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Knock down of the *Arabidopsis thaliana* chloroplast protein disulfide isomerase 6 results in reduced levels of photoinhibition and increased D1 synthesis in high light

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Running title: The chloroplast AtPDI6 affects photoinhibition and D1 cycle

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FOOTNOTES

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Abstract

A chloroplast protein disulfide isomerase (PDI) was previously proposed to regulate the translation of the unicellular green alga *Chlamydomonas reinhardtii* chloroplast *psbA* mRNA, encoding the D1 protein, in response to light. Here we show that AtPDI6, a member of the 13 *Arabidopsis thaliana* PDI genes, plays a role in the chloroplast as well. We found that AtPDI6 is targeted and localized to the chloroplast. Interestingly, AtPDI6 knock down plants displayed higher resistance to photoinhibition than wild-type plants when both were exposed to ten-fold increase in light intensity. The AtPDI6 knock down plants also displayed higher rate of D1 synthesis under similar light intensity. The increased resistance to photoinhibition could not be rationalized by changes in antenna or by non-photochemical quenching. Thus, the increased D1 synthesis rate, which could keep a larger fraction of the D1 pool active under light stress, might led to the decrease in photoinhibition. These results suggest that while the D1 synthesis rates observed in wild type plants under high light intensities are elevated, repair could potentially occur faster. The findings implicate AtPDI6 as an attenuator of D1 synthesis, modulating photoinhibition in light regulated manner.

INTRODUCTION

Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily, and classically characterized as a catalyst of oxidative protein folding in the endoplasmic reticulum (ER) (Creighton 1984, Gilbert 1998). However, a growing body of evidence indicates that PDIs are localized to additional subcellular compartments and carry out regulatory functions in animals (Barbouche *et al.* 2003, Ellerman *et al.* 2006, Laurindo *et al.* 2008, Popescu *et al.* 2010) and plants (Andeme Ondzighi *et al.* 2008, Wang *et al.* 2008). RB60, the PDI of the unicellular algae *Chlamydomonas reinhardtii*, was found to be localized to the chloroplast and to take part in the translational regulation of the *psbA* mRNA, encoding the photosystem II core protein D1 (Kim and Mayfield 1997, Trebitsh *et al.* 2000, Trebitsh *et al.* 2001). The Arabidopsis PDI5 has been localized to protein storage vacuoles and lytic vacuoles as well as to the ER (Andeme Ondzighi *et al.* 2008), and the Arabidopsis PDI2 localized to the ER and nucleus compartments (Cho *et al.* 2011). Immuno-labeling using *C. reinhardtii* RB60 antisera detected an Arabidopsis chloroplast protein matching in size to the predicted molecular weight of several of the Arabidopsis PDI-like gene products (Kulp *et al.* 2006). Proteomic studies suggested that four PDI homologs are localized to chloroplasts (Kleffmann *et al.* 2006, Sun *et al.* 2009). A zinc finger protein with PDI-like activity, that does not share homology with PDIs, has also been found to localize to the thylakoid membranes in Arabidopsis (Shimada *et al.* 2007).

The *C. reinhardtii* PDI, RB60, was reported to serve as a redox sensor of a protein complex implicated in the light-regulation of the translation of *psbA* mRNA (Danon and Mayfield 1991, Kim and Mayfield 1997, Trebitsh *et al.* 2000). RB60 was shown to react specifically with cysteine residues of RB47, another member of the *psbA* mRNA binding complex (Alergand *et al.* 2006). It was proposed that RB60 might function as an attenuator,

diminishing *psbA* mRNA translation by oxidizing the regulatory cysteines of RB47 under low light, and stimulating translation by reducing RB47 cysteines under high light (Alergand *et al.* 2006). While the exact mechanism by which RB60 affects *psbA* translation is yet to be determined, evidence thus far suggest a pivotal role for PDI in the light regulated synthesis of D1.

Photoinhibition has originally been defined as a "strong light destruction of the photosynthetic apparatus" (Kok 1956). Photoinhibition affects both photosystems I and II. While photosystem II is sensitive to photoinhibition under all excess light conditions in all known photosynthetic organisms, photosystem I is damaged mostly under cold stress in chill sensitive plants (Sonoike 2011, Tikkanen *et al.* 2014). The primary target of photoinhibition is the reaction center D1 protein, yet prolonged exposure could result in the degradation of additional photosystem II proteins (Adir *et al.* 2003, Aro *et al.* 1993a, Keren and Krieger-Liszkay 2011). To counter the damage and retain photosynthetic activity, the damaged D1 must be degraded, de novo synthesized and integrated into the reaction center, a process termed D1 turnover. D1 turnover was found to occur also under low light irradiance, and its rate was reported to be positively correlated with light intensities (Jansen *et al.* 1999). Under high light intensities, when the capacity for repair does not match the rate of D1 degradation, photodamage accumulates increasing photoinhibition (Adir *et al.* 2003, Aro *et al.* 1993a, Keren and Krieger-Liszkay 2011). While in its classical view, photoinhibition is considered as a deleterious event; new ideas on the role of this process suggest an adaptive role for this phenomenon (Adams *et al.* 2013, Greer *et al.* 1991, Juvany *et al.* 2013, Long *et al.* 1994, Raven 2011).

Here we show that AtPDI6 is targeted to chloroplasts using stable transgenic plants and transient expression assays. Interestingly, we found in two independent AtPDI6 T-DNA insertion

lines, that lowering AtPDI6 expression level led to increased resistance to photoinhibition under high light intensity. Our findings further suggest that the higher resistance to photoinhibition might be caused by the increased D1 synthesis rates observed in these plants. These data lead us to speculate that similarly to RB60 AtPDI6 might be involved in the attenuation of D1 synthesis and could, in turn, regulate the onset of photoinhibition when Arabidopsis plants are exposed to high light intensity.

Results

Localization of AtPDI6 to *A. thaliana* chloroplasts

To investigate whether similarly to *C. reinhardtii*, one or more of the actively expressed PDI paralogs of *A. thaliana* (Houston *et al.* 2005, Meiri *et al.* 2002) are localized to the chloroplast, we assayed their subcellular targeting identities by GFP fusion assay. Using confocal laser microscopy, we compared the fluorescence of the transiently expressed PDIs fused with GFPK at their carboxy terminus in *A. thaliana* protoplasts to the chlorophyll autofluorescence as we previously assayed for RB60 (Levitan *et al.* 2005). To avoid mislocalization due to over-accumulation of expressed proteins we imaged only protoplasts displaying the earliest signal of GFP fluorescence. The green fluorescence pattern detected for the GFP fusion protein of the product of gene At1g77510 (AtPDI6:GFPK) overlapped in part the red autofluorescence of the chlorophyll (Fig. 1A), suggesting that it might be targeted to chloroplasts *in vivo*. Yet, the GFP fluorescence was detected outside of the chloroplasts as well. Previously, we have shown that RB60 is dually localized to the chloroplast and the ER (Levitan *et al.* 2005). Thus, to investigate whether AtPDI6 might also be targeted to the ER, we compared the fluorescence, in the same cell, of the AtPDI6:GFPK signal detected outside the chloroplasts with that of an ER-localized

YFPK (Fig. 1A, Chit:YFP). Notably, the fluorescence of AtPDI6:GFPK detected outside of chloroplasts matched that of the ER marker, suggesting that AtPDI6 resembles RB60 in its localization to both the chloroplast and the ER (Levitan *et al.* 2005).

To further investigate the chloroplast role of AtPDI6, transgenic plants stably expressing AtPDI6 with a carboxy terminal hemagglutinin tag (AtPDI6:HA) were generated. Intact chloroplasts from AtPDI6:HA or from wild type plants were purified on a Percoll density gradient and lysed with denaturing buffer. The protein extracts were separated by gel electrophoresis and blotted onto a membrane for an immunoblot analysis. The loading of chloroplast proteins was demonstrated by staining of the large subunit of Rubisco (LSU, Fig. 1B, bottom panel). The anti-HA antibody did not react with the protein extracts of wild type plants, indicating the lack of nonspecific epitopes. In contrast, the anti-HA antibody recognized AtPDI6:HA in the total protein extract and in the intact chloroplast protein extract of the AtPDI6:HA transgenic plants, further substantiating the chloroplast localization of AtPDI6 (Fig. 1B, top panel). To rule out the possibility that the detection of AtPDI6:HA in the chloroplast extract is due to contaminating ER microsomes, we assayed the protein extracts with antibody specific to the ER marker BiP (Fig. 1B, middle panel). BiP was decorated by the specific antibody in the total protein extracts from both wild type and AtPDI6:HA plants but not in the corresponding extracts from the isolated chloroplasts, indicating that the purified chloroplasts are free of ER microsomes. Notably, the localization of AtPDI6 to chloroplasts is also supported by its identification in two proteomic studies of chloroplast proteins by independent research groups (Kleffmann *et al.* 2006, Sun *et al.* 2009).

To discern the subchloroplast partition of AtPDI6, intact chloroplasts purified from AtPDI6:HA plants were homogenized and separated by centrifugation into the soluble stroma

and the membrane fractions. Immunoblot assay of the two fractions using anti-HA serum indicated that AtPDI6 is mostly soluble (Fig. 1C), colocalized with the LSU of RUBISCO rather than with the membrane localized photosystem II core protein D1.

AtPDI6 is an active PDI

AtPDI6 has been identified as a PDI-like protein according to its amino acid sequence (Meiri *et al.* 2002). A detailed analysis of the conserved domains and motifs of *AtPDI6* open reading frame using the program Interpro Scan (EMBL-EBI) (Zdobnov and Apweiler 2001) predicts a classical PDI with the four consensus domains (a, b, b', a') and a carboxy-terminal ER retention signal KDEL (Kulp *et al.* 2006, Tian *et al.* 2006) (Fig. 2A). The two redox active thioredoxin domains, a and a', contain the classic PDI active site motif Cys-Gly-His-Cys (CGHC). To characterize the biochemical activity of AtPDI6, the protein containing a N-terminal His_6 tag was expressed in *Escherichia coli* and purified using nickel affinity matrix. The activity of the purified AtPDI6 was then tested in the catalytic oxidative refolding of reduced denatured RNaseA, an assay that depends on both the oxidase and the isomerase activities of PDI (Lundstrom *et al.* 1992). The catalytic activity, measured by the hydrolysis of the RNaseA substrate cCMP, of 3 µM recombinant AtPDI6 in reactions containing 15µM reduced denatured RNaseA was similar to that catalyzed by 3µM of the control bovine PDI (Fig. 2B), indicating that similarly to RB60 AtPDI6 is a bona fide PDI capable of both transferring and isomerizing protein disulfides.

AtPDI6 deficiency reduces the rate of photoinhibition

To elucidate AtPDI6 function *in vivo*, two independent homozygous T-DNA insertion lines in the *AtPDI6* locus were isolated. SAIL_430_A04, denoted *pdi6-1*, contains a T-DNA insertion in

the 10th exon of AtPDI6, and SALK_026561, denoted *pdi6-2*, contains an insertion in the 5' UTR of the gene (Fig. 3A). The degree by which the *AtPDI6* mRNA level is suppressed in these two lines was determined by qRT-PCR of total RNA extracted from each of the insertion lines and from wild type plants (Fig. 3B). *AtPDI6* mRNA levels were found to be significantly reduced in *pdi6-1* and *pdi6-2* plants, indicating that the insertion lines are adequate for studying the phenotypic consequences of lowering the expression of AtPDI6.

RB60, the chloroplast localized PDI of *C. reinhardtii*, was reported to act as a regulator in the light regulated translation of *psbA* mRNA, encoding the photosystem II core protein D1 (Danon and Mayfield 1994, Trebitsh *et al.* 2000, Trebitsh *et al.* 2001). To investigate whether AtPDI6 affects photosystem II dynamics under high light stress, wild type, *pdi6-1* and *pdi6-2* leaf disks were subjected to 850 µmol photons $\cdot m^{-2} \cdot sec^{-1}$ light intensity (about 10 times growth light intensity). The maximal quantum yield of photosystem II (Fv/Fm), corresponding to the portion of active photosystem II reaction centers (Maxwell and Johnson 2000), was calculated from chlorophyll fluorescence measurements taken at 30 minutes intervals (Fig. 4A). Each fluorescence measurement was carried out after 15 minutes of dark adaptation, allowing complete relaxation of non-photochemical quenching (NPQ). Notably, following a 60 minutes exposure to high light, the Fv/Fm decrease in *pdi6-1* and *pdi6-2* leaf disks was significantly lower than the decrease measured for wild type leaf disks, indicating that these plants retain a higher proportion of their reaction centers active. This suggests a higher resistance to photoinhibition in AtPDI6 knock down plants (Fig. 4A).

The lower susceptibility of the AtPDI6 knock down plants to the photoinhibitory conditions might be a result of either a reduced damage to photosystem II, enhanced repair of photosystem II, or a combination of the two. Thus, to assay whether the resistance to

photoinhibition of AtPDI6 knock down plants might be caused by higher photosystem II repair rate, a similar experiment was carried out in the presence of the chloroplast protein synthesis inhibitor lincomycin. The photoinhibition rates, expectedly higher in comparison to the experiment with no lincomycin, were similar for wild type and AtPDI6 knock down plants in the presence of lincomycin (Fig. 4B). This finding suggests that photosystem II repair might be the main factor contributing to the reduced photoinhibition of pdi6-1 and pdi6-2 plants.

A reduced damage to photosystem II during exposure to high light conditions could also be a result of lower excitation pressure by a smaller antenna/photosystem II ratio (Melis *et al.* 1998, Neidhardt *et al.* 1998), or by activation of fast acting photoprotective mechanisms such as NPQ (Li *et al.* 2009). To consider a further effect due to differences in antenna size, we compared the chlorophyll contents and [chlorophyll a]/[chlorophyll b] ratios of wild type and knock down plants (Fig. 5A,B). We found that the total chlorophyll concentration in *pdi6-1, pdi6-2* and wild type plants was similar (Fig. 5A). Moreover, there was no difference in the [chlorophyll a]/[chlorophyll b] ratio (Fig. 5B). The similar chlorophyll contents and similar [chlorophyll a]/[chlorophyll b] ratio indicated a similar composition of reaction centers and antenna proteins, suggesting that the reduced photoinhibition of AtPDI6 knock down plants was not a result of a change in antenna architecture.

To evaluate the short term effects of NPQ induction and relaxation mechanisms, chlorophyll fluorescence kinetics of dark-adapted AtPDI6 knock down plants and of wild type plants were measured during and after exposure to either 80 or 800 µmol photons·m^{-2}·sec^{-1} actinic light (Fig 5C). The relaxation of NPQ in the dark was evaluated according to (Quick *et al.* 1989). Similar NPQ kinetics were observed on a short time scale of a few minutes for wild type, *pdi6-1* and *pdi6-2* leaves during the induction and the relaxation periods under both low light and

high light experiments (Fig. 5C). These findings together with the strong dependency of the phenotype on chloroplast protein synthesis indicate that the reduced levels of photoinhibition in the AtPDI6 knock down plants are largely linked to photosystem II repair and D1 protein turnover.

AtPDI6 knock down plants have increased rates of D1 synthesis

Light induced damage has been attributed mainly to photosystem II, and in particular to the core protein D1 (Adir *et al.* 2003, Aro *et al.* 1993b, Barber and Andersson 1992). Thus, first we examined whether AtPDI6 affects the steady state amounts of D1 before and during high light treatment. Previous studies suggest that the overall levels of D1 in plants exposed to high light conditions decline in a slower rate than the loss observed to photosystem II activity (Keren *et al.* 1997, Ohad *et al.* 1985, Virgin *et al.* 1988). Immunoblot assays with anti-D1 antibodies of total protein extracts from wild type, *pdi6-1* and *pdi6-2* leaf disks were used to evaluate overall D1 levels. Serial dilutions of the protein extracts are presented, to help in the evaluation of D1 levels. Similarly to the results found in the literature, steady state D1 levels in wild type plants were found to be stable following 90 minutes and 210 minutes of the high light treatment (Fig. 6A), in parallel to a significant loss in photosystem activity (Fig. 4A). The addition of a chloroplast translation inhibitor was reported to enhance the decrease in overall D1 levels. However, the loss of reaction center activity was reported to occur faster than the loss of D1 under these conditions as well, and D1 was observed even after prolonged high light treatment (Aro *et al.* 2005, Yin *et al.* 2008). Here, in the presence of the chloroplast translation inhibitor lincomycin, D1 steady state levels in wild type leaf disks were found to decrease substantially after 210 minutes of high light treatment (Fig. 6A, Lin+). When comparing wild type and

AtPDI6 knock down leaf disks, no apparent difference in D1 steady state levels was observed both before and during the high light treatment (Fig. 6A). The similar overall D1 levels make it less likely for the higher resistance to photoinhibition to be caused by the distribution of the photon flux over larger number of photosystems.

Photoinhibition is said to occur when the D1 repair cycle no longer able to compensate for damage to photosystem II. In the chloroplast of *C. reinhardtii*, RB60 was reported to change the redox state of RB47 in a protein complex that binds to the *psbA* mRNA, ultimately resulting in a change in D1 translation rates as light intensity changes (Alergand *et al.* 2006, Danon and Mayfield 1994, Kim and Mayfield 1997, Trebitsh *et al.* 2000). The disappearance in the presence of lincomycin of the reduced sensitivity to photodamage in AtPDI6 knock down plants (Fig. 4B) further implicated the rate of de novo synthesis of D1 as a likely cause of the higher resistance to photoinhibition. Thus, the levels of D1 synthesis in wild type and AtPDI6 knock down plants were tested by metabolic pulse labeling using [^{35}S]-methionine during the high light treatment. Higher D1 synthesis rates were found in *pdi6-1* and *pdi6-2* leaf disks compared to wild type, following 90 minutes of exposure to high light (Fig. 6B). The increased synthesis of D1 was not the result of a change in D1 transcript levels, as *psbA* mRNA levels appeared to be similar under growth light and high light conditions in wild type, *pdi6-1* and *pdi6-2* leaf disks (Fig. 6C). These findings advocate that *psbA* mRNA translation is stimulated to a higher degree in *pdi6-1* and *pdi6-2* than in wild type plants. Presumably, as overall D1 levels remain similar in AtPDI6 knock down plants, the higher rate of synthesis of the D1 might result in a larger fraction of active photosystem II reaction centers, thereby conferring higher tolerance of high light stress.

AtPDI6 mRNA levels increase in high light

To better understand the role of *AtPDI6,* we wished to determine whether the expression of *AtPDI6* is regulated by high light stress. qRT-PCR analysis showed that the steady state level of *AtPDI6* mRNA in leaf disks isolated from wild type plants increased significantly after a 75 minutes exposure to high light intensity (850 $\mu mol \cdot m^{-2} \cdot sec^{-1}$), 2.8 times higher than after a same period of normal growth light (70 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) (Fig. 7). These finding suggest that *AtPDI6* is a high light response gene.

Discussion

The localization of a catalytically active PDI, AtPDI6, to the chloroplasts of the higher plant *A. thaliana* (Fig. 1 and 2) prompted us to investigate whether AtPDI6 knock down strains might implicate a chloroplast role for the protein. As the algal chloroplast PDI, RB60, has been shown to be involved in the regulation of translation of *psbA* mRNA (Kim and Mayfield 1997, Trebitsh, *et al.* 2000), we were specifically interested in determining whether AtPDI6 affects the D1 repair cycle and as result photosystem II activity. Our findings that diminished levels of AtPDI6 conferred resistance to photoinhibition under high light conditions (Fig. 4A) probably due to higher D1 repair rates (Fig. 4B and 6) were in support of the hypothesized role of the protein. The increased D1 synthesis in the knock down strains over its typical high level in wild-type plants might further suggest that AtPDI6 might act as an attenuator, repressing or activating D1 synthesis in response to light and redox signals. Yet, until the exact mechanism by which AtPDI6 carries out this phenomenon is determined, the possibility that the reduced photoinhibition sensitivity and increased synthesis rate of D1 are secondary effects of the

disturbance of AtPDI6 function in chloroplasts of AtPDI6 knock down plants cannot be ruled out.

Photoinhibition has been characterized as a state of stress in plants exposed to high irradiance levels (Adir *et al.* 2003). As the rate of damage is positively correlated with light intensity, it has been proposed that photoinhibition occurs when the rate of photosystem II repair cannot compensate for the rapid damage (Edelman and Mattoo 2008, Jansen *et al.* 999). Inhibition of D1 synthesis was shown to be a major factor in photoinhibition (Murata *et al.* 2007, Nishiyama *et al.* 2006, Nishiyama *et al.* 2011). This has been supported by observations in the field, manifested in a transient mid-day decrease in variable fluorescence values (Adams 1988, Bolhar-Nordenkampf *et al.* 1991, Demmig-Adams *et al.* 1989). Enhanced rate of D1 synthesis as light intensity increases has been observed in numerous studies. Intriguingly, the results presented here suggest that under the experimental conditions AtPDI6 knock down plants were able to repair photosystem II faster than wild type. This suggests that the capacity for repair in wild type plants might be even greater than the high rates observed in previous studies (Aro *et al.* 1993a, Edelman and Mattoo 2008, Jansen *et al.* 1999, Ohad *et al.* 1984, Trebitsh *et al.* 2000), and that AtPDI6 might take part in attenuating the repair mechanisms.

What might be the possible benefits of repairing photosystem II reaction centers at a lower rate than the one observed in AtPDI6 knock down plants for wild type Arabidopsis plants? A speculative explanation might be that this phenomenon could help alleviate the high light stress. Exposure to high irradiance has been suggested to result in excessive production of reducing photosynthetic equivalents, which, in turn, could inflict deleterious oxidative stress on chloroplasts and on the cell as a whole (Barber and Andersson 1992). Thus, it is not entirely surprising that plant cells employ a plethora of protective mechanisms (Li *et al.* 2009) which

come at the expense of photosynthetic efficiency. Chloroplasts are able to dissipate excessive excitation energy as heat by activating NPQ (Muller *et al.* 2001). Excitation energy can also be redistributed between the two photosystems via the state transition mechanism (Rochaix 2007), promoting cyclic electron flow that does not yield net production of reducing equivalents. Excessive reducing equivalents can be removed by photorespiration (Brestic *et al.* 1995, Guan *et al.* 2004, Kozaki and Takeba 1996) and the Mehler reaction (Asada 1999, Asada *et al.* 1974, Biehler and Fock 1996). In addition, under high light intensities, carbon fixation reactions could reach maximal rate even with less functional photosystems (Long *et al.* 1994). It has been suggested that accumulation of inactive photosystem II centers during photoinhibition might protect as quenchers the remaining active reaction centers (Greer *et al.* 1991). Photoinhibition of photosystem II has also been proposed to occur in a regulated manner, protecting photosystem I from damage by decreasing the linear electron flux (Sonoike 2011). Hence, what may be viewed as damage to the photosystem II might also be seen as avoidance through inactivation, blurring the distinction between protection and damage in photoinhibition (Long *et al.* 1994).

In this study, our results revealed that AtPDI6 functions in the regulation of D1 synthesis under high light. We postulate that while the D1 synthesis rates observed in wild type plants under high light intensities are high, repair could potentially occur even faster and photoinhibition could be ameliorated to some extent. We speculate that the observed attenuation of D1 synthesis by AtPDI6 during exposure to high light might function as a photoprotective mechanism.

Methods

Plant material and growth conditions

A. thaliana ecotype Colombia 0 plants were used in this study. All the plants used in this study were grown under short day conditions (10/14 hours light/dark cycles of 70 µmol photons·m^{-2}·sec^{-1}, at 22°c), following cold treatment for homogenous germination (2 days at 4°c). When specified, plants were grown in a greenhouse, at 22°c. Greenhouse grown plants were grown in March and April in Israel, and so daytime was between 11.3 and 13 hours/day. In the greenhouse maximal light intensities of 500-600 µmol photons·m^{-2}·sec^{-1} have been measured around noon.

Plants used for high light experiments were grown on 1% agar Nitsch medium (Duchefa, www.duchefa.com) supplemented with 2% sucrose in polycarbonate 107mm·107mm·96mm culture boxes with a "LIFEGUARD 40mm" culture box lid (Sigma, www.sigmaaldrich.com), or in Petri dishes on the same medium. All other plants in this study were grown on soil.

Construction of fusion proteins and fluorescent microscopy

The *AtPDI6* open reading frame was amplified by using an *A. thaliana* cDNA library and ligated through SmaI sites, into a pUC18-GFPK containing vector, yielding a fusion protein upstream and in frame to a GFP5 open reading frame that contained the ER retention signal KDEL at its C-terminus (AtPDI6:GFPK). AtPDI6:GFPK was expressed under the control of the cauliflower mosaic virus 35S promoter. The ER marker chitinase YFP fusion protein was constructed as described in (Levitan, Trebitsh, Kiss, Pereg, Dangoor and Danon 2005). Transient expression in *A. thaliana* protoplasts was conducted as described in (Kovtun *et al.* 2000, Levitan *et al.* 2004). Fluorescence images were obtained by using a confocal laser scanning microscope Olympus

Fluoview FV500, as described previously (Levitan, Trebitsh, Kiss, Pereg, Dangoor and Danon 2005).

Transgenic plants stably expressing AtPDI6:HA

The *AtPDI6* open reading frame was amplified out to an *A. thaliana* cDNA library (primers: AtPDI6 up SalI: 5'-GTCGACATGGCGTTTAAGGGTTTCG-3'; AtPDI6 down NheI: 5'-GCTAGCTGCGGCCGTTTCCTCGGT-3') and cloned in frame to a C terminal 3·HA tag + c-Myc tag+stop codon, into a pART7 vector containing an upstream 35S promoter. The construct containing the promoter and the tagged *AtPDI6* was sub-cloned into a pBART binary vector and stably transformed into *A. thaliana* plants by floral dipping. Transgenic plants were selected according to their resistance to the herbicide BASTA.

Isolation of intact chloroplasts

Intact chloroplasts were isolated according to (Aronsson and Jarvis 2002), with modifications. 5 gr of leaf blades from 4 weeks old plants were homogenized in 50 ml grinding buffer (0.3M sorbitol, 20mM HEPES-KOH pH=8.0, 5mM $MgCl_2$, 5mM EDTA, 5mM EGTA, 10mM $NaHCO_3$) by a polytron homogenizer (Kinematica, Switzerland, www.kinematica-inc.com) by 2-3 3 sec pulses at ≈50% max speed. Homogenized leaves were filtered by gauze cloth followed by filtration by 2 layers of Miracloth (Calbiochem, www.emdchemicals.com). The homogenate was centrifuged (600 g, 10 min, 4°c, break off). The pellet re-suspended in 5 ml chloroplast wash buffer (0.3M sorbitol, 10mM HEPES-KOH pH=8.0, 10mM $MgCl_2$, 10mM NaCl) and loaded on a two-step Percoll (GE Healthcare Life Sciences, www.gelifesciences.com) gradient in a 15 ml Corex tube. The bottom step of the gradient was comprised of 1.47 ml Percoll mix (95% w/v

Percoll, 3% w/v PEG-8000, 1% w/v Ficoll, 1% w/v BSA) and 0.53 ml gradient mix (0.75M sorbitol, 25mM HEPES-KOH pH=8, 10mM EDTA), and the top step of 4.21 ml Percoll mix and 5.79 ml gradient mix. Intact chloroplasts were spun down (10000 g, 15 min, 4°c, break off, swing-out rotor) and gently collected from the interface between the two steps of the gradient. Percoll traces were removed by washing chloroplasts with chloroplast wash buffer and followed by centrifugation (1000 g, 10 min, 4°c, break off). Pellet was suspended in 200µl-500µl chloroplast wash buffer and kept at -80°c.

Protein electrophoresis and Western blot analysis

Plant material was homogenized in protein extraction buffer (10mM Tris pH=7.5, 1mM EDTA, 1mM NaCl, 10µl/ml protease inhibitor cocktail (Sigma)) using acid washed glass beads (Sigma) in an amalgamator (Securamate, Silmet, Israel, www.silmetdental.com). Protein samples were incubated in protein sample buffer (10% glycerol, 1% SDS, 50mM β-mercaptoethanol, 15mM DTT, 75mM Tris pH 6.8, 1mM EDTA, 0.01% Pyronin Y). Samples for AtPDI6:HA localization were incubated for 5 minutes at 95°c prior to loading onto the gel. Samples for anti-D1 analysis were incubated over-night at room temperature prior to lading. Protein samples were separated by SDS-PAGE and electro-blotted onto nitrocellulose membranes (Whatman, www.whatman.com). Equal protein loadings were determined for all experiments by amido black staining of the blotted membranes. Primary antibodies used in this study: mouse anti HA (Sigma), rabbit anti maize BiP (generously provided by E. Herman), rabbit anti psbA (Agrisera, Sweden, www.agrisera.com). Secondary antibodies used were goat anti mouse conjugated to horseradish peroxidase and goat anti rabbit HRP. Chemiluminescese assays were preformed using EZ-ECL (Biological Industries, Israel, www.bioind.com).

Semi quantitative RT-PCR and quantitative RT-PCR

RNA extraction was preformed using Tri-Reagent (Molecular Research Center, www.mrcgene.com) according to manufacturers instructions. cDNA was generated using Superscript II reverse transcriptase (Invotrogen, www.invitrogen.com).

For the generation of cDNA used for semi-quantitative RT-PCR, oligo dT was used as the primer for nuclear encoded genes and an *A.t. psbA* down primer (5'-TCCATTTGTAGATGGAGCCTCAAC-3') was used for the chloroplast encoded *psbA* mRNA. cDNA levels were equalized according to actin cDNA levels, determined by actin up (5'-GGTTTTGCTGGGGATGATGC-3') and actin down (5'-CATTGAAAGTCTCAAACATGATTTGA-3') primers. AtPDI6 cDNA was amplified using AtPDI6 up SalI and AtPDI6 down NheI primers. *psbA* cDNA was amplified using A.t. *psbA* up primer (5'- CGGCGGTCCTTATGAACTAA-3'). Amplifications were done at low cycle numbers after calibration, to ensure the amplification results are still unsaturated.

For qRT-PCR, cDNA was generated by random priming, using a random hexamer mix (Applied Biosystems, www.appliedbiosystems.com). All qRT-PCR reactions were done using an Applied Biosystems 7300 Real-Time system at its default parameters. Power SYBR Green Master Mix (Applied Biosystems) was used for all reactions. Primer pairs for qRT-PCR reactions were calibrated by standard curves so R^2 values were greater then 0.98 and a slope between -3 and -3.6. In all qRT-PCR reactions RNA levels were equilibrated using Actin 7, as micro-array data suggests its levels are not affected by varying light conditions (primers: ACT7 F: 5'-AGTGGTCGTACAACCGGTATTGT-3'; ACT7 R: 5'-GAGGAAGAGCATACCCCTCGTA-3' ; AtPDI6 F: 5'- CCCTTGGTGTGGTCACTGC-3';

AtPDI6 R: 5'- CATCGATCTTGGCGAGAGC-3'). All primers were used at a final concentration of 0.5µM.

Recombinant PDI6 expression and purification

Mature AtPDI6 (mPDI6), AtPDI6 lacking its N terminal signal peptide as predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson *et al.* 2007), was amplified out of an *A. thaliana* cDNA library (primers: mPDI6 up BamHI: 5'-GGATCCAGGAATTTGTCTTGACTCTC-3'; mPDI6 down KpnI: 5'-GGTACCCTACAGCTCGTCCTTTGCGG-3') and subcloned into a pQE-30 expression vector (Qiagen, www.qiagen.com) containing an in-frame N terminal 6·His tag. M-15 *Escherichia coli* (Qiagen) transformed with the mPDI6:pQE-30 construct were grown in Luria Bertani medium at 37°c to a mid-logarithmic state (OD600=0.4-0.6), after which PDI6 expression was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Recombinant mPDI6 purification was preformed as described in (Alergand, Peled-Zehavi, Katz and Danon 2006). Following purification, recombinant mPDI6 storage buffer was changed by dialysis to phosphate buffered saline (Sigma, www.sigmaaldrich.com) in 20% glycerol and stored at -80°c.

In vitro determination of PDI activity

mPDI activity was determined as described in (Gilbert 1998), with modifications. 15µM reduced denatured ribonuclease A (rdRNaseA) were refolded by 3µM of either recombinant mPDI6 or bovine PDI (Sigma), or in the absence of PDI, in a 96 well plate with a final reaction volume of 300µl. cCMP hydrolysis was followed by measuring the change in absorbance at 296nm every

10 sec for 45 minutes. PDI activity was deduced by calculating the second derivative of the absorbance.

High light exposure, chlorophyll fluorescence measurement and chlorophyll concentration determination.

Maximal photosystem II quantum yield values were calculated for dark-adapted leaf disks during prolonged exposure to high light. Leaf disks (7 mm diameter) were cut out of 6 weeks old plants and suspended on tap water with or without 1.4 mg/ml lincomycin. Leaf disks were left in total darkness for 2 hours prior to exposure to high light (850 µmol photons·m^{-2}·sec^{-1}). Following every 30 minutes of exposure to high light, leaf disks were dark adapted for 15 minutes, after which chlorophyll fluorescence was measured and Fv/Fm values calculated using a Maxi version Imaging-PAM (Heinz Walz GmbH, www.walz.com). Chlorophyll concentration was determined for 6 weeks old plants as described in (Porra *et al.* 1989). Chlorophyll fluorescence levels during the induction of illumination were measured from dark-adapted intact leaves using the Dual-PAM 100 system (Heinz Walz GmbH). Actinic light (set to 56 or 850 µmol photons·m^{-2}·sec^{-1}) was switched on 40 seconds after F_0 and Fm determination, for 240 sec. Saturating pulses (600 msec of 10000 µmol photons·m^{-2}·sec^{-1}) were administered at 20 sec intervals. Photosystem II quantum yield (Y(II)) and non photochemical quenching (NPQ) levels were calculated according to (Maxwell and Johnson 2000): $Y(II)=(F_m-F)/F_m$ $NPQ=(F_m^0-F_m)/F_m$.

In vivo labeling of chloroplast proteins

In vivo labeling of chloroplast proteins was done according to (Aro, McCaffery and Anderson 1993a) with modifications. The lower epidermis of leaf disks was gently scraped with coarse sand paper. Leaf disks were subjected to high light treatment as describes above. Following 60 minutes of high light treatment, cycloheximide and Tween-20 were added to a final concentration of 20µg/ml and 0.4% respectively. 15 minutes after the addition of cycloheximide and Tween-20, a 15 minute metabolic pulse labeling was induced by the addition of L-[^{35}S] methionine redivue solution (GE Healthcare Life Sciences) to a final concentration of 60µCi/ml, after which leaf disks were promptly frozen in liquid N_2. Proteins were extracted, separated by SDS-PAGE and electro-blotted onto a nitrocellulose membrane as described above. Labeled proteins were visualized by autoradiography. Densitometry measurements were calculated by ImageJ (NIH).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number NM_106400. The AGI locus identifier for AtPDI6 is At1g77510.

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Figure Legends

Figure 1. AtPDI6 is localized to chloroplasts. **A.** AtPDI6 fused to a C terminal GFP and the ER retention signal KDEL was transiently expressed in Arabidopsis protoplasts and imaged using confocal microscopy (AtPDI6:GFPK). The auto-fluorescence of the chlorophyll (Chlps) and the fluorescence of a YFP fused ER marker (ER) were imaged as well, and the merge images (merged) illustrate the chloroplast and ER localization of AtPDI6. **B.** Western blot analysis of total cell proteins (tot) and intact chloroplasts (chlps) from wild type plants and transgenic plants stably expressing AtPDI6 fused to a C terminal HA tag (AtPDI6:HA). Immunoblot assay using anti-HA Ab shows that AtPDI6:HA is localized to chloroplasts. Anti-BiP Ab, an ER marker, indicates that chloroplasts are free from ER contamination. Staining of the Rubisco large subunit (LSU) indicates the loading of soluble chloroplast proteins. **C.** Immunoblot assay using anti-HA sera shows that AtPDI6:HA is localized in the chloroplast stroma (sol), like the Rubisco large subunit, rather than to thylakoids (mem) like the D1 protein.

Figure 2. AtPDI6 is an active PDI. A. AtPDI6 domain architecture is predicted by InterPro Scan to be that of a classical PDI, with two redox active thioredoxin domains containing the canonical CGHC active site, two redox-inactive thioredoxin folds and an ER retention signal. **B.** Oxidative re-folding of 15µM reduced denatured Ribonuclease A by 3µM recombinant AtPDI6 (squares), 3µM bovine PDI (triangles) and no PDI (diamonds), was followed by the hydrolysis of cCMP creating a change in absorbance at 269nm. The AtPDI6 oxido-isomerase activity in vitro suggests an oxidative role in chloroplasts.

Figure 3: Characterization of two AtPDI6 knock down lines. A. Schematic representation of *AtPDI6* gene structure showing the T-DNA insertion sites of SALK_026561 (*pdi6-2*, an insertion in the 5' UTR) and SAIL_430_AD4 (*pdi6-1*, an insertion in the 10th exon of *AtPDI6*). Bulky segments represent exons, thin segments represent introns. **B**. Quantitative RT-PCR analysis showed that *AtPDI6* transcript levels in *pdi6-1* and *pdi6-2* plants are lower than those in wild type.

Figure 4: AtPDI6 knock down plants display resistance to photoinhibition with no apparent change in chlorophyll content. A. Leaf disks of AtPDI6 knock down lines (full triangles and full squares) retain higher Fv/Fm values during exposure to high light intensity (850 µmol m^{-2} sec^{-1}) corresponding to a higher percent of their photosystem II reaction centers active, as compared to wild type leaf disks (full circles). **B.** A similar experiment with the addition of 1.4 mg/ml lincomycin (a chloroplast translation inhibitor). Photoinhibition rates were found to be similar for wild type (empty circles) and AtPDI6 knock down lines (empty triangles and squares).

Figure 5: AtPDI6 knock down and wild type plants have similar antenna characteristics and NPQ mechanisms. A. Chlorophyll concentrations in leaves of AtPDI6 knock down plants were found to be similar to that of wild type plants. **B.** [chlorophyll a]/[chlorophyll b] ratio was found to be similar as well. Both results indicate that the higher resistance to high light of the AtPDI6 knock down plants is less likely to be the effect of a change in antenna architecture or antenna/photosystem ratio. **C.** Chlorophyll fluorescence characteristics of AtPDI6 knock down and wild type plants in a light treatment followed by relaxation in the dark. NPQ values, calculated according to (Maxwell and Johnson 2000), show similar kinetics in the leaves of wild type and AtPDI6 knock down plants in low light and high light during the first 1000 seconds of illumination and during the relaxation period in the dark.

Figure 6. AtPDI6 deficiency enhances D1 turnover in high light. A. Western blot analysis of total leaf proteins with D1 antiserum. Serial dilutions are presented to aid in quantification. D1 levels are similar in wild type and AtPDI6 knock down plants before and after exposure to 90 minutes and 210 minutes of high light in the absence and the presence of lincomycin (-Lin and +Lin), suggesting the overall amount of photosystem II reaction centers is similar. **B**. Metabolic pulse labeling with [S^{35}]-methionine shows that the synthesis rate of D1 is higher in AtPDI6 knock down plants than in wild type plants during exposure to high light. Top Panel. Autoradiogram of pulse labeled D1. Protein staining of the Rubisco large subunit (LSU) with amido black is shown to demonstrate protein loading. Bottom panel. Averages of three independent pulse-labeling experiments analyzed by densitometry. Error bars indicate standard error. **C**. RT-PCR analysis shows that *psbA* mRNA (encoding D1) levels are similar in wild type and AtPDI6 knock down plants under normal growth light conditions (GL) and in response to

high light (HL), indicating that the higher D1 synthesis rates are due to higher translation rates of *psbA* mRNA in AtPDI6 knock down plants.

Figure 7. AtPDI6 is a high light response gene. *AtPDI6* steady state mRNA levels were determined following a 90 minute exposure to low light or high light by qRT-PCR. *AtPDI6* mRNA levels were found to increase about 2.8 fold following exposure to high light.