Exploring the oxidative side of chloroplast redox regulation in *Arabidopsis thaliana*

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ABSTRACT

Plants are subjected to changes in light conditions, so they have a mechanism for redox regulation of photosynthetic enzymes that allows them to acclimate to different light intensity situations. The redox regulation is mainly based on the activation (reduction) of photosynthetic enzymes during the day and their inactivation (oxidation) at night. This study focuses on analyzing the mechanism of oxidative redox regulation and the role of an atypical thioredoxin (TRX) called Cdsp32 (chloroplastic drought-induced stress protein of 32 kDa), whose function is still unknown. It is thought that Cdsp32 may act together with atypical TRXs to activate 2-Cysteine-Peroxiredoxin (2cp), which allows the detoxification of hydrogen peroxide (H_2O_2) to water (H_2O). Furthermore, a protein called NADPH TRX reductase C (NTRC) may control the activity of Cdsp32. Therefore, from previous articles, it is understood that Cdsp32 reduces and activates the 2cp protein. In addition, it is known that NTRC also reduces the 2cp. However, the function of Cdsp32 in the oxidative redox regulation and its functional relationship with 2cp and NTRC are still unknown. By confocal microscopy, it was observed that Cdsp32 is localized in the chloroplasts of mesophyll cells, and co-localized with 2cp. The levels and redox status of Cdsp32 in *Arabidopsis thaliana* wild-type, *2cpab* and *ntrc* were studied by Western Blot. It was observed that 2cp and NTRC may be functionally related with Cdsp32, as the lack of these proteins is balanced by a higher amount of Cdsp32. Moreover, in mutants deficient of 2cp or NTRC, Cdsp32 is reduced in light and dark conditions, so the redox status of Cdsp32 does not change in the light-to-dark transition. In addition, the effect of Cdsp32 overexpression on the transgenic lines *wt/P_{DEX}-Cdsp32-HA* and *2cpab/P_{DEX}-Cdsp32-HA* were analyzed under two types of growth conditions: with dexamethasone (DEX) and without DEX. DEX induces the overexpression of Cdsp32. Under conditions with DEX, the transgenic lines *wt/P_{DEX}-Cdsp32-HA* were albino. When we analyzed the phenotypic effect, we observed that Cdsp32 overexpression induces the premature senescence. The most important objective of the study was to analyze the effect of Cdsp32 on the redox status of photosynthetic enzymes. In the dark-to-light transition, the overexpression of Cdsp32 (*35S-Cdsp32-cmyc*) in *Nicotiana benthamiana* leaves does not affect the redox status of the enzymes. The redox status of enzymes was similar

in control and the gene construct *35S-Cdsp32-cmyc*. The photosynthetic enzymes were reduced (therefore activated) in light and oxidized (therefore inactivated) in dark. However, in the light-to-dark transition, the overexpression of Cdsp32 accelerates the reduction and delays oxidation of target enzymes (FBPase and ATPc). Overall, these results suggests that Cdsp32 does not function as an oxidant TRX.

Keywords: photosynthesis, redox regulation, chloroplast, light, dark, enzymes, thioredoxin

INTRODUCTION

Redox regulation is a universal regulatory mechanism present in all types of organisms, from bacteria and fungi to plants and animals, that promotes energy saving and cellular energy exchange (Geigenberger et al., 2021). Redox regulation is based on post-translational changes in the redox state of cysteine residues, which can rapidly and reversibly alter protein functions, thereby modulating biological processes (Meng et al., 2021). The thiol group of cysteines is very sensitive to oxidizing conditions, so it can react with hydrogen peroxide and be oxidized rapidly (Cejudo et al., 2019). When it is reduced, it can react with another cysteine to form a disulphide bridge (Cejudo et al., 2019).

In plants, redox regulation is a central element in adjusting metabolism and development to the prevailing environmental conditions (Telman et al., 2020). Thiol-based redox regulation is essential to ensure that chloroplast metabolism responds rapidly to changes in light intensity (Yoshida et al., 2015). Redox reactions enable the regulation of photosynthesis and photorespiration processes, as it allows the reductive activation of photosynthetic enzymes under light conditions, and their rapid oxidative inactivation in darkness (Yoshida et al., 2019). Our study is focused on the mechanism of redox regulation in the model plant *Arabidopsis thaliana*. Thiol-dependent redox regulation begins when the electron transport chain, during the day, captures photons of light and sends reducing power to ferredoxin (FDX) (Serrato et al., 2021) (Figure 1). At this point, two different pathways can occur: the FDX-FTR-TRX pathway and the NTRC pathway. NTRC is an "NADPH-dependent TRX reductase C with a joint TRX domain" (Cejudo et al., 2019). Previous articles have shown that the NTRC-dependent redox regulatory pathways play an essential role in proper plant development and photosynthetic efficiency (Yoshida and Hisabori, 2016).

Thioredoxins (TRXs), which play a central role in the redox regulation, are small polypeptides that present a folding called TRX-fold (Cejudo et al., 2021). There are two types of TRXs: typical (TRXs *m*, *f*, *x*, *y*, *z*) and atypical TRXs (TRX L2, ACHT 1 and ACHT 4). In the FDX-FTR-TRX pathway, FDX is reduced and sends its electrons to typical TRXs, with the action of the enzyme FDX-dependent TRX reductase (FTR) (Serrato et al., 2021). Typical thioredoxins, such as TRXs m, TRXs f and TRXs x, reduce target enzymes, which become activated (Serrato et al., 2021). However, in the dark, the target enzymes are oxidized (therefore inactivated) by atypical TRXs such as TRX L2, ACHT 1 and ACHT 4, which transfer reducing power to 2-Cys PRX (2cp). The 2cp protein enables the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) (Figure 1). Cdsp32 (chloroplastic drought-induced stress protein of 32 kDa) (Rey et al., 2005) is an atypical TRX which may also act together with atypical TRX L2, ACHT 1 and ACHT 4 in the inactivation of chloroplast enzymes. Therefore, Cdsp32 may be involved in this process of

oxidation of the target enzymes (such as ATPc and FBPase). The 2cp protein may be the target of the Cdsp32 protein and NTRC may control the activity of Cdsp32 (Figure 1).

Our study focuses on studying the function of the Cdsp32 protein in the oxidative side of chloroplast redox regulation. The aim of the study is to provide a deeper understanding of the role of Cdsp32 in the redox regulation (mainly in the oxidative side of the redox regulation), its effect in the redox status of putative target enzymes (such as ATPc and FBPase) and its functional relationship with 2cp, which is a target protein of Cdsp32, and NTRC, which may reduce Cdsp32. To this end, several transgenic lines have been used: two lines overexpressing the Cdsp32 protein in a wild-type genetic background (*wt/P_{DEX}-Cdsp32-HA*), and another line overexpressing the Cdsp32 protein in a *2cpab* genetic background (*2cpab/P_{DEX}-Cdsp32-HA*). These transgenic lines are inducible by dexamethasone (DEX), which is a synthetic glucocorticoid. In addition, the *2cpab* (lacks 2cp protein) and *ntrc* mutants have been studied. All these mutant and transgenic lines allow the study and characterization of the Cdsp32 protein. The chloroplast localization of Cdsp32 and co-localization with 2cp have been defined by confocal microscopy. In addition, the levels of Cdsp32 in *Arabidopsis thaliana* adult plants and seedlings were analyzed by Western Blot. To identify the redox status of Cdsp32 in wild-type and mutant lines *2cpab* and *ntrc*, Western Blot was performed using the alkylating agent MMPEG24. On the other hand, the effect of Cdsp32 overexpression on seedlings and adult plants was studied. Finally, the effect of Cdsp32 on the redox status of photosynthetic enzymes under light and dark conditions was analyzed by alkylation assay with IAA (iodoacetamide).

MATERIALS AND METHODS

Biological material and growth conditions

Arabidopsis thaliana wild-type (ecotype Columbia), the mutant lines *2cpab* and *ntrc*, and the transgenic lines *wt/P_{DEX}-Cdsp32-HA* and *2cpab/P_{DEX}-Cdsp32-HA,* were used in the study. Plants were grown in soil substrate in growth chambers at 22ºC and a light intensity of 125 μE m^{-2} s^{-1} under different photoperiods: long-day conditions (16-hour light/8-hour darkness); and short-day conditions (8-hour light/16-hour darkness). Seedlings were grown in a phytotron in plates with two different culture media: MS (Murashige and Skoog) medium and MS with DEX (30 μM). To study the effect of Cdsp32 in adult plants, *Arabidopsis* wild-type, the mutant lines *2cpab* and the transgenic lines *wt/P_{DEX}-Cdsp32-HA* and *2cpab/P_{DEX}-Cdsp32-HA* (lines 2 and 5) were sprayed with two different solutions: control solution (0.1% Tween) and solution with DEX (DEX 30 μM + 0.1% Tween). The plants were subjected to these conditions for 14 days before Imaging-PAM.

Bacterial strains of the species *Agrobacterium tumefaciens* containing a T-DNA engineered with different gene(s) of interest (*35S-Cdsp32-cmyc*, *35S-2cpA-CFP-HA* and *35S-Cdsp32-YFP-HA*) (Table 1) were used in the study. They were grown at 28ºC and agroinfiltrated in *Nicotiana benthamiana*. *Nicotiana* was cultivated in soil substrate in growth chambers under 16-hour light/8-hour darkness photoperiod at 22 ºC and a light intensity of 125 μE m^{-2} s^{-1}.

Agroinfiltration in *Nicotiana benthamiana* plants

Foliar infiltration of Agrobacterium strains in *Nicotiana* allows transient expression of the transgene and thus facilitates the study of proteins of interest in plants. The co-expression of the strain p19 suppresses the gene silencing mechanism of *Nicotiana*, so all bacterial strains were mixed with the strain p19 (Jay et al., 2023). First, inocula of the bacteria strains were prepared with LB medium containing the antibiotics rifampicin (50 mg/ml), gentamicin (20 mg/ml), and kanamycin (50 mg/ml), these antibiotics allow only these strains of bacteria to grow. The cultures remained in the incubator at 28°C (optimum temperature for growth) for 24 hours. The absorbance of liquid cultures was measured at 600 nm, and tubes of strains were prepared with the p19 mixture. The tubes were centrifuged for 5 minutes at 5000 rpm at 4°C and the pellet was resuspended in agroinfiltration buffer (10 mM MES pH 5.7, 10 mM $MgCl_2$, 200 µM acetosyringone). The tubes were incubated for 2 hours. Finally, the strains were agroinfiltrated on *Nicotiana* with a syringe.

Confocal microscopy

Nicotiana benthamiana plants were used 3 days after infiltration with Agrobacterium. This technique was performed on the bacterial strains with Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP): p19 + *35S-Cdsp32-YFP-HA*, p19 + *35S-2cpA-CFP-HA*, and p19 + *35S-Cdsp32-YFP-HA* + *35S-2cpA-CFP-HA*. These fluorescent proteins provide information of the localization of 2cpA (to be observed in cyan) and Cdsp32 (to be observed in yellow). Leaf discs were obtained using a punch and observed under the confocal microscope. Several images of the mesophyll cells were obtained: bright field, photo of the fluorescent protein, image of the chlorophyll (in red) and the merge.

Protein extraction and western blot analysis

For protein extraction, plant leaves were ground in a mortar using liquid nitrogen and extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40 (v:v)) with protease inhibitors. Protein extracts were quantified using the Bradford method and separated on polyacrylamide gels. Gels were transferred to nitrocellulose membranes, stained with Ponceau staining as loading control and incubated with primary antibodies: anti-Cdsp32, anti-HA, anti-cmyc, anti-NTRC, anti-2cp, anti-FBPase, and anti-ATPc. For detecting each protein, the nitrocellulose membranes were incubated in the presence of a secondary antibody conjugated to the anti-rabbit horseradish peroxidase (HRP), except for the anti-cmyc membrane, which was incubated with a secondary antibody conjugated to the anti-mouse antibody.

Alkylating assays

For the alkylating assays with methyl-maleimide-(polyethylene glycol)24 (MMPEG24), leaves were weighed (approximately 30-40 mg) and grounded in a mortar using liquid nitrogen and alkylation buffer (2% SDS, 100 mM Tris-HCl pH 7.8, 5% glycerol and 8M urea) with MMPEG 10 mM. The sample control is used without MMPEG (only alkylation buffer). After trituration, 3.5 volumes of 10% TCA were added and homogenized. 100 µl was taken from the mortar and

incubated for 20 minutes on ice. It was centrifuged at maximum rpm for 10 minutes at 4°C and the supernatant was discarded. 200 µl of acetone was added to the pellet and centrifuged at maximum rpm for 10 minutes at 4°C. The pellet was removed and placed in a fume hood for 2 minutes. The pellet was resuspended in 100 µl alkylation buffer (2% SDS, 100 mM Tris-HCl pH 7.8, 5% glycerol and 8M urea) and incubated 20 minutes at room temperature (RT). It was heated to 70°C for 5 minutes and centrifuged at RT for 5 minutes at maximum rpm, and the supernatant was transferred to PCR strips. Protein extracts were separated on polyacrylamide gels, transferred on nitrocellulose membranes, stained with Ponceau staining as loading control and incubated with primary antibodies: anti-FBPase and anti-Cdsp32. For detecting each protein, the nitrocellulose membranes were incubated in the presence of a secondary antibody conjugated to the anti-rabbit horseradish peroxidase (HRP).

For the Alkylation with iodoacetamide (IAA), leaves were weighed (approximately 30-40 mg) and grounded in a mortar using liquid nitrogen and 3.5 volumes of alkylation buffer (2% SDS, 100 mM Tris-HCl pH 7.8, 5% glycerol and 8M urea) with IAA 60 mM. It was incubated at RT for 20 minutes and heated for 5 minutes at 70ºC. It was centrifuged at maximum rpm at room temperature for 15 minutes. Then the supernatant was transferred to PCR strip and loaded. Protein extracts were separated on polyacrylamide gels. Gels were transferred to nitrocellulose membranes, stained with Ponceau staining as loading control and incubated with primary antibodies: anti-NTRC, anti-2cp, anti-Cdsp32, anti-FBPase, anti-HA, anti-cmyc and anti-ATPc. For detecting each protein, the nitrocellulose membranes were incubated in the presence of a secondary antibody conjugated to the anti-rabbit horseradish peroxidase (HRP).

Determination of photosynthetic parameters

The measurements were performed with an IMAGING-PAM chlorophyll fluorometer on plants adapted to dark for 20-30 minutes. Applying saturation pulses of actinic light, the different parameters were calculated. Photosynthetic parameters Y(II) (Quantum Yield of Photosystem II), Y(NPQ) (Non-Photochemical Quenching) and ETR (Electron Transport Rate) were measured. In the first place, the yield or quantum yield of PSII (Y(II)) indicates the energy that plants used for biochemical purposes (photosynthesis). Secondly, the Non-Photochemical Quenching (NPQ) is energy that is not used for biochemical purposes and is dissipated by fluorescence or as heat. Finally, the electron transport rate (ETR) was measured by the gradual increase of light intensity. Using GraphPad software, the graphs of induction-recovery curve (IRC) and light curve (LC) could be obtained.

RESULTS AND DISCUSSIONS

Determination of Cdsp32 localization and co-localization with 2cpA

To determine the localization of Cdsp32 and co-localization with 2cpA, confocal microscopy was used. Leaves of *Nicotiana benthamiana* infiltrated with bacterial strains of *Agrobacterium tumefaciens* were used in the study. Each strain has a T-DNA engineered with different constructs: "*35S-Cdsp32-YFP-HA*" and "*35S-2cpA-CFP-HA*". Cdsp32 protein was bound to a Yellow Fluorescent Protein (YFP) and 2cpA protein was bound to a Cyan Fluorescent Protein (CFP). Chlorophyll signal corresponds to chloroplasts, usually placed on the walls of the mesophyll, as the vacuole is very large and pushes them towards the wall. Mesophyll cells have many small chloroplasts in the wall (Figure 2). Using confocal microscopy, it was observed that the Cdsp32 protein (bound to the Yellow Fluorescent Protein, YFP) localize in chloroplasts of mesophyll cell of *Nicotiana* leaves (Figure 2A). In addition, the 2cpA protein (bound to the Cyan Fluorescent Protein, CFP) is also localized in chloroplasts (Figure 2B). Finally, to study the co-localization of the proteins, the mesophyll cells of a leaf expressing the *35S-Cdsp32-YFP-HA* and *35S-2cpA-CFP-HA* gene constructs were analyzed by confocal microscopy. It was found that there is co-localization between the Cdsp32 and 2cpA protein (Figure 2C) because both yellow and cyan signals overlap throughout the chloroplast. However, *Agrobacterium tumefaciens* has not transformed all mesophyll cells, so there are cells that do not express neither blue nor yellow signal. Moreover, not all chloroplasts will be marked by both proteins, some will only show the blue signal or the yellow signal. For example, in the image "2cpA-CFP" in Figure 2C, where the cells in the corners of the image do not express the yellow signal. Therefore, confocal microscopy results show that both Cdsp32 and 2cpA proteins are localized in the chloroplast. In addition, Cdsp32 co-localize with 2cpA, which may indicate that the proteins have related functions in the same biochemical process. Therefore, Cdsp32 may act with 2cp in thiol-dependent redox regulation.

Levels of Cdsp32 in wt and the mutants *2cpab* and *ntrc*

Cdsp32 and 2cp may be functionally related, however, it is not clear whether there is a functional relationship between NTRC and Cdsp32. Therefore, we first studied the Cdsp32 levels in *Arabidopsis* wild-type and mutant lines *2cpab* and *ntrc* in seedlings and adult plants. To study the Cdsp32 levels in seedlings, three culture plates were prepared with MS medium: wild-type, *2cpab* and *ntrc*. Seedlings were harvested after one week of growth under long-day conditions and stored in eppendorfs at -80°C. Then Western Blot was performed with primary antibodies: anti-Cdsp32, anti-NTRC, and anti-2cp (Figure 3A). In the results of the Western Blot with anti-Cdsp32, the different lines studied (wt, *2cpab* and *ntrc*) present the Cdsp32 protein (Figure 3A). However, there is apparently less Cdsp32 in the mutant line *ntrc*, but this is due to the fact that there is less load as show in the Ponceau staining. On the other hand, the *ntrc* mutant does not contain the NTRC protein and *2cpab* does not have the 2cp protein (Figure 3A). Therefore, it is confirmed that they are *ntrc* and *2cpab* mutants, as they lack the NTRC and 2cp proteins.

To analyze the Cdsp32 levels at the adult stage, plants were grown in soil substrate during 3-5 weeks in growth chambers under at 22ºC and a light intensity of 125 $\mu E\ m^{-2}\ s^{-1}$ under different photoperiods: long-day (LD) conditions and short-day (SD) conditions. Western Blot protein analysis showed that the mutant lines *2cpab* and *ntrc* contain more Cdsp32 protein than wild-

type in SD conditions. However, there are not clear differences between the wild-type and mutant lines in LD conditions (Figure 3B). Based on the results, it can be concluded that, in adult plants, the lack of NTRC or 2cp proteins is compensated by an increased amount of Cdsp32 protein (Figure 3B). Therefore, these proteins may be functionally related, as the lack of one protein is compensated by a higher amount of the other.

Analysis of the redox status of Cdsp32 in wt and the mutants *2cpab* and *ntrc* in adult plants

Arabidopsis wild-type and the mutant lines *2cpab* and *ntrc* were used to perform Western Blot of Alkylation with MMPEG. Plants were cultivated under long-day conditions (16-hour light/8-hour darkness) and were adapted to dark. Then, dark-adapted plants were subjected to light for 30 minutes. For each line (wt, *2cpab* and *ntrc*), two different plants adapted to darkness (D) and plants subjected to light for 30 minutes (L) were harvested. On the membrane with the anti-Cdsp32 antibody, the redox state is similar (completely reduced) in the different lines studied (wild-type, *2cpab* and *ntrc*) and in the different conditions (dark and light) (Figure 4A), unlike other targets enzymes, such as FBPase (Figure 4B). On the membrane with anti-FBPase, which is used in the study as a control, it is observed that, in the dark conditions, FBPase protein is oxidized, whereas in the light, it is reduced. However, there are differences in the mutant lines studied. The *2cpab* mutant contains a higher percentage of reduction in light conditions (91%) than the wild-type (43%), while in the *ntrc* mutant, FBPase is completely oxidized in light (Figure 4B). The results conclude that, regardless of the conditions (dark or light) and the mutant lines (*ntrc* or *2cpab*), Cdsp32 protein is completely reduced. Therefore, the lack of the NTRC protein or the lack of 2cp does not influence the redox state of Cdsp32.

Effect of Cdsp32 overexpression on plants at early stage of development

The *Cdsp32* mutant could not be used for the study because its homozygous form is lethal, so Cdsp32 overexpression lines were studied. This experiment was performed to compare the phenotype of *Arabidopsis* seedlings with Cdsp32 overexpression respect to seedlings with wt-levels of Cdsp32, in two different genetic backgrounds: wild-type and *2cpab* mutant background, which provides information on the phenotypic effect of plants lacking 2cp and overexpressing Cdsp32.

Seedlings of wild-type, mutant *2cpab* and transgenic lines *wt/P_{DEX}-Cdsp32-HA* and *2cpab/P_{DEX}-Cdsp32-HA* were grown with dexamethasone (+DEX) and without dexamethasone (-DEX) (Figure 5A). DEX induces the overexpression of Cdsp32 in the transgenic lines *wt/P_{DEX}-Cdsp32-HA* and *2cpab/P_{DEX}-Cdsp32-HA*. Therefore, the band corresponding to Cdsp32 is detected in the growth conditions with DEX, and not in the conditions without DEX (Figure 5A). Wild-type seedlings show green cotyledons, but the transgenic lines *wt/P_{DEX}-Cdsp32-HA* in the growth conditions with DEX are albino (Figure 5A). On the other hand, the mutant lines *2cpab*, which lack the 2cp protein, present green cotyledons. In addition, the transgenic lines *2cpab/P_{DEX}-Cdsp32-HA* are not affected when Cdsp32 is overexpressed but grow less under non-DEX conditions (lacking 2cp, and Cdsp32 is not overexpressed). Therefore, overexpression of Cdsp32 in the wild-type genetic background results in an absence of chlorophyll and thus albinism (Figure 5A). However, the overexpression of Cdsp32 in the *2cpab* mutant background (*2cpab/P_{DEX}-Cdsp32-HA*) does not affect the production of chlorophyll, so seedlings have green

cotyledons (Figure 5A). It could be concluded that the effect of Cdsp32 depends on 2cp. Cdsp32 is a thioredoxin that could act on the 2cp protein by allowing 2cp to change from its oxidized form (dimeric form) to its reduced form (monomeric form) (Cejudo et al., 2018).

Western Blot was performed on *Arabidopsis* wild-type and transgenic lines *wt/P_{DEX}-Cdsp32-HA* with primary antibodies: anti-HA (which only recognizes exogenous Cdsp32) and anti-Cdsp32 (which recognizes endogenous and exogenous Cdsp32) (Figure 5B). On the membrane with anti-HA, it is observed that only one band appears, corresponding to Cdsp32-HA in *wt/P_{DEX}-Cdsp32-HA* with DEX. Therefore, the transgenic lines *wt/P_{DEX}-Cdsp32-HA* expression is induced under conditions with DEX (Figure 5B). On the membrane with anti-Cdsp32, there is one band corresponding to the wild-type treated with DEX. Moreover, there is apparently less Cdsp32 in the wild-type without DEX, but because there is less load as show in the Ponceau staining (Figure 5B). Secondly, there is one band corresponding to Cdsp32-HA in *wt/P_{DEX}-Cdsp32-HA* under non-DEX conditions, because *wt/P_{DEX}-Cdsp32-HA* presents the endogenous Cdsp32. In addition, it is observed that there are two bands in transgenic lines *wt/P_{DEX}-Cdsp32-HA* under conditions with DEX: the top band corresponds to Cdsp32-HA, which has a higher molecular weight because of the HA tag, and the bottom band corresponds to the endogenous Cdsp32 protein. Moreover, on the membrane with anti-2cp, bands corresponding to 2cp were detected in all samples. However, there is apparently less 2cp in the wild-type without DEX and the transgenic *wt/P_{DEX}-Cdsp32-HA* with DEX, but due to the fact that there is less load as show in the Ponceau staining (Figure 5B).

On the other hand, Western Blot was performed with *Arabidopsis* wild-type, *2cpab* and *2cpab/P_{DEX}-Cdsp32-HA* seedlings (Figure 5C). In the membrane with anti-Cdsp32, there are bands in all samples, so endogenous Cdsp32 is expressed in the different lines studied (wt, *2cpab* and *2cpab/P_{DEX}-Cdsp32-HA*) (Figure 5C). On the membrane with anti-Cdsp32-HA, there is only one band corresponding to Cdsp32-HA in the transgenic line *2cpab/P_{DEX}-Cdsp32-HA* under DEX conditions, indicating that DEX induces the overexpression of Cdsp32-HA (Figure 5C). Finally, in the membrane with anti-2cp, bands corresponding to 2cp in the wild-type under DEX and non-DEX conditions were detected (Figure 5C), and there are not bands corresponding to 2cp in the mutant lines. The result confirms that the mutant lines *2cpab* and the transgenic lines *2cpab/P_{DEX}-Cdsp32-HA* lack the 2cp protein. Therefore, it cannot be concluded whether the lack of phenotypic effect on the *2cpab/P_{DEX}-Cdsp32-HA* transgenic lines (Figure 5A) is because it requires the presence of 2cp or Cdsp32 overexpression is not sufficiently induced, since the Cdsp32 detected levels were lower than in the wt background.

Effect of Cdsp32 overexpression on adult plants

To analyze the effect of Cdsp32 overexpression on adult plants, *Arabidopsis* transgenic lines *wt/P_{DEX}-Cdsp32-HA* were studied. *Arabidopsis* wild-type, mutant lines *2cpab* and transgenic lines *wt/P_{DEX}-Cdsp32-HA* (lines 2 and 5) were grown on soil and sprayed with two different solutions: control (0.1% Tween) and DEX (30 µM DEX + 0.1% Tween). After 9 days, plants of the transgenic lines *wt/P_{DEX}-Cdsp32-HA* under growth conditions with DEX became paler and smaller (Figure 6B) compared to control plants (Figure 6A). Then, after 14 days, necrotic leaves appeared in DEX-treated plants whereas plants under non-DEX conditions grew normally (Figure 6A, B). Therefore, these results indicate that the overexpression of Cdsp32 induces the premature senescence.

Imaging-PAM analysis was performed on wild-type (WT) and transgenic lines *wt/P$_{DEX}$-Cdsp32-HA* (2 and 5) after 3 days and 14 days under conditions with DEX (Figure 7A). Plants were adapted to 30 minutes of darkness before measurements were taken. Three parameters related to the photosynthetic activity of the samples can be obtained: Y(II) and Y(NPQ), corresponding to the respective quantum yields of the photochemical activity of PSII (Y(II)) and the dissipation of energy as heat (Y(NPQ)). Regarding Y(II), the first value corresponds to the maximum efficiency of PSII (Fv/Fm, which is the value of Y(II) when the plant is dark adapted). Plants use 70-80% of the light they receive, so the maximum quantum efficiency of PSII is between 0.7-0.8 in all samples. The photosynthetic efficiency of photosystem II (PSII) in the transgenic lines reaches the levels of wild-type plants and is not altered. In addition, the photosynthetic efficiency of PSII in transgenic line 5 (*wt/P$_{DEX}$-Cdsp32-HA*) reaches higher values in the different conditions: with and without DEX (Figure 7B and 7C). On the other hand, we analyzed the parameter Y(NPQ), which indicates the energy received by the plant that is not used in photochemistry and is dissipated in the form of heat as a defense mechanism against excessive light. When the actinic light used in the induction-recovery curve is similar to or lower than the growth light, there is no light stress and NPQ is not observed. Only a transient NPQ peak is observed when the light is switched on, which corresponds to the time it takes for the Calvin cycle to be activated after illumination and therefore the energy cannot be used in photochemistry. The main cause of this transient NPQ peak is the cyclic flux. After 3 days, the transgenic lines adequately perform this defense mechanism against excess light (except for the transgenic line 5, which emits hardly any energy in the form of heat) (Figure 7B). However, after 14 days, Y(NPQ) values decrease in the mutants with respect to the wild-type, especially in transgenic line 5. Moreover, at 14 days, the maximum NPQ reached by the mutants was lower than that reached by the wild-type (Figure 7C). Finally, in the light curve (LC), the different lines have similar ETR (Electron Transport Rate) values (Figure 7D). Overall, these results indicate that Cdsp32 does not affect the photosynthetic efficiency of PSII, because there are similar results in wt and transgenic lines *wt/P$_{DEX}$-Cdsp32-HA*, even the transgenic line 5 reaches higher values with and without DEX. However, the Cdsp32 affects the defense mechanism against excess light (NPQ). After 3 days, the transgenic line 5 emits hardly any NPQ energy, but the transgenic line 3 performs this defense mechanism adequately. At 14 days, the maximum NPQ of the transgenic lines (3 and 5) is lower than wild-type. Finally, Cdsp32 does not affect the electron transport rate.

Effect of Cdsp32 on the redox status of photosynthetic enzymes

The main objective of the research was to study the effect of Cdsp32 on the redox state of photosynthetic enzymes during the day and night. It has not been studied in *Arabidopsis* because the Cdsp32 overexpression induces plant senescence (Figure 6B). Moreover, we couldn't study the *Cdsp32* mutant because its homozygous form is lethal. Therefore, agroinfiltration of gene constructs *35S-Cdsp32-cmyc* was performed on a leaf of *Nicotiana benthamiana*, allowing transient expression of Cdsp32. The tag cmyc is small and easy to handle, so it was decided to focus on the effect of the *35S-Cdsp32-cmyc* strain on *Nicotiana*. To confirm that Cdsp32-cmyc is expressed, Western Blot was performed with the primary antibody anti-cmyc (Figure 8). On the membrane with anti-cmyc, there is only one band corresponding to Cdsp32-cmyc in leaves infiltrated with the gene construct *35S-Cdsp32-cmyc*, so agroinfiltration worked properly.

To analyze the effect of Cdsp32 on the activation (reduction) of enzymes in light conditions, *Nicotiana* leaves were agroinfiltrated with *Agrobacterium tumefaciens* containing gene constructs for *35S-Cdsp32-cmyc*. After infiltration, plants were grown under long-day conditions (16-hour light/8-hour darkness) for 3 days. Samples were harvested after 8 hours of darkness (D), and after 30 minutes at a light intensity of 175 μE m^{-2} s^{-1} and 22ºC (L). Leaves infiltrated and not infiltrated (control) of each type of sample were collected. Then Western Blot was performed with the primary antibodies: anti-FBPase, anti-ATPc and anti-2cp (Figure 9). On the membrane with anti-FBPase, the control and *35S-Cdsp32-cmyc* present similar results, the FBPase enzyme is reduced by approximately 70% in the presence of light. However, in the dark, FBPase is oxidized, as expected (Figure 9A). Secondly, on the membrane with anti-ATPc, both control and *35S-Cdsp32-cmyc* are completely reduced in light conditions. In the dark, the ATPc enzyme is oxidized (Figure 9B). Therefore, the photosynthetic enzymes are oxidized in dark conditions, as it was expected. Finally, on the anti-2cp membrane, the 2cp protein can be in dimeric (oxidized) or monomeric (reduced) form (Cerveau et al., 2019). Under light conditions, 2cp in leaves infiltrated with *35S-Cdsp32-cmyc* is more reduced (9%) than in the control (6%). However, they are completely oxidized in dark (Figure 9C). Therefore, it could be concluded that Cdsp32 does not affect the redox status of photosynthetic enzymes in the transition from night to day. The redox status of the enzymes FBPase and ATPc do not change in the transition dark-to-light in leaves agroinfiltrated. During the day, Cdsp32 does not act on the photosynthetic enzymes, so it does not reduce or oxidize the enzymes. In addition, 2cp is the target of Cdsp32, as Cdsp32 reduces and activates the 2cp protein (Cejudo et al., 2018). For this reason, the overexpression of Cdsp32 results in a higher reduction of 2cp compared to the control under light conditions.

To analyze the effect of Cdsp32 on how enzymes are oxidized in dark, *Nicotiana* leaves were agroinfiltrated with *Agrobacterium tumefaciens* containing constructs for *35S-Cdsp32-cmyc*. Plants were grown under long-day conditions (16-hour light/8-hour darkness). Dark-adapted plants were subjected to high light (HL) for 20-30 minutes at a light intensity of 500 μE m^{-2} s^{-1} and 28ºC. Two leaves infiltrated and not infiltrated (control) of each type of sample were collected 3 days after agroinfiltration. Then plants were subjected to dark for 5 minutes (D_5), 10 minutes (D_{10}) and 15 minutes (D_{15}), and two leaves of each type of sample were collected. Western Blot was performed with the primary antibodies: anti-FBPase, anti-ATPc and anti-2cp (Figure 10). FBPase became oxidized progressively from light to dark. Under high light conditions, the control, and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc* are completely reduced. After 5 minutes of darkness, FBPase is more reduced (24%) in the leaf agroinfiltrated than the control (11%). Then, after 10 minutes of darkness, FBPase is completely oxidized in the control and in infiltrated leaves (Figure 10A). On the membrane with anti-ATPc, both infiltrated and not infiltrated leaves are completely reduced. After 5 minutes of darkness, the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc* is almost completely reduced (90%), while the control is partially oxidized (reduction of 65%). At 10 minutes, ATPc in the not infiltrated leave (control) is fully oxidized, while the ATPc in the leaf infiltrated with gene construct is still reduced (24%) (Figure 10B). At 15 minutes, all the samples are completely oxidized. Finally, on the membrane with anti-2cp, 2cp in the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc* oxidized more slowly than the control in dark (Figure 10C). The 2cp in the leaf infiltrated with gene construct *35S-Cdsp32-cmyc* presents a higher percentage of reduction because it detects more monomers (Figure 10C).

Therefore, we conclude that Cdsp32 is involved in the oxidative side of chloroplast redox regulation, as Cdsp32 overexpression significantly affects the oxidation process of enzymes in the dark. In the transition from day to night, Cdsp32 does not affect the redox status of photosynthetic enzymes. The overexpression of Cdsp32 delays oxidation of FBPase and ATPc. These results suggest that Cdsp32 does not function as an oxidant Trx because, during the night, Cdsp32 delays oxidation of target enzymes. Cdsp32 overexpression affects the redox status of 2-Cys PRX, resulting in a higher reduction of the 2cp. This result confirms that 2cp is a target protein of Cdsp32, i.e., Cdsp32 reduces the 2cp protein.

CONCLUSIONS

The objective of this study was to understand the role of Cdsp32 in the oxidative regulation of chloroplast enzymes. This study aimed to test the following main hypotheses. The first hypothesis was that NTRC and Cdsp32 are functionally related. Secondly, to analyze the effect of Cdsp32 on the redox status of target enzymes, such as FBPase and ATPc. Experiments have confirmed that Cdsp32 protein reduces 2cp, since overexpression of Cdsp32 results in a higher percentage of reduction of 2cp, both in the light and in the dark conditions. In addition, 2cp and NTRC are functionally related with Cdsp32, because the lack of NTRC or 2cp results in a higher amount of Cdsp32. Finally, it has been demonstrated that during the day, Cdsp32 does not affect the redox status of the target enzymes. However, during the night, Cdsp32 affects the redox status of photosynthetic enzymes, as Cdsp32 delays the oxidation of FBPase and ATPc. Therefore, it can be concluded that Cdsp32 acts in the oxidative side of chloroplast redox regulation.

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Table 1. **Agrobacterium strains used in this study**.

Construct	**Protein**	**Tag**	**Purpose**
35S-Cdsp32-cmyc	Cdsp32	Cmyc	Redox studies
35S-2cpA-CFP-HA	2cpA	CFP-HA	Confocal microscopy
35S-Cdsp32-YFP-HA	Cdsp32	YFP-HA	Confocal microscopy

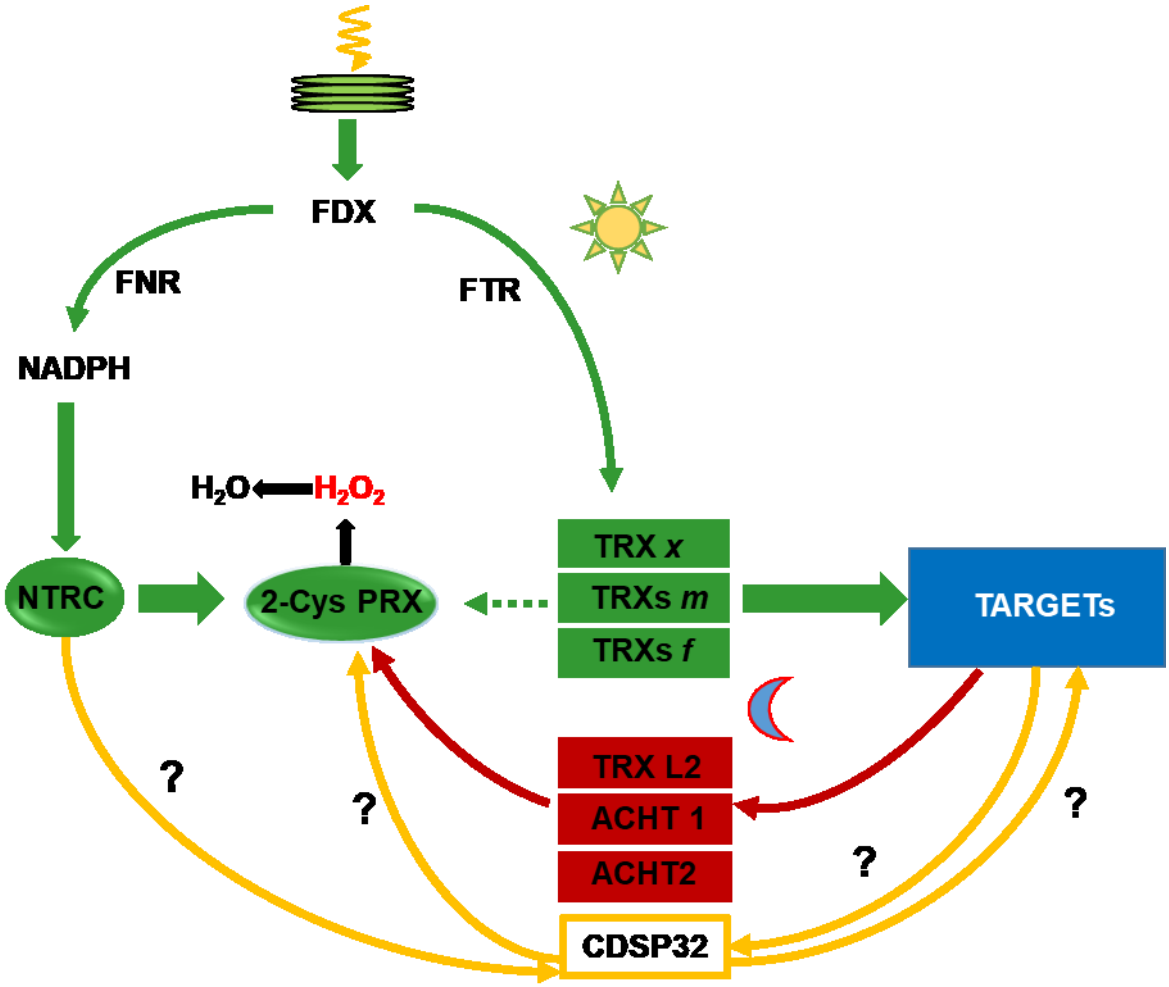

Figure 1. **Proposed model of the thiol-dependent redox regulation of photosynthetic enzymes in the chloroplast**. There are two different pathways: the FDX-FTR-TRX pathway and the NTRC pathway. During the day (green arrows), typical TRXs (m, f and x) reduce and activate target enzymes. The 2-Cys PRX protein is predominantly reduced by NTRC and, to a lesser extent (green dashed arrows), by typical TRXs. In dark conditions (red arrows), target enzymes are oxidized and inactivated by atypical TRXs (TRX L2, ACHT 1 and ACHT2), which reduce 2-Cys PRX, allowing the conversion of H_2O_2 to H_2O. The function of the atypical TRX Cdsp32 (yellow arrows) is still unknown. Image obtained and edited from Casatejada et al., 2023.

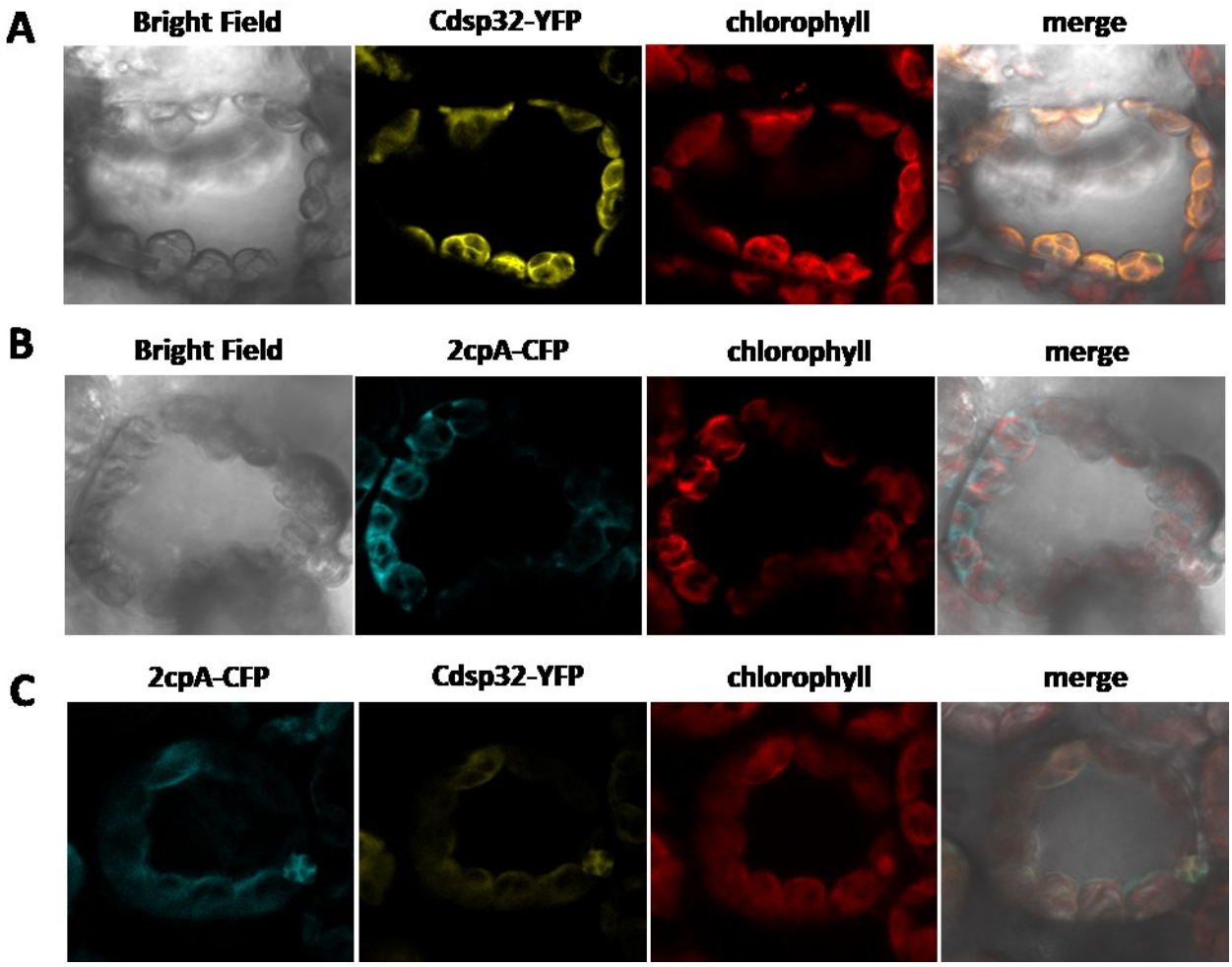

Figure 2. **Confocal microscopy images of *Nicotiana benthamiana* leaves agroinfiltrated with *35S-2cpA-CFP-HA* and *35S-Cdsp32-YFP-HA***. **A**) Images of a mesophyll cell agroinfiltrated with the gene construct *35S-Cdsp32-YFP-HA*. **B**) Images of a mesophyll cell agroinfiltrated with the gene construct *35S-2cpA-CFP-HA*. **C**) Images of a mesophyll cell agroinfiltrated with the gene constructs *35S-2cpA-CFP-HA* and *35S-Cdsp32-YFP-HA.* CFP is Cyan Fluorescent Protein and YFP is Yellow Fluorescent Protein.

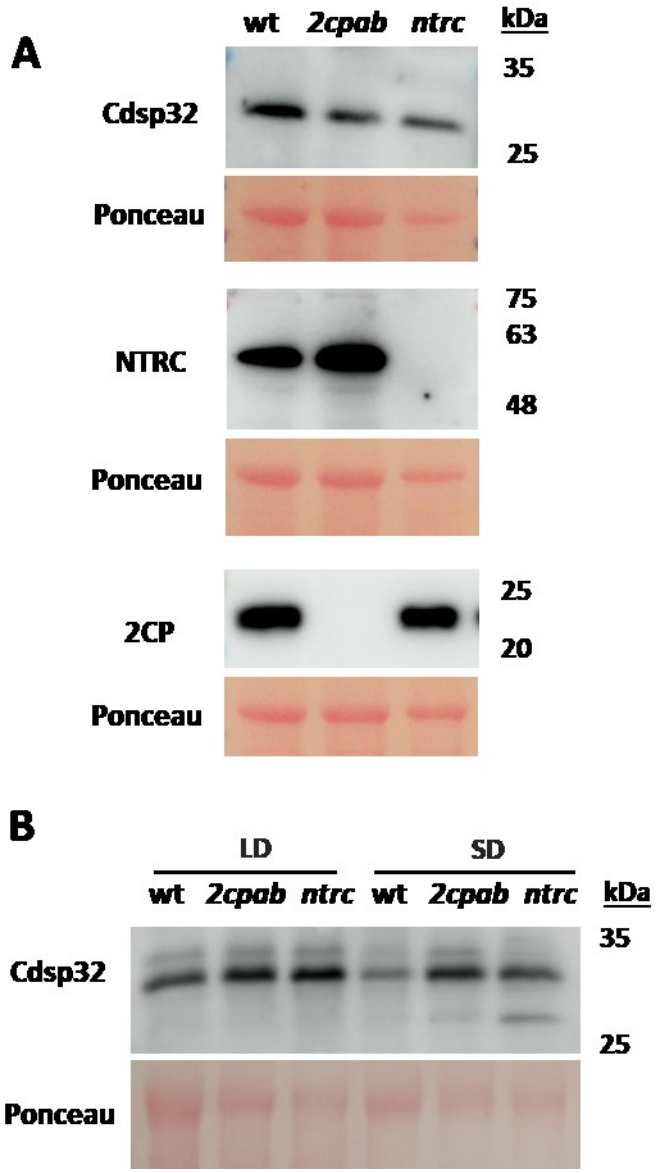

Figure 3. **Levels of Cdsp32 in wt and the mutants *2cpab* and *ntrc*. A**) Levels of Cdsp32 in wt, *2cpab* and *ntrc* seedlings grown in MS for 6 days. Below each membrane is shown the staining of the membrane with Ponceau as a loading control. **B**) Levels of Cdsp32 in wt, *2cpab* and *ntrc* plants. Plants were cultivated under long-day (LD) conditions (16-hour light/8-hour darkness); or short-day (SD) conditions (8-hour light/16-hour darkness).

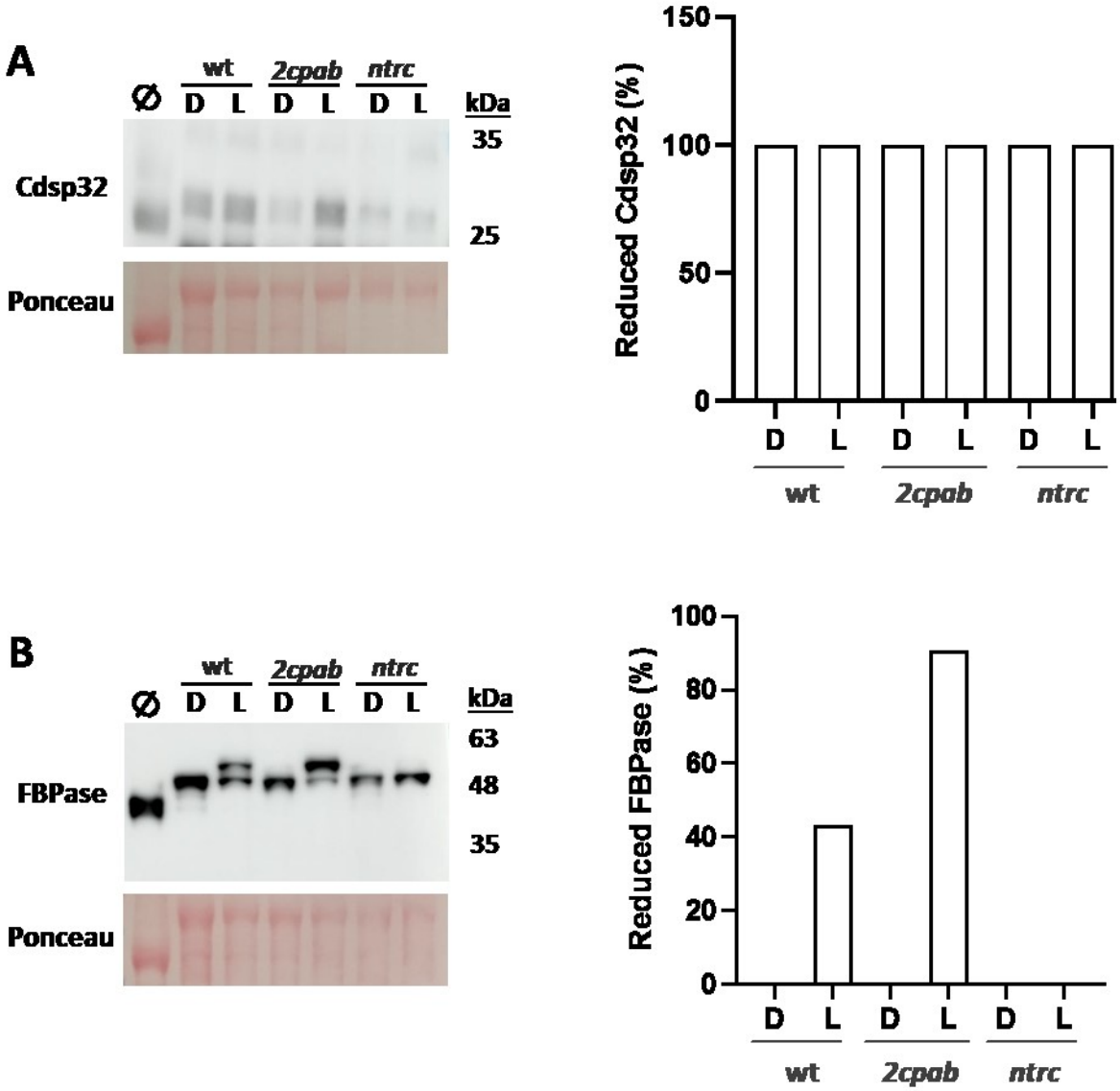

Figure 4. **Analysis of the redox status of Cdsp32 in wt and the *2cpab* and *ntrc* mutant plants**. For each genotype (wt, 2cpab and ntrc), two different samples were analyzed: plants adapted to darkness (D) and dark-adapted plants that have been subjected to light for 30 minutes (L). Below each membrane is shown the staining of the membrane with Ponceau as a loading control. **A**) Determination of the redox status of Cdsp32 in MMPEG alkylation assays. The graphic represents the percentage of reduction of Cdsp32. **B**) Determination of the redox status of FBPase in MMPEG alkylation assays. The graphic represents the percentage of reduction of FBPase.

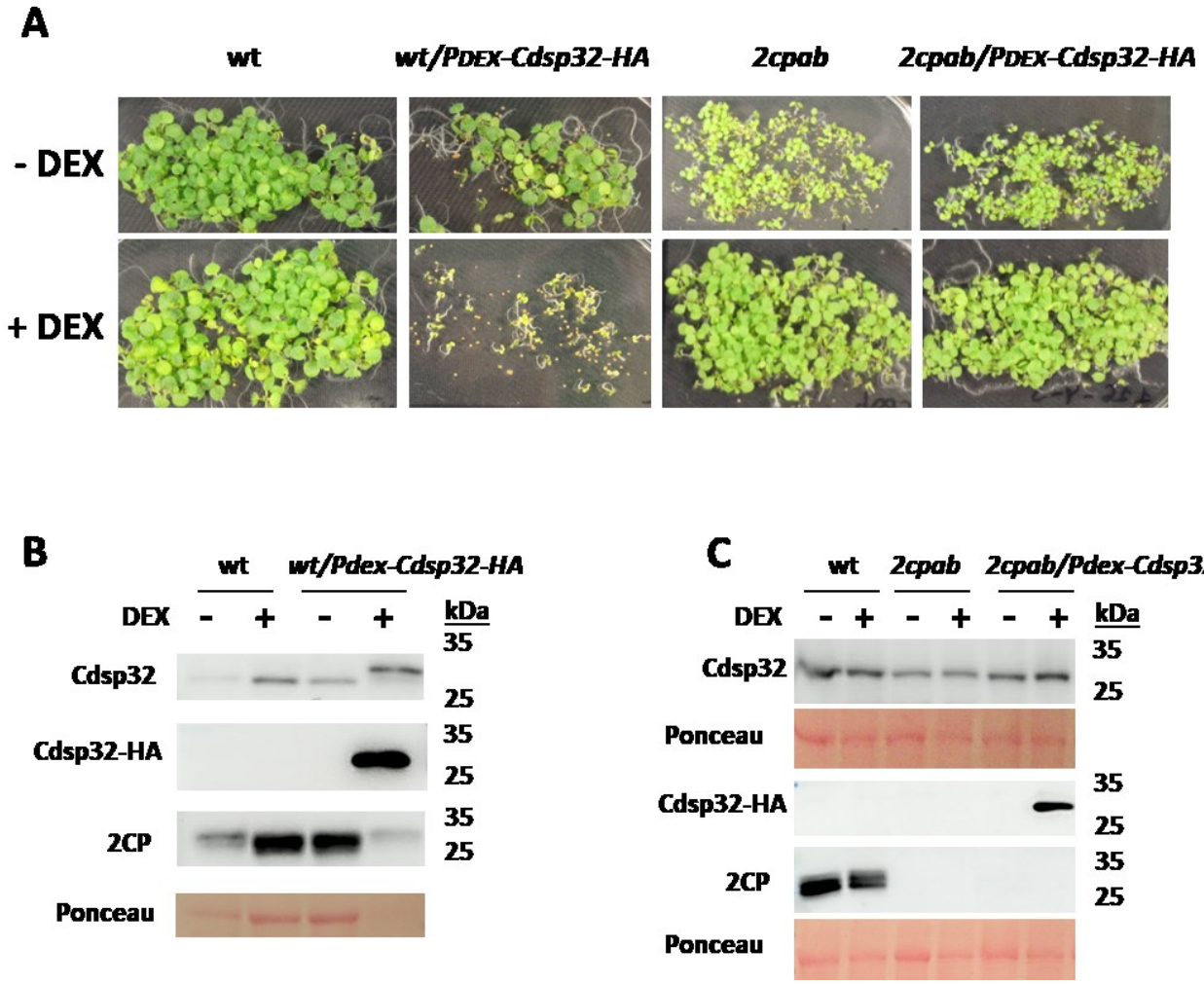

Figure 5. **Effect of Cdsp32 overexpression at early stage of plant development**. Seedlings of *Arabidopsis thaliana* wild-type, the mutant line *2cpab* and the transgenic lines *wt/P$_{DEX}$-Cdsp32-HA* and *2cpab/P$_{DEX}$-Cdsp32-HA* were subjected to two types of growth conditions: with dexamethasone (+DEX) and without dexamethasone (-DEX) for 6 days. **A**) Growth phenotype of the seedlings with and without DEX. **B**) Western Blot analysis on the levels of Cdsp32, Cdsp32-HA and 2cp in wt and *wt/P$_{DEX}$-Cdsp32-HA* seedlings. **C**) Western Blot analysis on the levels of Cdsp32, Cdsp32-HA and 2cp in wt, *2cpab* and *wt/P$_{DEX}$-Cdsp32-HA* seedlings. Below each membrane is shown the staining of the membrane with Ponceau as a loading control.

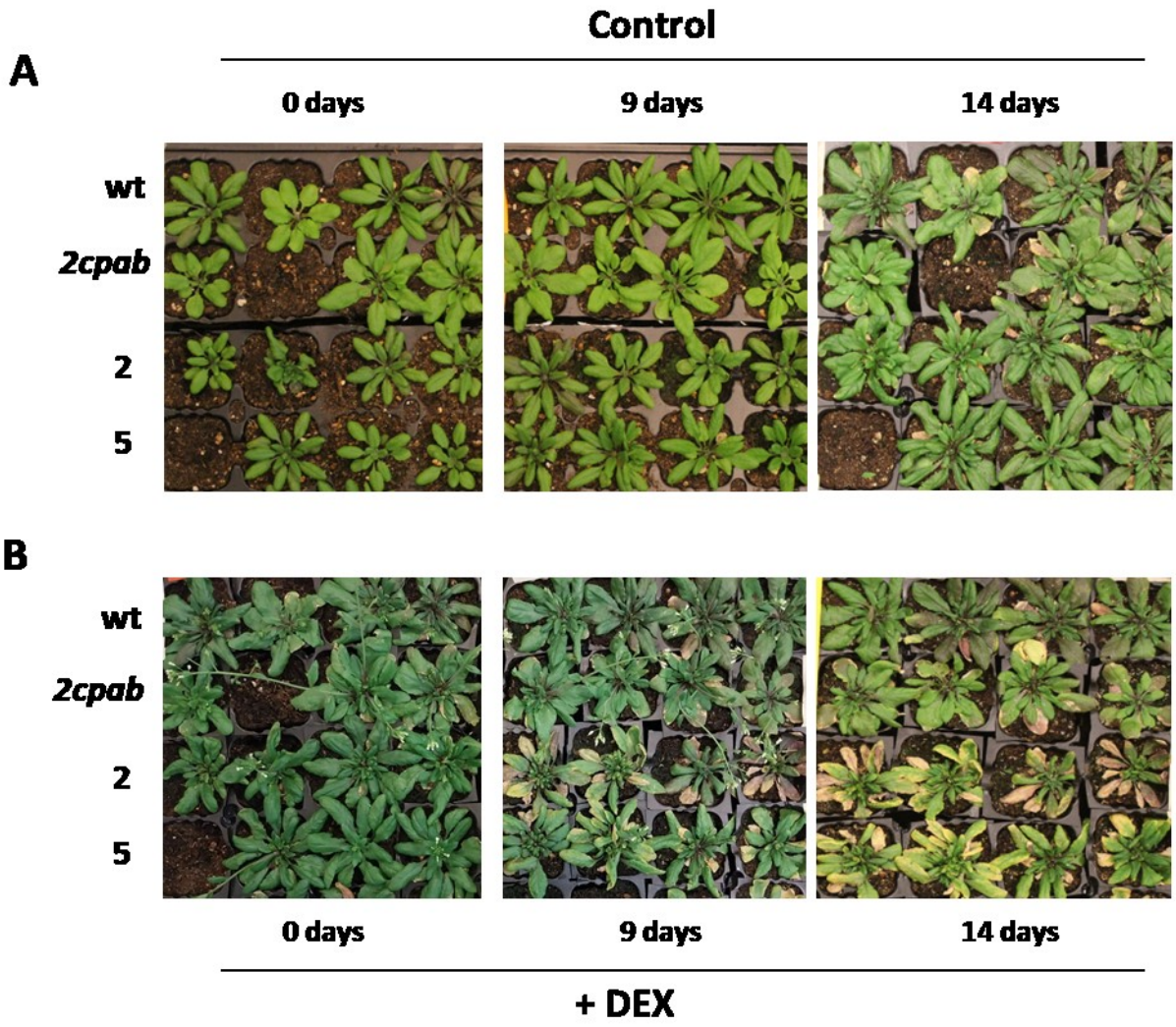

Figure 6. **Effect of Cdsp32 overexpression on adult plants**. *Arabidopsis thaliana* wild-type, mutant *2cpab* and transgenic *wt/PDEX-Cdsp32-HA* (2 and 5) plants were sprayed with 0.1% Tween (**A**) and solution with DEX (0.1% Tween + 30 µM DEX) (**B**). Photos were taken before spraying, 9 days after spraying and 14 days after spraying.

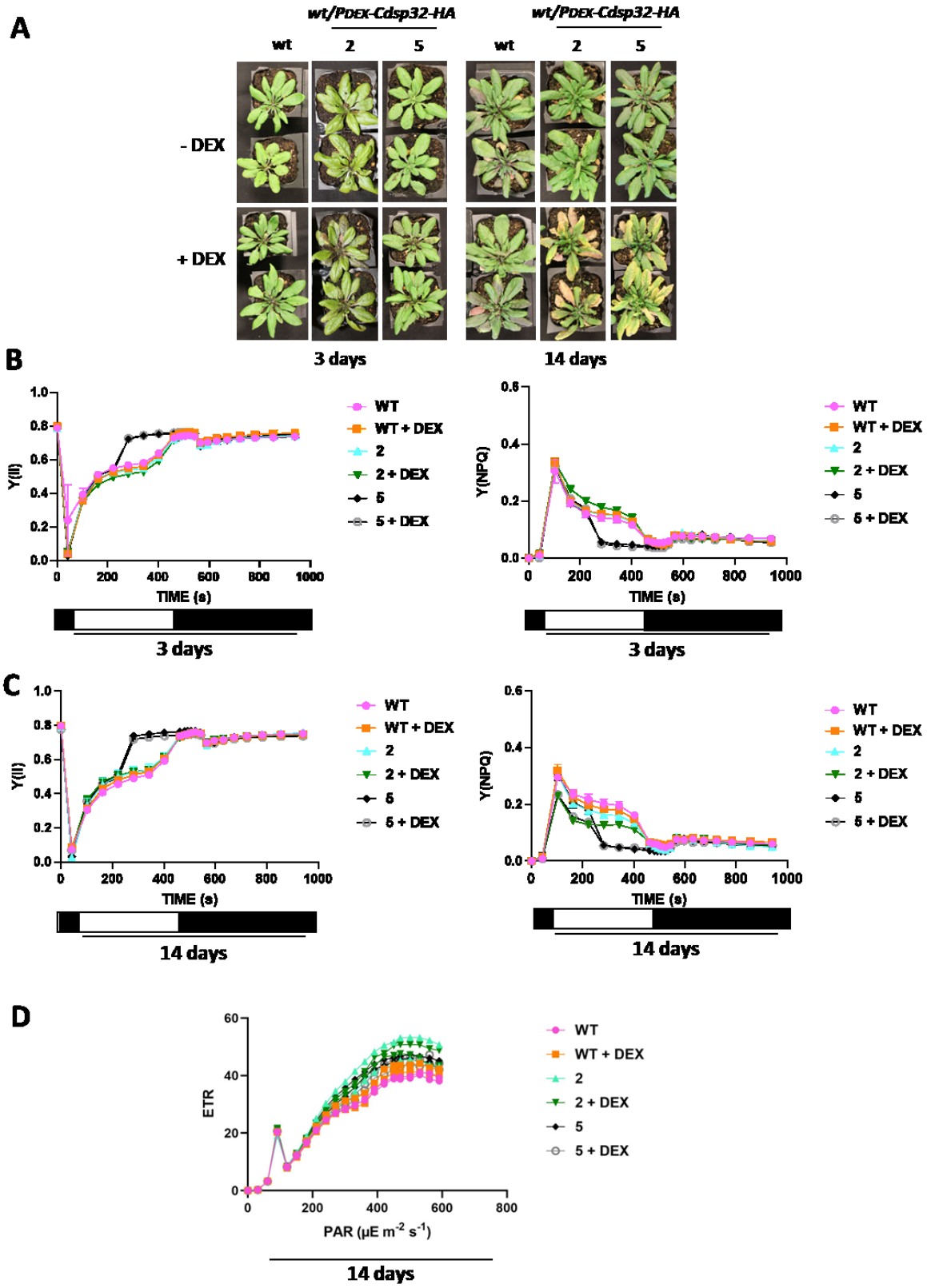

Figure 7. **Effect of Cdsp32 overexpression on photosynthetic parameters**. **A**) *Arabidopsis thaliana* wild-type, mutant *2cpab* and transgenic *wt/PDEX-Cdsp32-HA* (2 and 5) were sprayed with control solution and solution with DEX, and they were subjected to these conditions for 14 days. **B**) Y(II) and Y(NPQ) values after 3 days in these conditions. **C**) Y(II) and Y(NPQ) values after 14 days in these conditions. **D**) ETR parameter in a Light Curve (LC).

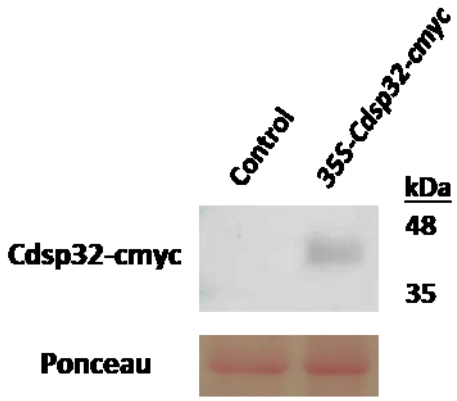

Figure 8. **Agroinfiltration of *Nicotiana benthamiana* leaves with *35S-Cdsp32-cmyc***. Western Blot analysis of *Nicotiana* leaves infiltrated or not (control) with *Agrobacterium* strain containing the Cdsp32-cmyc construct. Below each membrane is shown the staining of the membrane with Ponceau as a loading control.

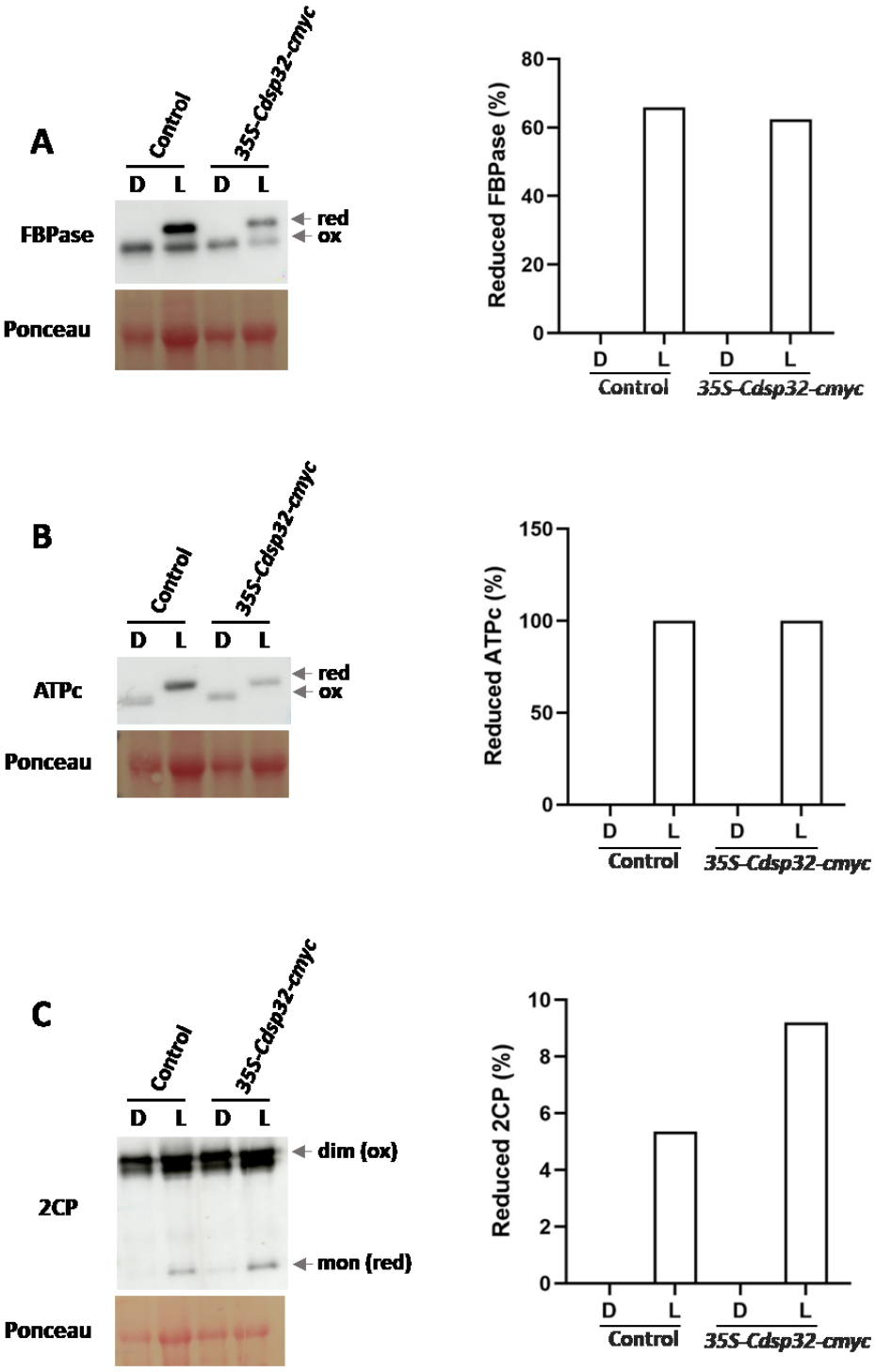

Figure 9. **Effect of Cdsp32 on the redox status of photosynthetic enzymes in the dark-to-light transitions**. **A**) Western Blot analysis on the redox status of FBPase in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. **B**) Western Blot analysis on the redox status of ATPc in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. **C**) Western Blot analysis on the redox status of 2cp in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. Below each membrane is shown the staining of the membrane with Ponceau as a loading control. Band quantification was performed using the "Gel Analyzer" software and graphic was performed using GraphPad software.

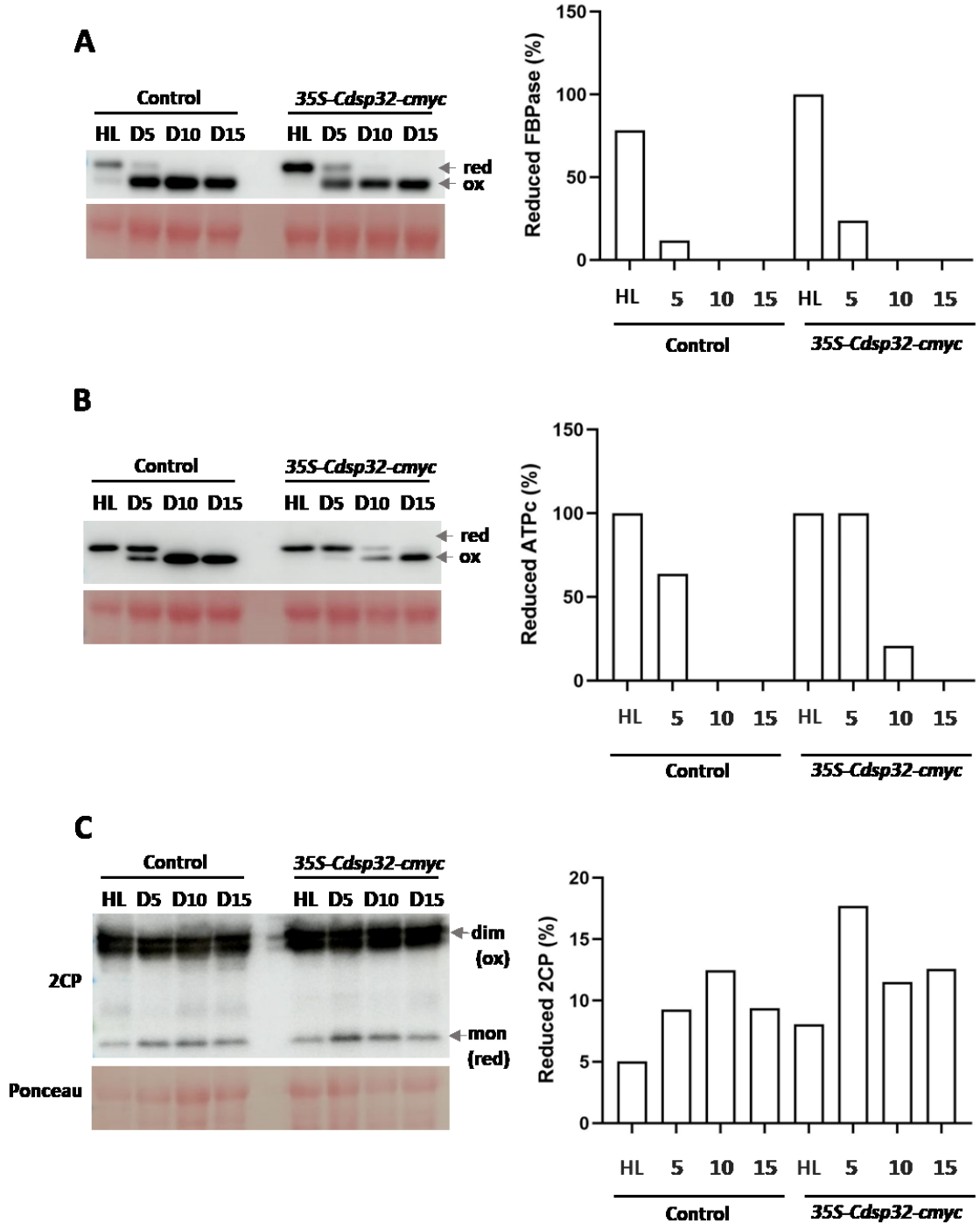

Figure 10. **Effect of Cdsp32 on the redox status of photosynthetic enzymes in the light-to-dark transitions**. **A**) Western Blot analysis on the redox status of FBPase in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. **B**) Western Blot analysis on the redox status of ATPc in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. **C**) Western Blot analysis on the redox status of 2cp in the control and the leaf infiltrated with the gene construct *35S-Cdsp32-cmyc*. Below each membrane is shown the staining of the membrane with Ponceau as a loading control. Band quantification was performed using the "Gel Analyzer" software and graphic was performed using GraphPad software.