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The effect of irradiance and redox-modifying reagents on the 52 kDa protein disulfide isomerase of *Arabidopsis* chloroplasts

D.-P. LU and D.A. CHRISTOPHER[1]

Department of Molecular Biosciences and Bioengineering, University of Hawaii, 1955 East-West Rd., Agsciences 218, Honolulu, HI 96522, USA

Abstract

Immunoblot analysis was used to assess the effects of light and redox-modifying chemicals on the 52 kDa protein disulfide isomerase (PDI) from chloroplasts of *Arabidopsis thaliana*. A monoclonal antiserum was used that preferentially cross-reacts with the 52 kDa relative to the 65 kDa isoform of PDI. The PDI-52 was most abundant in leaves, flowers, stems and seeds, but was undetected in roots. PDI-52 formed a ~220 kDa protein complex on blue native gels, indicating that it associates with either itself or other proteins in chloroplasts. Light decreased the levels of PDI-52 by 80 %, relative to the control protein (the CF1 subunit of chloroplast ATP synthase). Treatment with dithiothreitol decreased the content of the 52 kDa protein by half. In contrast, when the reduction of plastoquinone is blocked by DCMU, or when the plants are treated with phosphate, PDI-52 contents increased by 1.5 to 2-fold relative to CF1. The effect of the chemical treatments coincided with the effect of the light/dark cycle and implied that light decreased PDI-52 protein content by way of the cellular redox environment.

Additional key words: disulfide bonds, oxidizing-reducing environment, starch biogenesis.

Introduction

Protein disulfide isomerases (PDIs) were originally identified as proteins in the lumen of the endoplasmic reticulum (ER) that catalyzed the reversible formation and isomerization of disulfide bonds needed for the proper folding, assembly, activity and secretion of numerous enzymes and structural proteins (Freedman *et al.* 1994, Frand and Kaiser 1998, Aslund and Beckwith 1999, Tu *et al.* 2000, Wedemeyer *et al.* 2000). PDIs modify disulfide bonds *via* their integral redox-active thioredoxin-domains (Kanai *et al.* 1998, Motohashi *et al.* 2001). In animals and yeast, PDIs have also been shown to be located in the nucleus, cytoplasm, mitochondria and extracellular environment (Couet *et al.* 1996, Wilson *et al.* 1998, Lahav *et al.* 2000, Rigobello *et al.* 2001, Turano *et al.* 2002), where they serve as chaperones and important subunits in protein complexes (Bennett *et al.* 1988, Freedman *et al.* 1994, John and Bulleid 1994, Lamberg *et al.* 1996, Ferrari and Soling 1999, Grillo *et al.* 2002, Lumb *et al.* 2002). In these complexes, PDIs regulate cell viability (Ferrari and Soling 1999), ion homeostasis (Honscha *et al.* 1993), transcription (Markus and Benezra 1999), and cell differentiation (Ohtani *et al.* 1993, Fornes and Bustos-Obregon 1994).

In plants, the traditional function of PDIs is to assist with the folding, assembly and segregation of storage proteins during the biogenesis of protein bodies from the ER of the seed endosperm (Shimoni *et al.* 1995, Li and Larkins 1996, Takemoto *et al.* 2002). The expression of some PDI genes is upregulated by the unfolded protein response (Martinez and Chrispeels 2003). In the unicellular green alga, *Chlamydomonas reinhardtii,* a 60 kDa PDI was found in chloroplasts to be associated with at least three other proteins (Danon and Mayfield 1994, Trebitsh *et al.* 2001). PDI-60 is proposed to regulate light-induced translation and synthesis of the *psbA* gene product, the D1 membrane protein of photosystem (PS) 2 (Danon and Mayfield 1994, Trebitsh *et al.* 2000). Activation of the PDI-RB60 is proposed to occur *via* the ferredoxin-thioredoxin system (Trebitsh *et al.* 2000, Buchanan and Balmer 2005). Numerous regulatory processes and feedback modulation in chloroplasts are mediated by the ferredoxin-thioredoxin system and the redox state of the plastoquinone pool (Buchanan and Balmer 2005, Pfannschmidt and Liere 2005). Like-

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Abbreviations: DTT - dithiothreitol; CF1 - beta subunit of chloroplast ATP synthase; DCMU - dichlorophenyl)-1,1-dimethylurea; LSU Rubisco - large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; PDI - protein disulfide isomerase; PS - photosystem.
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[1] Corresponding author; fax: 808-956-3542, e-mail: dchr@hawaii.edu

wise, redox has been shown to regulate cation channels processes outside the chloroplast (Chen *et al.* 2004).

The *Arabidopsis* genome has at least 12 *pdi* genes (Meiri *et al.* 2002). We recently used immunolabeling combined with fluorescence and electron microscopy to determine that two immunorelated PDI isoforms of 52 and 65 kDa (PDI-52 and PDI-65) were localized to the chloroplast and associated with the biogenesis of transitory starch in leaves (Lu and Christopher 2006). The antisera preferentially reacted with the PDI-65 and also reacted with recombinant PDIs derived from *in vitro* translation of pdi cDNAs (Lu and Christopher 2006). The contents of PDI-65 increased in response to light, however the levels of PDI-52 could not be measured due to the weaker binding of the antisera to PDI-52. Here we describe the use of a new monoclonal antisera, which preferentially cross-reacts with PDI-52, to investigate the chloroplast fractions and tissues in which PDI-52 resides, and to measure the response of PDI-52 content to light and chemicals that modify the redox state of the chloroplast and overall cellular environment.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L. cv. Columbia (wild type) were purchased from *Lehle Seeds* (Round Rock, TX, USA). Seeds were planted in flats containing water-saturated *Jiffy* potting mix (Bentonville, AR, USA) and chilled (5 °C) overnight (14 h). Plants were exposed to a 14-h photoperiod (cool-white fluorescent tubes, irradiance of 80 μmol $m^{-2}s^{-1}$) at temperature of 22 to 26 °C for 28 d. To obtain dark-adapted seedlings, the 28-d-old plants were placed in complete darkness for 3 d. Following dark-adaptation, plants were exposed to the cool white light (described above) for 0, 5, 10 or 24 h before harvesting. Tissue was harvested by quick freezing in liquid nitrogen or was used fresh for chloroplast isolation. All manipulations of dark-grown plants were performed in complete darkness or under a dim green safelight as previously described (Chun *et al.* 2001).

Isolation and fractionation of chloroplasts: Intact chloroplasts were isolated from 4-week-old *Arabidopsis* leaves on Percoll gradients as described (Hoffer and Christopher 1997). The purified intact chloroplasts were carefully washed to avoid contamination with cytoplasmic proteins and then counted under a light microscope with a haemocytometer. The chloroplasts were resuspended in lysis buffer (10 mM Tris HCL, 10 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride, PMSF) and vortexed. The sample was centrifuged at 12 000 *g* for 5 min. The resulting supernatant was removed and saved as the stromal phase. The pellet containing thylakoids, envelope, starch and some nucleic acids, was resuspended in lysis buffer, loaded on 50 % Percoll and centrifuged at 5 000 *g* for 5 min. The thylakoids tightly banded in the percoll were removed, washed and saved. The grey-white pellet was saved as the starch phase.

Antisera: The monoclonal antisera PDI-52 (mouse) to the *Chlamydomonas* chloroplast PDI-RB60 (Trebitsch *et al.* 2001) were generously provided by Dr. Avihai Danon (The Weizmann Institute of Science, Rehovot, Israel). Polyclonal antisera (rabbit) to the CF1 ATPase and D2 photosystem 2 subunit were generously provided by Dr. J. Mullet (Texas A & M University, College Station, TX, USA) and described in Christopher and Mullet (1994).

SDS-PAGE and immunoblot analysis: Samples of chloroplast subfractions were used on an equal chloroplast number basis. Protein samples were incubated in sample buffer (2 % SDS, 0.1 M DTT, 10 % glycerol, 50 mM Tris-HCl, pH 6.8) and separated with 10 % (m/v) SDS-PAGE on polyacrylamide mini-gels. After electrophoresis, gels were stained with Coomassie blue and air-dried on gel drying films (*Promega,* Madison, WI, USA), or transferred to *PROTRAN* pure nitrocellulose membrane (*Perkin Elmer Life Sciences*, Boston, MA, USA) with a *Bio-Rad* (Hercules, CA, USA) min-transblot apparatus in transfer buffer (25 mM Tris-OH, pH 8.3, 96 mM glycine, 15 % methanol). The blots were blocked for 1 h at reverse transcription (RT) with 5 % non-fat milk in 1× Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1 % *Tween 20*. PDI-52 and CF1 antisera were used at a 1:3000 dilution, while the D2 antisera was used at a 1:400 dilution. The blots were probed with different primary antibodies for 1 h at RT, followed by incubation with anti-rabbit or anti-mouse IgG supplied in the ECL kit (*Amersham Biosciences,* Pittsburgh, PA, USA) for 1 h at RT. Washes after treatment with primary antisera were done with 1× Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.2 % *Tween 20*. Chemiluminescence was generated and detected according to the manu-facturer's instructions (*Amersham Biosciences*) by on X-ray film. Bands were quantitated by densitometry using a *Kodak EDAS 290* system.

Immunoelectron microscopy: *Arabidopsis* leaves from 4-week grown plants (described above) were harvested at 4 h of the light period. They were fixed with 0.5 % glutaraldehyde plus 4 % paraformaldehyde in 0.1 M Na-cacodylate buffer containing 2 mM $CaCl_2$, pH 7.4, for 2.5 h under gentle vacuum, washed in 0.1 M cacodylate plus 2 mM $CaCl_2$ for 2 × 15 min, at RT. Tissue was dehydrated in a graded series of ethanol, embedded in *LR White* (medium grade), and polymerized at 51 °C. Ultra-thin (60 - 80 nm) sections were obtained on a *Reichert Ultracut E* (Wetzlar, Germany) ultra-microtome. Sections were treated with and without 5 % Na-meta-periodate for 10 min and then washed three times with deionized H_2O for 3 min intervals. Samples were blocked

with 1× Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1 % *Tween 20* (TBST) and 5 % non-fat dry milk for 1 h at RT. Primary antisera were added at 1:1000 dilution and further incubated for 1 h at RT. Sections were washed in three times in TBST with 5 % milk for 5 min intervals. The secondary antibody was 10 nm gold-conjugated goat anti-mouse H+L (#15751, *Ted Pella, Inc*., Redding, CA, USA). It was used at 1:100 dilution for a 1 h incubation. Sections were washed three times for 5 min intervals with TBST/5 % milk, once for 5 min in TBST, and twice for 5 min in deionized H_2O. They were subsequently stained with 5 % uranyl acetate and 3 % lead citrate. The samples were viewed on an electron microscope (*LEO 912 EFTEM*, Oberkochen, Germany) at 100 kV.

Native protein gel electrophoresis: Blue native gel electrophoresis was performed as described (Cline and Mori 2001). The total chloroplast proteins were solubilized in buffer (12.5 mM BisTris-HCl (pH 7.0), 10 % (m/v) glycerol, 2.5 % (m/v) *Triton X-100 (Sigma-Aldrich*, St. Louis, MO, USA), agitated for 30 min at 4 °C. Samples were then centrifuged at 100 000 *g* for 30 min at 4 °C. The supernatant was combined with 1/10 BN sample buffer (100 mM BisTris-HCl, 0.5 M 6-amino-caproic acid, pH 7.0, 30 % sucrose, and 5 % (m/v) *Brilliant Blue G* (*Sigma-Aldrich*)) and loaded onto a native 5 to 14 % polyacrylamide gradient gel in a *Hoefer Mighty Small* electrophoresis unit (*Amersham Pharmacia Biotech*, San Francisco, USA). Electrophoresis was performed at 80 V and 4 °C. The cathode buffer (0.05 M Tricine, 15 mM BisTris) containing 0.01 % *Brilliant Blue G* was used initially and replaced by cathode buffer lacking dye after the top 1/2 to 2/3 of the gel was covered with dye.

DTT, DCMU and phosphate treatments and total protein extraction: 4 week-old plants (grown as described above) were carefully washed and transferred to flasks containing sterile H_2O or sterile H_2O plus either 2 mM or 10 mM DTT, 0.1 or 1.0 μM 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU, *Sigma-Aldrich*), 10 mM or 30 mM NaH_2PO_4 (pH 5.8). The plants in flasks were incubated under white light (cool-white fluorescent, 80 μmol $m^{-2}s^{-1}$) at 22 to 26 °C and shaken at the speed of 100 rpm for 10 h. After treatment, plants were harvested for chloroplast isolation and total cell protein extraction. The total protein was extracted as described (Shimoni *et al*. 1995). Protein concentrations were determined by the DC protein assay reagents (*Bio-Rad,* Hercules, CA, USA).

Results and discussion

As a prerequisite to investigate the effects of plant development, light and redox on PDI-52 levels, we determined the specificity of the PDI antisera to the two PDI isoforms in *Arabidopsis* chloroplasts. Immunoblot analysis was carried out on *Arabidopsis* chloroplast proteins obtained from intact chloroplasts purified on Percoll gradients. As controls, for the separation of the stromal and membrane sub-fractions, we used antisera against an integral thylakoid membrane protein, the D2 subunit of PS 2 (which is intrinsic to the bilayer). As previously observed (Lu and Christopher 2006), the anti-PDI antisera recognized two immunochemically related PDIs of 52 and 65 kDa in the purified chloroplast protein subfractions (Fig. 1). However, this new antisera had a selective preference for the 52 kDa PDI isoform (Fig. 1). Detection of the 65 kDa isoform could be eliminated by washes of 0.2 % *Tween 20* at higher stringency and such washes were used in subsequent immunoblot analyses (Figs. 2, 3 and 4). The majority (80 %) of the 52 kDa PDI was located in the stromal phase, while 20 % was in the proteins associated with starch (Fig. 1*A*,*B*). This is consistent with a PDI that exists at the stromal starch interface as detected by immuno-microscopy (Lu and Christopher 2006). However, the distribution of the PDI-52 in the stroma (Fig. 1) was significantly higher than the content of PDI-65 in this chloroplast fraction. The immunoblot analysis with the D2 antisera indicated minimal contamination of stroma with thylakoid proteins, although trace thylakoid proteins were detected in the starch protein fraction.

Immunoblot analysis was conducted on total cellular proteins from different tissues to determine the tissue in which chloroplast PDI-52 kDa accumulated (Fig. 2*A*). PDI-52 kDa was most abundant in leaves followed by flowers, green siliques and stems. Virtually no PDI was detected in roots, indicating the presence and function of this PDI in the aerial parts of the plant. Coomassie blue staining of the LSU of Rubisco was used as a control and indicated that all tissues except roots had the LSU of Rubisco present, suggesting that the capacity to fix carbon was present in the same tissues in which PDI-52 was detected. Native blue gel electrophoretic analysis of chloroplast proteins was used to determine if the PDI-52 was associated with proteins or a monomer (Fig. 2*B*). PDI-52 was consistently detected in a ~220 kDa complex (Fig. 2*B*). This experiment indicated that the 52 kDa protein associated with at least one protein in chloroplasts of green aerial tissues.

The PDI-52 and PDI-65 associated with various fringe regions of the starch grain in green chloroplasts (Lu and Christopher 2006). The biogenesis of transitory starch in the leaves is regulated by light (Zeeman *et al.* 2002). However, the response of PDI-52 to light has not been measured in plants. Therefore, we used immunoblot analysis to measure the contents of the 52-kDa PDI, relative to the contents of the CF1 protein, in chloroplasts of dark-adapted plants that were exposed to light for various time intervals (Fig. 3*A*). Coomassie staining of the LSU of Rubisco was also used as a gel loading control (Fig. 3*A*). The level of PDI-52 increased 15 to

20 % during dark-adaptation, but decreased over 50 % after 10 and 24 h light relative to the CF1 and the before dark (BD) controls (Fig. 3*B*). This indicated that light decreased the abundance of PDI-52, while darkness slightly increased PDI-52 levels. The substantial decrease of PDI-52 in the light suggests that it is required for activities in darkness.

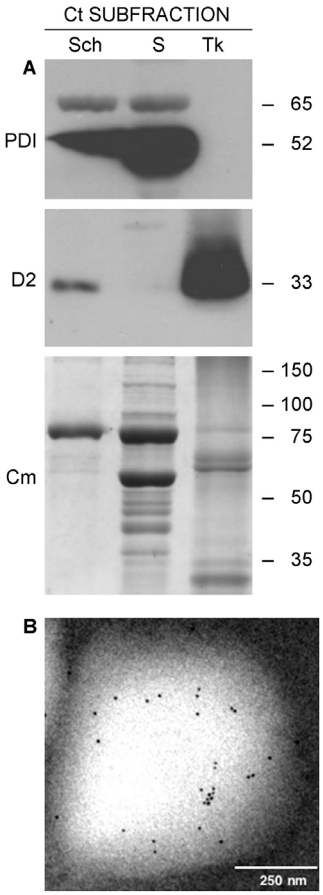

Fig. 1. Immunodetection of PDI-like proteins in purified chloroplasts of *Arabidopsis*. *A* - Purified intact chloroplasts were sub-fractionated into starch (Sch), stroma (S) and thylakoid membrane (Tk) phases, proteins were extracted and subjected to immunoblot analysis. Protein subfractions were loaded on an equal chloroplast number basis. Each panel represents a replica immunoblot hybridized with either PDI-52 monoclonal antibody or polyclonal antisera against the CF1 subunit of the chloroplast ATPase. The bottom panel is a representative coomassie (Cm) stained gel of proteins separated via SDS-PAGE. Numbers to the right of the panels denote protein Mr in kDa. *B* - an electron micrograph of an *Arabidopsis* starch grain immuno-labeled with PDI-52 antiserum and 10 nm gold secondary antibody. Size is indicated in nm.

We investigated the nature of the metabolic signals underlying the light-mediated decrease in the levels of PDI-52 (Fig. 4). We chose three chemicals that can alter the cellular redox potential similar to the effect of light-dark cycles. DTT reduces disulfide bonds in proteins and especially the vicinal dithiols of PDIs (Danon and Mayfield 1994) and causes a general increase in the cellular reducing environment. DCMU is an inhibitor of photosynthetic electron transport of PS 2, and affects the chloroplast redox environment through the plastoquinone pool, which is a different mechanism than the redox effects of DTT (Surpin *et al.* 2002). Phosphate can stimulate transitory starch breakdown in leaves, which is enhanced in the dark (Smith *et al.* 2003, Ritte *et al.* 2004). The CF1 protein served as an internal standard chloroplast protein for comparison to the levels of the PDI-52 (Fig. 4). Two different concentrations of each chemical were initially used (Fig. 4*A*) for a 24 h treatment period. PDI-52 was virtually undetected after treatment with 10 mM DTT. DCMU and phosphate treatments both increased PDI-52 contents relative to CF1 after 24 h. Each of the chemicals produced a more pronounced effect with increasing concentration (Fig. 4*A*).

Secondary effects could contribute to an artifactual effect on plant viability when a chemical is used for 24 h. Therefore, a single lower concentration of each chemical was used for a shorter treatment period (6 h) in the next experiment (Fig. 4*B*,*C*). Treatment with 2 mM DTT decreased the levels of PDI-52 by half, while treatments with 0.1 μM DCMU and 10 mM phosphate increased the

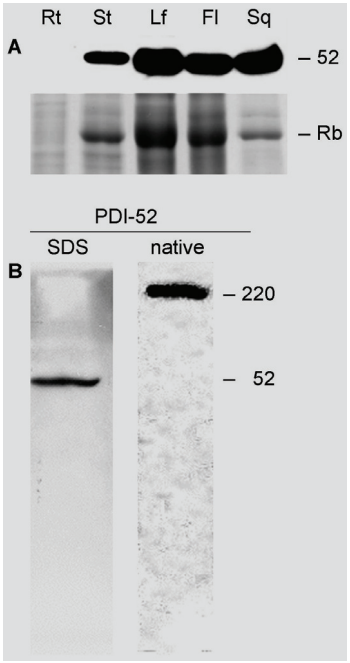

Fig. 2. Immunoblot analysis of tissue distribution and protein association of PDI-52. *A* - Equal amounts of protein extracted from *Arabidopsis* roots (Rt), leaves (Lf), flowers (Fl), stem (St), and green siliques (Sq) were separated on protein gels via SDS-PAGE and subjected to immunoblot analysis with the anti-PDI-52 antiserum. The flower sample contained the entire inflorescence (petals, stamens, pistil and sepals). Rb indicates the coomassie stained band corresponding to the large subunit of Rubisco. *B* - Analysis of protein complex formation *via* native blue gel electrophoresis. Antiserum against PDI-52 was hybridized to equally loaded chloroplast proteins separated by SDS-PAGE and native blue gel electrophoresis. The estimated Mr of the proteins detected in kDa is indicated to the right of the panel.

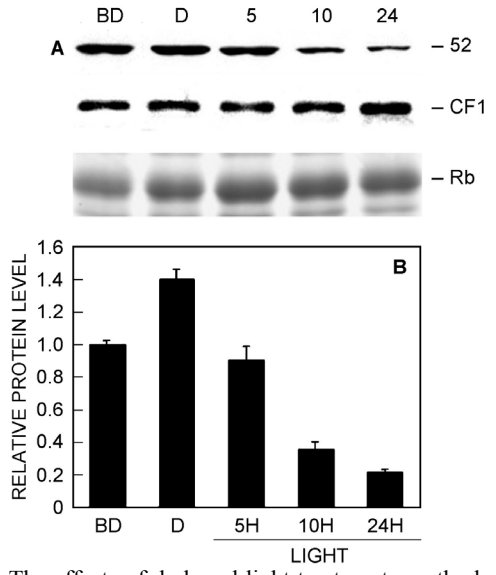

Fig. 3. The effects of dark and light treatments on the levels of the 52 kDa PDI in chloroplasts. *A* - Immunoblot analysis of PDI-52 the CF1 protein of ATPase. Chloroplasts were isolated from 3 week-old plants before dark-adaptation (BD), after 3 d dark-adaptation (D), and after 3 d dark-adaptation followed by exposure to either 5, 10 or 24 h light. The coomassie (Cm) stained gel is shown as a control for protein loading with the LSU or Rubisco labeled (Rb). *B* - Quantification of the relative protein levels (RPL) of PDI-52 in panel *A* represented on an equal CF1 protein level basis. Values indicate the mean ± SD of three independent experiments.

levels of the PDI-52 by over 60 % (Fig. 4*B*,*C*). These results indicate that the chemicals acted on redox signaling pathways that modulated PDI-52 levels.

The results suggest that the cellular redox environment influences the content of the PDI-52. In addition, we interpreted the light-induced decrease in PDI-52 levels as occurring *via* redox signaling pathway(s). Furthermore, the increase in PDI-52 levels in response to inhibition of photosynthetic electron transport by DCMU could mimic the effect of the dark cycle. These responses to light and redox were reminiscent of several other redox-regulatory responses in chloroplasts (Buchanan and Balmer 2005, Pfannschmidt and Liere 2005), in particular, regulation of nitrate reductase activity (Giordano *et al.* 2005) and photosynthesis gene transcription and mRNA stability in response to the redox state of the plastoquinone pool (Salvador and Klein 1999, Alfonso *et al.* 2000).

Phosphate is known to decrease starch biosynthesis and enhance its degradation, which occurs in the dark (Smith *et al.* 2003). The association of PDI-52 with starch (Fig. 1, and Lu and Christopher 2006) and an increase in the content of the PDI-52 in response to the treatment with phosphate, implied a function of PDI-52 in starch breakdown. Tiessen *et al.* (2002) reported that the activity of a key enzyme in starch biosynthesis, ADP-glucose pyrophosphorylase, was controlled by post-translational redox modification. In this mechanism, reduction of the intermolecular disulfide bonds in the enzyme stimulates its activity, and promotes starch biosynthesis, while re-formation of these bonds via oxidation decreases enzyme activity (Tiessen *et al.* 2002).

The signaling components underlying the redox modulation of ADP-glucose pyrophosphorylase are not yet known, but could involve the well-described thioredoxins (Buchanan 1991, Motohashi *et al.* 2001, Cho *et al.* 1999) and/or one of the PDIs described here. Depending on the protein species, there are one or two thioredoxin domains in each PDI, that can assist in the

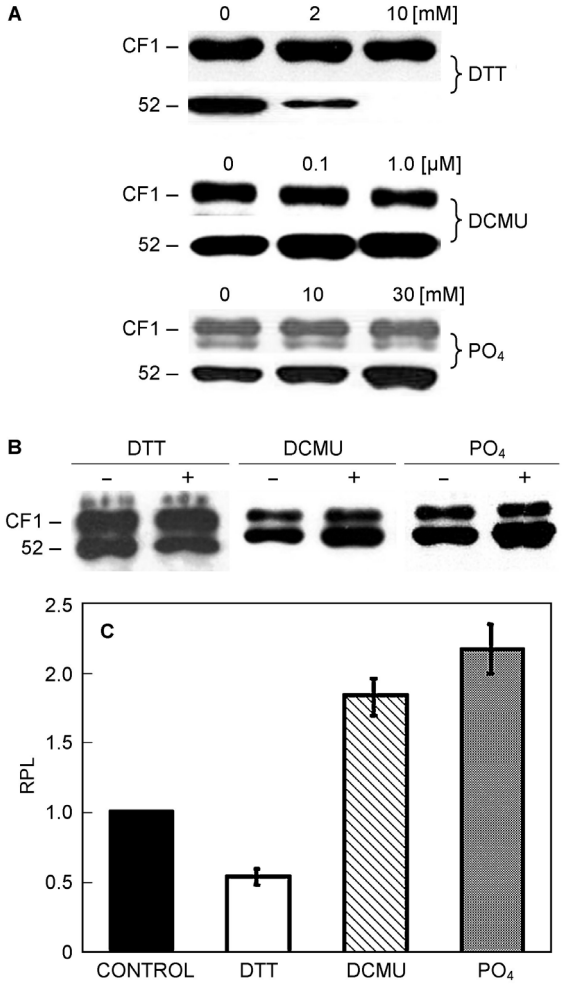

Fig. 4. The effect of treatments with redox modifying reagents and phosphate on the level of the PDI-52 in chloroplasts. *A* - Immunoblot analysis of PDI-52 relative to the internal control, the CF1 protein of ATPase. 4-week-old plants were treated with H_2O (0) or with one of the three chemicals DTT (2 and 10 mM), DCMU (0.1 and 1.0 μM) or phosphate (10 and 30 mM) for 24 h. *B* - Immunoblot analysis as in panel *A* except the PDI and CF1 antisera were hybridized simultaneously to the blot and the chemical treatment of four week-old plants was for only 6 h. H_2O control (-) or with one of the three chemicals (+) DTT (2 mM), DCMU (0.1 μM) or phosphate (10 mM). *C* - Quantification of the relative protein levels (RPL) of the PDI-52 kDa in panel *B* represented on an equal CF1 protein level basis. The control (no-chemical treatment) was set at a value of one. Values of the chemical treatments indicate the mean ± SD of three independent experiments.

formation and cleavage of disulfide bonds in protein targets (Freedman *et al.* 1994). Thus, the 52 kDa PDI described here and the 65 kDa PDI described previously (Lu and Christopher 2006) could assist with protein folding, or enzyme regulation, in chloroplasts and we suggest that candidate enzymes are those associated with starch metabolism (Smith *et al.* 2003). We show that the 52 kDa protein interacts with proteins to form a 220 kDa complex. Isolation of the proteins that interact with the PDI-52 will be required to decipher the role of these proteins in starch biogenesis and redox regulation in chloroplasts of mature leaves.

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