of this work was to analyze the relevance of plastid redox regulation at initial stages of plant development, i.e., during embryogenesis and seed formation, focusing on the roles of NTRC and 2-Cys Prxs, the enzymes playing a key function controlling redox homeostasis in leaf chloroplasts [19]. Though at lower level than in leaves, NTRC and 2-Cys Prxs are expressed in developing embryos, the contents of these enzymes increasing as embryogenesis progresses (Fig. 1A and B). Moreover, transgenic plants expressing GFP-tagged fusions of these proteins under their endogenous gene promoters confirmed this pattern of expression and showed the plastid localization of these enzymes in developing embryos. At early globular stage, the expression of 2-Cys Prxs was low and no NTRC expression was detected, however, the expression of these genes increased at heart and torpedo stages (Fig. 1A–C and S2), when embryo chloroplasts differentiate [6,9,52]. A remarkable difference between NTRC and 2-Cys Prxs was their pattern of localization within embryo plastids since 2-Cys Prxs are detected as speckles, whereas NTRC fluorescence entirely overlapped with chlorophyll autofluorescence (Fig. 1C and S2), which is consistent with the localization of these enzymes in mesophyll protoplasts and cells reported by previous studies [53,54]. Thus, the raise of expression of NTRC and 2-Cys Prxs coincides with embryo chloroplast differentiation suggesting that the function of these enzymes at these early stages becomes more relevant in embryos containing differentiating chloroplasts.

In leaves, NTRC and 2-Cys Prxs act as a redox couple, hence the functions of these enzymes in maintaining the chloroplast redox balance are tightly linked [40]. Strikingly, NTRC and 2-Cys Prxs appear to exert a different contribution at early stages of plant development as shown by the analysis of developing seeds devoid of each of these enzymes. By 10 dpa, most seeds in siliques of the *2cpab* mutant displayed abnormal development (Fig. 2A and B) and produced a high proportion of mature dry seeds with abnormal size and morphology (Fig. 2C), which showed impaired post-germinative growth (Fig. 2E and F). Conversely, the siliques of the *ntrc* mutant contained exclusively green seeds, indistinguishable from those of the WT (Fig. 2A and B) and generated WT-like mature seeds (Fig. 2C), which showed no alteration of post-germinative development (Fig. 2E and F). Therefore, the severe impairment of seed development in the *2cpab* mutant and the almost WT seed development of the *ntrc* mutant uncovers remarkable differences in the operation of these plastid redox systems in developing seeds and leaves in which the lack of NTRC causes severe effects on photosynthesis performance and plant growth [25,29,30]. The lack of effect of NTRC on seed development is somehow unexpected because it is well established that embryos of developing seeds perform photosynthesis [5–7]. Thus, it would be expected that redox regulatory mechanisms playing relevant roles in adjusting photosynthetic metabolism in mature chloroplasts would perform similarly in embryo photosynthesis. However, unlike leaves, which are directly exposed to sunlight, the light reaching developing embryos is filtered through the seed coat and pericarp, thereby being of lower intensity and enriched in the green and far-red wavelengths of the spectrum [5]. Thus, the different effect of NTRC in embryos and leaf chloroplasts suggests that the activity of NTRC is unnecessary under the light conditions of embryo photosynthesis, whereas

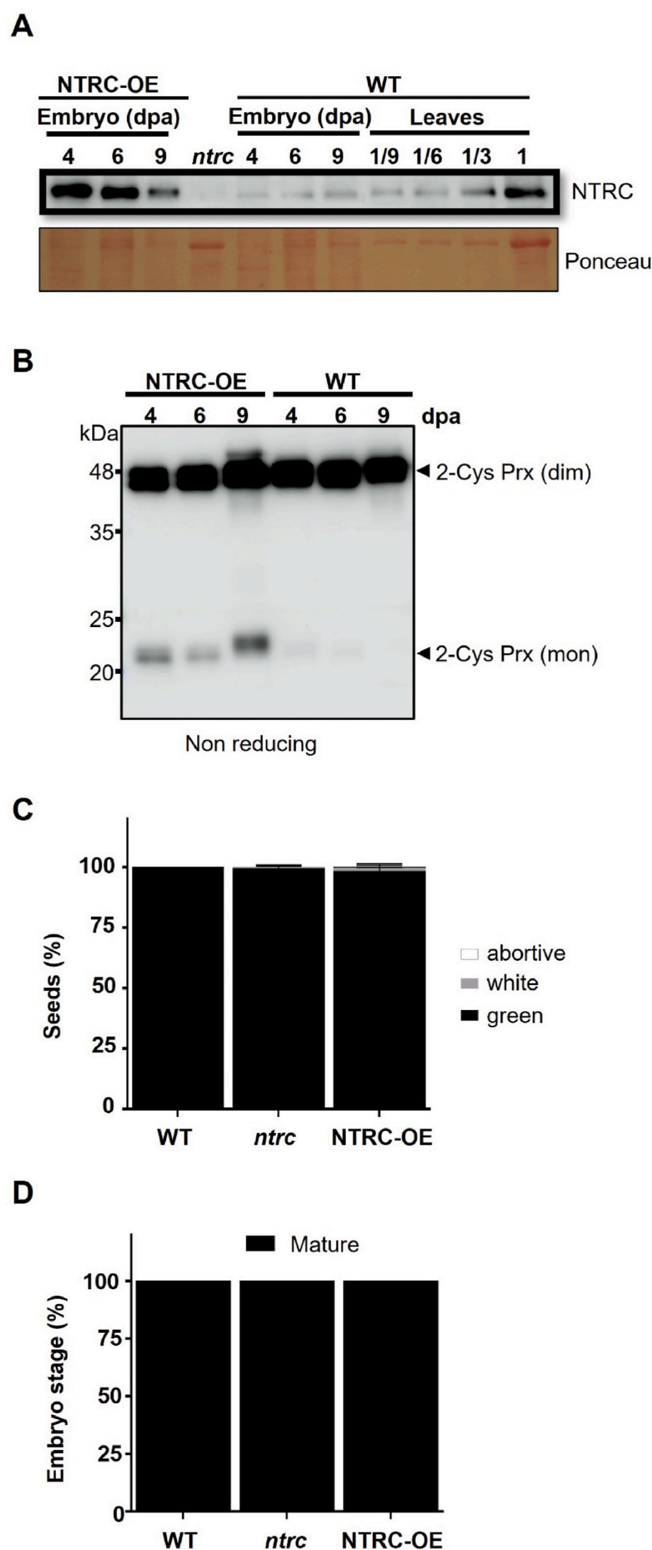


Fig. 4. The effect of plastid redox imbalance on seed and embryo development. (A) Western blot analysis of the levels of NTRC in extracts from embryos of WT and NTRC-OE plants harvested at 4, 6, and 9 dpa and rosette leaves from WT plants grown under long day conditions for 3 weeks. Embryo protein extracts,13 μg, aliquots of 1.44 μg (1/9), 2.17 μg (1/6), 4.33 μg (1/3) and 13 μg (1) of WT leaf extracts and 13 μg of *ntrc* leaf extracts (negative control), as indicated, were subjected to SDS–PAGE under reducing conditions. Even loading was monitored by Ponceau staining. (B) Western blot analysis of the levels of monomeric (mon) and dimeric (dim) forms of 2-Cys Prxs in WT and NTRC-OE embryos at 4, 6 and 9-dpa. Embryo protein extracts, 13 μg, were subjected by SDS–PAGE under non reducing conditions, transferred to nitrocellulose blots, and probed with anti-NTRC (A) or anti-2-Cys Prxs (B) antibodies. Molecular mass markers (kDa) are indicated on the left. (C) Percentages of green, white, and abortive seeds (C) and mature embryos (D) within siliques harvested at 10 dpa from WT and NTRC-OE plants. Values represent mean ± SEM from three replicates (siliques from different plants).

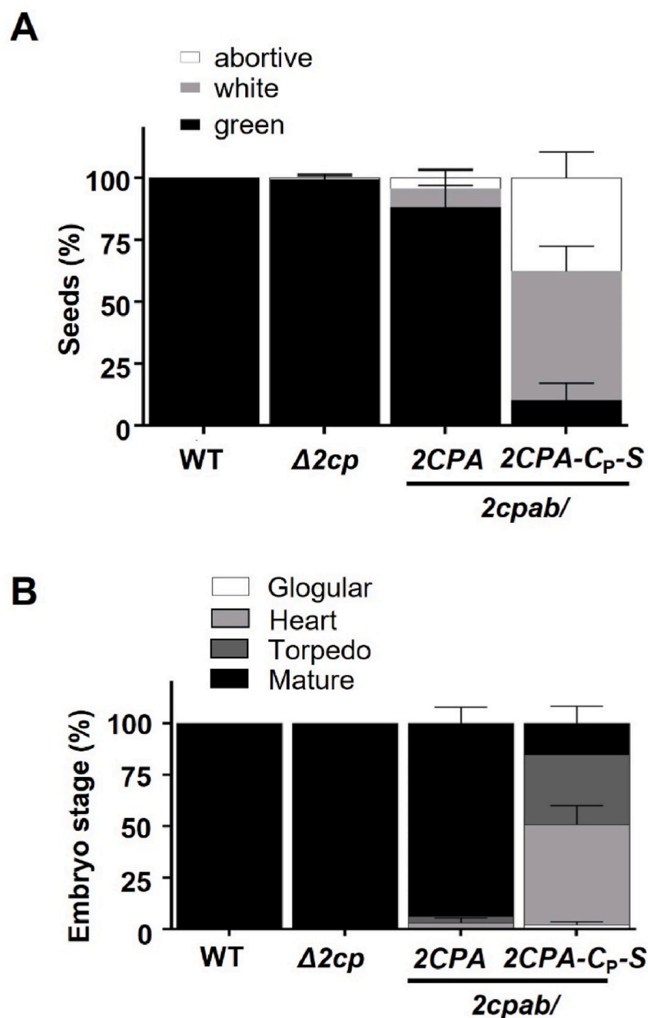


Fig. 5. Effect of decreased contents of 2-Cys Prx A and the mutation of the peroxidatic cysteine of the enzyme on seed and embryo development. Percentages of green, white, and abortive seeds (A) and developmental stage of embryos (B) within siliques harvested at 10 dpa from WT, the *Δ2cp* mutant and transgenic lines expressing the WT (2CPA) or mutant (2CPA-C_P-S) version of 2-Cys Prx A in the *2cpab* mutant background. Values represent mean ± SEM from five replicates (siliques from different plants).

it becomes essential under the more variable and stressful light conditions in mature leaves.

An intriguing result of this study is the heterogeneous seed phenotypes of the *2cpab* mutant ranging from almost WT-like to abortive seeds (Fig. 2A–C), which may indicate that the severity of the lack of 2-Cys Prxs at this developmental stage depends on the specific conditions of each of the developing seed in a silique. One such conditions might be the light intensity as suggested by the different effects of continuous light, short-day (Fig. S3) and long-day (Fig. 2B) on seed development of the *2cpab* mutant. Moreover, developing seeds devoid of plastid 2-Cys Prxs show delayed embryogenesis (Fig. 3A) and embryo arrest at heart and torpedo stages (Fig. 3B), when embryo chloroplasts differentiate. These stages of embryogenesis are also characterized by the increased synthesis and accumulation of lipids, in agreement with our results showing lower contents (Fig. 2D) and altered composition (Fig. S4) of fatty acids in abnormal seeds of the *2cpab* mutant. Altogether, these results suggest that the relevant role of 2-Cys Prxs during seed development is required for proper differentiation of embryo chloroplasts. It is known that chloroplasts biogenesis is essential for normal seed development and, consequently, mutations perturbing chloroplast functions often lead to embryo-defective phenotypes and production of abnormal seeds. In this regard, it is worth noting that the abnormal seeds observed in siliques of the *2cpab* mutant (Fig. 2A) resembles those of the Arabidopsis *mgd1* mutant, which contain traces levels of the major chloroplast lipid monogalactosyldiacylglycerol (MGDG), affecting embryo thylakoid membranes organization and chloroplast biogenesis [55].

Photosynthetic performance by chloroplasts from adult leaves is adjusted by reductive and oxidative signaling, in which 2-Cys Prxs play a central role [56]. The redox balance of 2-Cys Prxs is controlled by NTRC, which is the most efficient reductant of the enzyme [27,29,57]. Work performed in leaves has established that NTRC and 2-Cys Prxs exert opposing effects on the redox balance of chloroplast enzymes. In the light, the lack of NTRC causes under reduction of stromal enzymes, whereas the lack of 2-Cys Prxs causes their over reduction [32,58]. Similarly, in the dark, the lack of NTRC causes accelerated oxidation of these enzymes [47], whereas the lack of 2-Cys Prxs causes delayed oxidation [41–43]. Therefore, the negligible effect of NTRC on seed development (Fig. 2A–D) indicates that impairment of reductive signals within plastids do not interfere with proper embryo development, in contrast with the severe effect of the impairment of oxidative signals, mediated by 2-Cys Prxs. These findings raise the issue of the mode of action of plastid 2-Cys Prxs in embryogenesis and seed development, which was addressed in this work by analyzing the effect of plastid redox imbalance at these early stages of development. Arabidopsis lines overexpressing NTRC, which causes 2-Cys Prxs over reduction in plastid embryos (Fig. 4A and B), led to no significant alteration of seed development nor delayed embryogenesis (Fig. 4C and D), supporting the notion that the effect of 2-Cys Prxs at these developmental stages is independent of the redox balance of the enzyme.

It is known that 2-Cys Prxs are multifunctional enzymes that show peroxidase and chaperone activity [59], thus, an alternative possibility is that the function of 2-Cys Prxs in embryo plastids is exerted via its chaperone activity. In support of this notion, the chaperone activity of 2-Cys Prxs depends on the formation of aggregates [59–61], which is in line with the detection of 2-Cys Prx A and B as speckles (Fig. 1C and Fig. S2A), suggesting the presence of these enzymes in aggregates localized at defined locations in plastids of developing seeds. Moreover, the formation of 2-Cys Prx aggregates, hence the chaperone activity of the enzyme, requires the peroxidatic Cys residue at its active site [62,63]. The failure of the 2CPA-C_P-S mutant version of 2-Cys Prx A, in which the peroxidatic Cys residue was replaced by Ser, to recover normal embryogenesis and seed development when expressed in the *2cpab* mutant background (Fig. 5A and B) further supports a possible chaperone function of 2-Cys Prxs in plastid embryos, which might be more relevant when chloroplasts are differentiating and, thus, nuclear-encoded proteins translocated into the organelle are properly folded. However, whether the localization of 2-Cys Prxs A and B in speckles is due exclusively to the formation of aggregates or the participation of these enzymes in larger protein complexes requires further analysis. Previous reports have shown the relevance of chloroplast chaperones such as chaperonin 60α for embryogenesis [64]. Indeed, the Arabidopsis *schlepperless* mutant, which is defective in chloroplast chaperonin-60α, shows retardation of embryo development before of the heart stage [65], and a temperature-sensitive mutation affecting chaperonin-60α2 shows altered embryonic photosynthesis and delayed embryogenesis at restrictive low temperature [7]. Likewise, co-chaperones and chaperones such as chloroplast GrpE homologues (CGEs) and heat shock protein 90 (HSP90C), respectively, are essential for embryo viability [66,67], hence resembling the phenotypes of the *2cpab* mutant. The finding that Arabidopsis mutants defective in protein translocation to the chloroplast show abortive seeds [68,69] further shows the relevance of chloroplast differentiation in seed development. A possible function of 2-Cys Prxs as chaperones during embryo chloroplast differentiation could be the assembly of photosynthetic complexes, however, whether this effect is exerted directly or by aiding the folding

activity of other chaperones remains to be elucidated.

5. Conclusions

The analysis of seed development and embryogenesis in Arabidopsis mutants lacking NTRC or 2-Cys Prxs uncovers a relevant function of 2-Cys Prxs at these early stages of plant development. The severe effects of the lack of 2-Cys Prxs, and the almost WT seed phenotype of the *ntrc* mutant and the NTRC-OE line indicates that the activity of 2-Cys Prxs at these early stages of development is independent of NTRC, which reveals important differences in the operation of these redox systems in fully developed chloroplasts from leaves and differentiating chloroplasts during embryogenesis. Embryo arrest of the *2cpab* mutant at heart and torpedo stages suggests a relevant role of 2-Cys Prxs in chloroplast differentiation, which occurs at these stages of embryogenesis. Since this activity is independent of NTRC, a likely possibility is that the function of 2-Cys Prxs in embryogenesis is exerted via its chaperone activity.

Disclosures

Authors have no conflict of interest.

Funding

This work was supported by Grant PID2020-115156 GB-I00 funded by Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación/ 10.13039/501100011033. A.M.G-M. was supported by a FPI predoctoral contract (PRE2018-085831) from Ministerio de Ciencia e Innovación (Spain), J.J-L. by a post-doctoral contract from Consejo Superior de Investigaciones Científicas (Spain) and M.L.H by a post-doctoral contract from the VIPPI-Universidad de Sevilla.

Declaration of competing interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We acknowledge the technical assistance of Alicia Orea (Instituto de Bioquímica Vegetal y Fotosíntesis, Spain) for confocal microscopy, Carlos Parejo (Instituto de Bioquímica Vegetal y Fotosíntesis, Spain) for fatty acid analyses, and María Carmen Jiménez (Instituto de Ciencia de Materiales, Spain) for scanning microscopy. The anti-FBPase antibody was kindly provided by Dr. Sahrawy, Estación Experimental del Zaidín, CSIC, Spain.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102645.

References

[1] R.B. Goldberg, G. de Paiva, R. Yadegari, Plant embryogenesis: zygote to seed, Science 266 (1994) 605–614, https://doi.org/10.1126/science.266.5185.605.
[2] B.H. Le, et al., Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors, Proc. Natl. Acad. Sci. USA 107 (2010) 8063–8070, https://doi.org/10.1073/pnas.1003530107.
[3] M. Iwasaki, S. Penfield, L. Lopez-Molina, Parental and environmental control of seed dormancy in Arabidopsis thaliana, Annu. Rev. Plant Biol. 73 (2022) 355–378, https://doi.org/10.1146/annurev-arplant-102820-090750.
[4] C.A. ten Hove, K.J. Lu, D. Weijers, Building a plant: cell fate specification in the early Arabidopsis embryo, Development 142 (2015) 420–430, https://doi.org/10.1242/dev.111500.
[5] G. Allorent, et al., Adjustments of embryonic photosynthetic activity modulate seed fitness in Arabidopsis thaliana, New Phytol. 205 (2015) 707–719, https://doi.org/10.1111/nph.13044.
[6] J.T. Puthur, A.M. Shackira, P.P. Saradhi, D. Bartels, Chloroembryos: a unique photosynthesis system, J. Plant Physiol. 170 (2013) 1131–1138, https://doi.org/10.1016/j.jplph.2013.04.011.
[7] A. Sela, et al., Embryonic photosynthesis affects post-germination plant growth, Plant Physiol 182 (2020) 2166–2181, https://doi.org/10.1104/pp.20.00043.
[8] S.A. Ruuska, J. Schwender, J.B. Ohlrogge, The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes, Plant Physiol 136 (2004) 2700–2709, https://doi.org/10.1104/pp.104.047977.
[9] G. Allorent, F. Courtois, F. Chevalier, S. Lerbs-Mache, Plastid gene expression during chloroplast differentiation and dedifferentiation into non-photosynthetic plastids during seed formation, Plant Mol. Biol. 82 (2013) 59–70, https://doi.org/10.1007/s11103-013-0037-0.
[10] H. Rolletschek, L. Borisjuk, M. Koschorreck, U. Wobus, H. Weber, Legume embryos develop in a hypoxic environment, J. Exp. Bot. 53 (2002) 1099–1107, https://doi.org/10.1093/jexbot/53.371.1099.
[11] H. Rolletschek, et al., Evidence of a key role for photosynthetic oxygen release in oil storage in developing soybean seeds, New Phytol. 167 (2005) 777–786, https://doi.org/10.1111/j.1469-8137.2005.01473.x.
[12] B.B. Buchanan, The path to thioredoxin and redox regulation in chloroplasts, Annu. Rev. Plant Biol. 67 (2016) 1–24, https://doi.org/10.1146/annurev-arplant-043015-111949.
[13] B.B. Buchanan, A. Holmgren, J.P. Jacquot, R. Scheibe, Fifty years in the thioredoxin field and a bountiful harvest, Biochim. Biophys. Acta 1820 (2012) 1822–1829, https://doi.org/10.1016/j.bbagen.2012.07.006.
[14] P. Geigenberger, I. Thormählen, D.M. Daloso, A.R. Fernie, The unprecedented versatility of the plant thioredoxin system, Trends Plant Sci. 22 (2017) 249–262, https://doi.org/10.1016/j.tplants.2016.12.008.
[15] Y. Meyer, C. Belin, V. Delorme-Hinoux, J.P. Reichheld, C. Riondet, Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance, Antioxidants Redox Signal. 17 (2012) 1124–1160, https://doi.org/10.1089/ars.2011.4327.
[16] L. Nikkanen, J. Toivola, M.G. Díaz, E. Rintamäki, Chloroplast thioredoxin systems: prospects for improving photosynthesis, Philos. Trans. Royal Soc. London. Series B, Biological sciences (2017) 372, https://doi.org/10.1098/rstb.2016.0474.
[17] M. Zaffagnini, et al., Redox homeostasis in photosynthetic organisms: novel and established thiol-based molecular mechanisms, Antioxidants Redox Signal. 31 (2019) 155–210, https://doi.org/10.1089/ars.2018.7617.
[18] P. Schürmann, B.B. Buchanan, The ferredoxin/thioredoxin system of oxygenic photosynthesis, Antioxidants Redox Signal. 10 (2008) 1235–1274, https://doi.org/10.1089/ars.2007.1931.
[19] F.J. Cejudo, M.C. González, J.M. Pérez-Ruiz, Redox regulation of chloroplast metabolism, Plant Physiol 186 (2021) 9–21, https://doi.org/10.1093/plphys/kiaa062.
[20] L. Nikkanen, E. Rintamäki, Chloroplast thioredoxin systems dynamically regulate photosynthesis in plants, Biochem. J. 476 (2019) 1159–1172, https://doi.org/10.1042/BCJ20180707.
[21] F. Montrichard, et al., Thioredoxin targets in plants: the first 30 years, J. Proteonomics 72 (2009) 452–474, https://doi.org/10.1016/j.jprot.2008.12.002.
[22] L. Michelet, et al., Redox regulation of the Calvin-Benson cycle: something old, something new, Front. Plant Sci. 4 (2013) 470, https://doi.org/10.3389/fpls.2013.00470.
[23] J. Yu, et al., Plant chloroplast stress response: insights from thiol redox proteomics, Antioxidants Redox Signal. 33 (2020) 35–57, https://doi.org/10.1089/ars.2019.7823.
[24] A.J. Serrato, J. Fernández-Trijueque, J.D. Barajas-López, A. Chueca, M. Sahrawy, Plastid thioredoxins: a "one-for-all" redox-signaling system in plants, Front. Plant Sci. 4 (2013) 463, https://doi.org/10.3389/fpls.2013.00463.
[25] A.J. Serrato, J.M. Pérez-Ruiz, M.C. Spínola, F.J. Cejudo, A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana, J. Biol. Chem. 279 (2004) 43821–43827, https://doi.org/10.1074/jbc.M404696200.
[26] P. Bernal-Bayard, M. Hervás, F.J. Cejudo, J.A. Navarro, Electron transfer pathways and dynamics of chloroplast NADPH-dependent thioredoxin reductase C (NTRC), J. Biol. Chem. 287 (2012) 33865–33872, https://doi.org/10.1074/jbc.M112.388991.
[27] F. Alkhalfioui, M. Renard, F. Montrichard, Unique properties of NADP-thioredoxin reductase C in legumes, J. Exp. Bot. 58 (2007) 969–978, https://doi.org/10.1093/jxb/erl248.
[28] J.C. Moon, et al., Overexpression of Arabidopsis NADPH-dependent thioredoxin reductase C (AtNTRC) confers freezing and cold shock tolerance to plants, Biochem. Biophys. Res. Commun. 463 (2015) 1225–1229, https://doi.org/10.1016/j.bbrc.2015.06.089.
[29] J.M. Pérez-Ruiz, et al., Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage, Plant Cell 18 (2006) 2356–2368, https://doi.org/10.1105/tpc.106.041541.
[30] A. Lepisto, et al., Chloroplast NADPH-thioredoxin reductase interacts with photoperiodic development in Arabidopsis, Plant Physiol 149 (2009) 1261–1276, https://doi.org/10.1104/pp.108.133777.
[31] B. Naranjo, et al., The chloroplast NADPH thioredoxin reductase C, NTRC, controls non-photochemical quenching of light energy and photosynthetic electron

transport in Arabidopsis, Plant Cell Environ. 39 (2016) 804–822, https://doi.org/10.1111/pce.12652.
[32] I. Thormählen, et al., Thioredoxin f1 and NADPH-dependent thioredoxin reductase C have overlapping functions in regulating photosynthetic metabolism and plant growth in response to varying light conditions, Plant Physiol 169 (2015) 1766–1786, https://doi.org/10.1104/pp.15.01122.
[33] L.R. Carrillo, J.E. Froehlich, J.A. Cruz, L. Savage, D.M. Kramer, Multi-level regulation of the chloroplast ATP synthase: the chloroplast NADPH thioredoxin reductase C (NTRC) is required for redox modulation specifically under low irradiance, Plant J. 87 (2016) 654–663, https://doi.org/10.1111/tpj.13226.
[34] A. Lepisto, et al., Deletion of chloroplast NADPH-dependent thioredoxin reductase results in inability to regulate starch synthesis and causes stunted growth under short-day photoperiods, J. Exp. Bot. 64 (2013) 3843–3854, https://doi.org/10.1093/jxb/ert216.
[35] J. Michalska, H. Zauber, B.B. Buchanan, F.J. Cejudo, P. Geigenberger, NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts, Proc. Natl. Acad. Sci. USA 106 (2009) 9908–9913, https://doi.org/10.1073/pnas.0903559106.
[36] J.M. Pérez-Ruiz, M. Guinea, L. Puerto-Galán, F.J. Cejudo, NADPH thioredoxin reductase C is involved in redox regulation of the Mg-chelatase I subunit in Arabidopsis thaliana chloroplasts, Mol. Plant 7 (2014) 1252–1255, https://doi.org/10.1093/mp/ssu032.
[37] A.S. Richter, et al., Posttranslational influence of NADPH-dependent thioredoxin reductase C on enzymes in tetrapyrrole synthesis, Plant Physiol 162 (2013) 63–73, https://doi.org/10.1104/pp.113.217141.
[38] M. González, V. Delgado-Requerey, J. Ferrández, A. Serna, F.J. Cejudo, Insights into the function of NADPH thioredoxin reductase C (NTRC) based on identification of NTRC-interacting proteins in vivo, J. Exp. Bot. 170 (2019) 5787–5798, https://doi.org/10.1093/jxb/erz326.
[39] K. Yoshida, T. Hisabori, Two distinct redox cascades cooperatively regulate chloroplast functions and sustain plant viability, Proc. Natl. Acad. Sci. USA 113 (2016) E3967–E3976, https://doi.org/10.1073/pnas.1604101113.
[40] J.M. Pérez-Ruiz, B. Naranjo, V. Ojeda, M. Guinea, F.J. Cejudo, NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus, Proc. Natl. Acad. Sci. USA 114 (2017) 12069–12074, https://doi.org/10.1073/pnas.1706003114.
[41] V. Ojeda, J.M. Pérez-Ruiz, F.J. Cejudo, 2-Cys peroxiredoxins participate in the oxidation of chloroplast enzymes in the dark, Mol. Plant 11 (2018) 1377–1388, https://doi.org/10.1016/j.molp.2018.09.005.
[42] M.J. Vaseghi, et al., The chloroplast 2-cysteine peroxiredoxin functions as thioredoxin oxidase in redox regulation of chloroplast metabolism, Elife 7 (2018), https://doi.org/10.7554/eLife.38194.
[43] K. Yoshida, A. Hara, K. Sugiura, Y. Fukaya, T. Hisabori, Thioredoxin-like2/2-Cys peroxiredoxin redox cascade supports oxidative thiol modulation in chloroplasts, Proc. Natl. Acad. Sci. USA 115 (2018) E8296–E8304, https://doi.org/10.1073/pnas.1808284115.
[44] F.J. Cejudo, V. Ojeda, V. Delgado-Requerey, M. González, J.M. Pérez-Ruiz, Chloroplast redox regulatory mechanisms in plant adaptation to light and darkness, Front. Plant Sci. 10 (2019) 380, https://doi.org/10.3389/fpls.2019.00380.
[45] Y. Yokochi, Y. Fukushi, K.I. Wakabayashi, K. Yoshida, T. Hisabori, Oxidative regulation of chloroplast enzymes by thioredoxin and thioredoxin-like proteins in Arabidopsis thaliana, Proc. Natl. Acad. Sci. U.S.A. 118 (2021), e2114952118, https://doi.org/10.1073/pnas.2114952118.
[46] J. Awad, et al., 2-Cysteine peroxiredoxins and thylakoid ascorbate peroxidase create a water-water cycle that is essential to protect the photosynthetic apparatus under high light stress conditions, Plant Physiol 167 (2015) 1592–1603, https://doi.org/10.1104/pp.114.255356.
[47] V. Ojeda, J. Jiménez-López, F.J. Romero-Campero, F.J. Cejudo, J.M. Pérez-Ruiz, A chloroplast redox relay adapts plastid metabolism to light and affects cytosolic protein quality control, Plant Physiol 187 (2021) 88–102, https://doi.org/10.1093/plphys/kiab246.
[48] P. Pulido, et al., Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in Arabidopsis thaliana chloroplasts, J. Exp. Bot. 61 (2010) 4043–4054, https://doi.org/10.1093/jxb/erq218.
[49] S.J. Clough, A.F. Bent, Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana, Plant J. 16 (1998) 735–743, https://doi.org/10.1046/j.1365-313x.1998.00343.x.
[50] J. Feng, L. Ma, A method for characterizing embryogenesis in Arabidopsis, JoVE 126 (2017), https://doi.org/10.3791/55969.
[51] Y. Li-Beisson, et al., Acyl-lipid metabolism, Arabidopsis Book 11 (2013), e0161, https://doi.org/10.1199/tab.0161.
[52] R.I. Tejos, A.V. Mercado, L.A. Meisel, Analysis of chlorophyll fluorescence reveals stage specific patterns of chloroplast-containing cells during Arabidopsis embryogenesis, Biol. Res. 43 (2010) 99–111, https://doi.org/10.4067/S0716-97602010000100012.
[53] K. Kirchsteiger, J. Ferrández, M.B. Pascual, M. González, F.J. Cejudo, NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in Arabidopsis, Plant Cell 24 (2012) 1534–1548, https://doi.org/10.1105/tpc.111.092304.
[54] M. Muthuramalingam, et al., Multiple redox and non-redox interactions define 2-Cys peroxiredoxin as a regulatory hub in the chloroplast, Mol. Plant 2 (2009) 1273–1288, https://doi.org/10.1093/mp/ssp089.
[55] K. Kobayashi, M. Kondo, H. Fukuda, M. Nishimura, H. Ohta, Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis, Proc. Natl. Acad. Sci. USA 104 (2007) 17216–17221, https://doi.org/10.1073/pnas.0704680104.
[56] N. Lampl, et al., Systematic monitoring of 2-Cys peroxiredoxin-derived redox signals unveiled its role in attenuating carbon assimilation rate, Proc. Natl. Acad. Sci. USA 119 (2022), e2119719119, https://doi.org/10.1073/pnas.2119719119.
[57] J.C. Moon, et al., The C-type Arabidopsis thioredoxin reductase ANTR-C acts as an electron donor to 2-Cys peroxiredoxins in chloroplasts, Biochem. Biophys. Res. Commun. 348 (2006) 478–484, https://doi.org/10.1016/j.bbrc.2015.06.089.
[58] V. Ojeda, V.A. Nájera, M. González, J.M. Pérez-Ruiz, F.J. Cejudo, Photosynthetic activity of cotyledons is critical during post-germinative growth and seedling establishment, Plant Signal. Behav. 12 (2017), e1347244, https://doi.org/10.1080/15592324.2017.1347244.
[59] K.J. Dietz, Peroxiredoxins in plants and cyanobacteria, Antioxidants Redox Signal. 15 (2011) 1129–1159, https://doi.org/10.1089/ars.2010.3657.
[60] E.S. Lee, C.H. Kang, J.H. Park, S.Y. Lee, Physiological significance of plant peroxiredoxins and the structure-related and multifunctional biochemistry of peroxiredoxin 1, Antioxidants Redox Signal. 28 (2018) 625–639, https://doi.org/10.1089/ars.2017.7400.
[61] M. Liebthal, D. Maynard, K.J. Dietz, Peroxiredoxins and redox signaling in plants, Antioxidants Redox Signal. 28 (2018) 609–624, https://doi.org/10.1089/ars.2017.7164.
[62] J. Konig, et al., The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone, J. Exp. Bot. 64 (2013) 3483–3497, https://doi.org/10.1093/jxb/ert184.
[63] M. Liebthal, M.S. Kushwah, P. Kukura, K.J. Dietz, Single molecule mass photometry reveals the dynamic oligomerization of human and plant peroxiredoxins, iScience 24 (2021), 103258, https://doi.org/10.1016/j.isci.2021.103258.
[64] X. Ke, et al., Functional divergence of chloroplast Cpn60alpha subunits during Arabidopsis embryo development, PLoS Genet. 13 (2017), e1007036, https://doi.org/10.1371/journal.pgen.1007036.
[65] N.R. Apuya, et al., The Arabidopsis embryo mutant schlepperless has a defect in the chaperonin-60alpha gene, Plant Physiol 126 (2001) 717–730, https://doi.org/10.1104/pp.126.2.717.
[66] H. Inoue, M. Li, D.J. Schnell, An essential role for chloroplast heat shock protein 90 (Hsp90C) in protein import into chloroplasts, Proc. Natl. Acad. Sci. USA 110 (2013) 3173–3178, https://doi.org/10.1073/pnas.1219229110.
[67] P.H. Su, H.Y. Lin, Y.H. Lai, Two Arabidopsis chloroplast GrpE homologues exhibit distinct biological activities and can form homo- and hetero-oligomers, Front. Plant Sci. 10 (2019) 1719, https://doi.org/10.3389/fpls.2019.01719.
[68] A. Baldwin, et al., A molecular-genetic study of the Arabidopsis Toc75 gene family, Plant Physiol 138 (2005) 715–733, https://doi.org/10.1104/pp.105.063289.
[69] T. Inaba, et al., Arabidopsis tic110 is essential for the assembly and function of the protein import machinery of plastids, Plant Cell 17 (2005) 1482–1496, https://doi.org/10.1105/tpc.105.030700.