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Chloroplast protease/chaperone AtDeg2 holds γ_1 subunit of ATP synthase in an unaggregated state under high irradiance conditions in *Arabidopsis thaliana*

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Abstract

Little data on the role played *in vivo* by chloroplast protein AtDeg2 as a chaperone is available. Therefore, we sought for chloroplast proteins protected from high irradiance-induced interprotein aggregation *via* disulphide bridges by AtDeg2 acting as a holdase. To reach this goal, we performed analyses which involved comparative diagonal electrophoreses of lysates of chloroplasts isolated from wild type (WT) plants and transgenic plants *35S:AtDEG2$^{\Delta PDZ1}$-GFP* which expressed AtDeg2 lacking its chaperone activity but retaining the protease activity. The results of the analyses indicate that AtDeg2 acting as a holdase prevents a single chloroplast protein, *i.e.*, the γ_1 subunit of ATP synthase from long-term high irradiance-induced homodimerization mediated by disuplhide bridges and this allows us to better understand a complexity of physiological significance of AtDeg2 – the chloroplast protein of dual protease/chaperone activity.

Keywords: chaperone; Deg2; elevated irradiance; homodimerization; protease.

Introduction

Deg serine non-ATP hydrolyzing endopeptidases were first discovered in *E. coli* (Lipińska *et al*. 1988, Strauch and Beckwith 1988) and later demonstrated to be present in cells of organisms belonging to all domains of life (Ortega *et al*. 2009). The mature molecule of chloroplast serine endopeptidase AtDeg2 contains an N-terminal protease domain (with H159 D190 S268 triad as the catalytic site) as well as PDZ1 and PDZ2 domains shown to be involved in dimerization of trimeric units into non-active AtDeg2 hexamers, and suggested to be involved in rearrangement of trimeric units into enzymatically competent, higher oligomeric states of the protein (Sun *et al*. 2012). It has been shown recently that apart from being a non-ATP-hydrolyzing protease, chloroplast protein AtDeg2 owned chaperone activity as well, consisting in an ability to inhibit the aggregation of denatured lysozyme *in vitro* (holdase activity – Sun *et al*. 2012, Jagodzik *et al*. 2018) and to cause disaggregation of produced lysozyme aggregates (disaggregase activity – Jagodzik *et al*. 2018). It was demonstrated that PDZ1 and PDZ2 domains were

Highlights

- Under high light, *35S:AtDEG2ΔPDZ1-GFP* plants express AtDeg2 lacking chaperone activity
- *35S:AtDEG2ΔPDZ1-GFP* and WT plants show a very similar disulphide reductase activity
- AtDeg2 inhibits high light-induced homodimerization of γ_1 subunit of ATP synthase

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Abbreviations: DTT – dithiothreitol; GFP – green fluorescent protein; GSH – reduced glutathione; SDS-PAGE – sodium dodecylsulphate polyacrylamide gel electrophoresis.

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required for both holdase and disaggregase activities of AtDeg2 *in vitro* and protease domain (but not S268 in its catalytic site) contributed to these activities (Jagodzik *et al*. 2018). However, data concerning the role played by AtDeg2 *in vivo* as chaperone are rather limited. Namely, we have shown recently that under non-stressing conditions, AtDeg2 influenced a few phenotypic features of *Arabidopsis thaliana* through its chaperone activity in conjunction with the protease activity, *i.e*., the time when cotyledons were fully opened, the number of inflorescence branches, and seed length in plants which have terminated their generative development (Adamiec *et al*. 2018). To date, no chloroplast proteins, whose potential aggregation were inhibited by AtDeg2 or proteins which form aggregates subsequently disaggregated by AtDeg2, have been identified (neither under non-stressing conditions nor any abiotic stress). To fill this gap in our understanding of the functions performed by AtDeg2 *in vivo* due to its chaperone activity, we sought for chloroplast proteins protected from aggregation by AtDeg2 acting as a holdase, *i.e*., native protein substrates for holdase activity of AtDeg2. High irradiance stress is known to induce the accumulation of reactive oxygen species in plant cells (Hideg *et al*. 2002, Grabsztunowicz *et al*. 2015) which effectively promote oxidative modifications of sulfur-containing amino acid residues (Gill and Tuteja 2010) and induction of intra- or interprotein disulphide bridges is one of the forms of oxidative modifications of cysteine residue (Davies 2004, 2005). In fact, it was shown earlier that interprotein aggregates linked by disulphide bridges in chloroplasts may be induced by other stresses, *e.g*., in *Chlamydomonas reinhardtii* cells as a result of salt stress (Marín-Navarro and Moreno 2006) and in leaves of *A. thaliana* plants exposed to low-irradiance stress (Grabsztunowicz *et al*. 2015). Since both these stresses were demonstrated to induce the accumulation of reactive oxygen species in plant cells as well (Mittler *et al*. 2004, Grabsztunowicz *et al*. 2015), we expected that under conditions of high irradiance stress, some oxidatively modified chloroplast proteins might participate in the formation of aggregates held by disulphide bridges and hypothesized that a certain pool of these proteins might be protected from the aggregation by AtDeg2. Our analyses included WT plants and transgenic plants *35S:AtDEG2$^{\Delta PDZ1}$-GFP* that expressed AtDeg2 lacking both chaperone activities but retaining its protease activity (Adamiec *et al*. 2018). Our results indicate that AtDeg2 operates *in vivo* as holdase by inhibiting the formation of homodimers of γ_1 subunit of ATP synthase in chloroplasts of plants exposed to long-term high irradiance stress.

Materials and methods

Plant material and growth conditions: For a short-term high irradiance stress, *A. thaliana* ecotype Columbia WT plants and eight transgenic lines *35S:AtDEG2$^{\Delta PDZ1}$-GFP* were grown on sphagnum peat moss and wood pulp (*Agro Wit*, Poland) at 110 µmol(photon) m^{-2} s^{-1} (low irradiance) as described previously by Baranek *et al*. (2015) (except that the relative humidity was set at 70%) until the secondary stage 6.0 of ontogenesis (Boyes *et al*. 2001) was reached, and then the plants were transferred for 5 h to 400 µmol(photon) m^{-2} s^{-1}. For a long-term stress, the plants were grown until 6.0 stage was reached under the conditions described previously by Baranek *et al*. (2015) with the irradiance and relative humidity set at 400 µmol(photon) m^{-2} s^{-1} and 70%, respectively.

Plasmid construction: The vector construct for stable transformation (*35S:AtDEG2$^{\Delta PDZ1}$-GFP*) was prepared and the transformation performed exactly as described previously (Adamiec *et al.* 2018). Stable transformants were selected on MS media supplemented with gluphosinate ammonium (10 mg L^{-1}).

Isolation of chloroplast lysates and isolation of stromal fraction: Chloroplasts were isolated from leaves of WT plants and eight transgenic lines *35S:AtDEG2$^{\Delta PDZ1}$-GFP* exactly as described by us previously (Grabsztunowicz and Jackowski 2013). The lysates were prepared by suspending pelleted chloroplasts in a lysis buffer (which contained 50 mM Hepes-KOH, pH 7.8, 10 mM $MgCl_2$, and 10 mM iodoacetamide) and incubating them on ice for a few min. Chloroplast stromal fraction was prepared by subjecting chloroplast lysates to a series of differential centrifugations exactly according to Grabsztunowicz *et al*. (2015). The protein concentration was determined with *QuickStart™ Bradford 1× Dye Reagent* according to the manufacturer's recommendations (*BioRad*, USA).

SDS-PAGE, diagonal electrophoresis, immunoblotting, gel staining, protein quantitation and identification: Samples of chloroplast lysates containing equal amounts of protein were subjected to SDS-PAGE according to Laemmli (1970). After the electrophoresis, the polypeptides were electrotransferred on PVDF membranes and immunostained with monospecific primary antibodies (rabbit) raised against Deg2 C-terminal sequence (TQALDQGIGDSPVS) (*GenScript*, USA). The immunostained bands were quantitated by applying *ChemiDoc Imaging System* (*BioRad*, USA) and *Gelix One 1d Software ver. 4.1* (*Biostep*, Germany). Three individual blots representing two biological replicates were analyzed.

Chloroplast lysates were concentrated as described by Jackowski and Przymusiński (1995) and the concentrated lysates containing 120 µg of protein were subjected to diagonal electrophoresis exactly as described by Grabsztunowicz *et al*. (2015). After the diagonal electrophoresis the proteins were visualized by Blue Silver method according to Candiano *et al.* (2004). The highest protein amount of 120 µg was the amount allowing to avoid obscuring the spots lying close to the diagonal of the gel, by the diagonal itself. Individual spots were quantitated using *ChemiDoc Imaging System* and *PDQuest 2-D software* (*BioRad*, USA). The spot which migrated under the diagonal of the gel and was found to be more abundant in the chloroplast lysates of *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants than in

WT plants was excised and the protein identified by mass spectrometry at the Institute of Biochemistry and Biophysics of Polish Academy of Sciences in Warsaw, Poland. Six individual gels representing two biological replicates were analyzed.

Disulphide reductase activity was assayed by using insulin as a substrate as described by Holmgren (1979) with modifications introduced by Jeon and Ishikawa (2002). Briefly, bovine insulin was incubated for 30 min at 30°C in 100 mM sodium phosphate buffer, pH 7.0 containing 1 mM DTT, in the presence or in the absence of chloroplast stroma samples representing WT plants and *35S:AtDEG2$^{\Delta PDZ1}$-GFP* transgenic line *4.23*. An increase in the absorbance at 650 nm was determined (*Ultrospec 4000* UV/visible spectrophotometer, *Pharmacia Biotech*, Sweden), which was due to the aggregation of free insulin chains produced by reduction of interchain disulphide linkages, catalyzed by those proteins of the stroma samples which possessed disulphide reductase activity (Holmgren 1979). The absorption values at 650 nm were determined by performing four enzymatic assays representing two biological replicates.

Statistical analysis: Statistical significance of the differences in the level of the appropriate proteins resolved by SDS-PAGE and diagonal electrophoresis as well as the differences in the disulphide reductase activity between the appropriate samples were analyzed with the *Student*'s *t*-test at $p<0.05$. Prior to the *Student*'s *t*-test, the normality of data distribution was checked with the *Shapiro-Wilk* test and the equality of variances was determined by applying the *F* test (*Origin*, *OriginLab Corporation*, USA).

Results

Transgenic lines: To search for chloroplast proteins protected by AtDeg2 acting as a holdase from high irradiance-dependent interprotein aggregation *via* disulphide bridges, we applied *35S:AtDEG2$^{\Delta PDZ1}$-GFP* transgenic lines (represented by T2 progeny) generated in the *deg2-3* background (Luciński *et al*. 2011, Adamiec *et al*. 2018). The *35S:AtDEG2$^{\Delta PDZ1}$-GFP* transformants express – in fusion with GFP – the mutant AtDeg2 ΔPDZ1 protein which is proteolytically active but lacks holdase and disaggregase activities (Adamiec *et al*. 2018). We attempted to choose the line(s) for which the abundance of AtDeg2 ΔPDZ1-GFP fusion was under high irradiance stress the most similar to that of native AtDeg2 in WT plants. For this purpose, the immunoblot-based screening of AtDeg2 level was performed in lysates of chloroplasts isolated from leaves of WT plants and eight transgenic lines exposed to short- or long-term high irradiance stress (Fig. 1). The *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.31* and *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* lines were used for further analyses with respect to short-term high irradiance stress and long-term high irradiance stress, respectively, as the level of AtDeg2 ΔPDZ1-GFP fusions in these lines were not significantly different from those of AtDeg2 in WT plants under specific stress conditions whereas AtDeg2 ΔPDZ1-GFP levels in remaining lines tended to be significantly reduced.

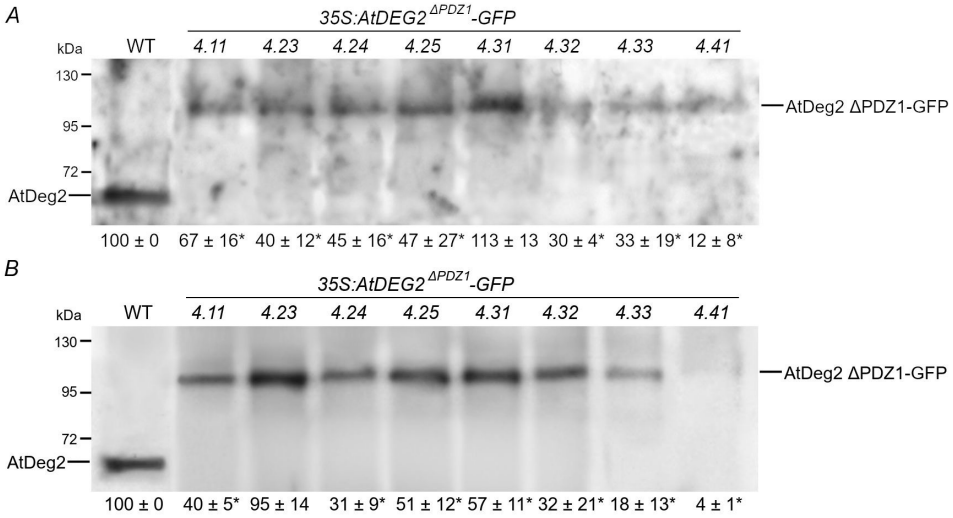

Fig. 1. Immunoblot analysis of the protein levels of AtDeg2 and AtDeg2 ΔPDZ1-GFP fusions in WT plants and eight transgenic *35S:AtDEG2$^{\Delta PDZ1}$-GFP* lines expressing mutated version of AtDeg2 which is proteolytically active but lacks its chaperone activities. 8 µg of protein of lysates of chloroplasts isolated from leaves of plants exposed to short-term high irradiance stress (*A*) or long-term high irradiance stress (*B*) was resolved by SDS-PAGE, electrotransferred onto PVDF membrane and immunodecorated with monospecific anti-Deg2 antibodies. The numbers indicated under the lanes show signal strength of AtDeg2 or AtDeg2 ΔPDZ1-GFP fusions quantified relative to the values ±SE determined for AtDeg2 in WT plants (100%). The signals were determined by scanning three blots generated through analyses of plants exposed to short-term stress and three blots generated through analyses of plants exposed to long-term stress. For each of the stresses the three blots represented two biological replicates. *The asterisks* indicate the data for which any genotype/WT plants differences were significant ($p<0.05$).

Identification of native protein substrate for holdase activity of AtDeg2: The search for chloroplast proteins, which may be protected by AtDeg2 through its holdase activity from forming interprotein aggregates held by disulphide bridges – possibly arising as a result of exposition of plants to prolonged high irradiance stress – was accomplished by the experimental approach based on diagonal electrophoresis, *i.e.*, a form of two-dimensional analysis useful for investigating the subunit composition of multi-subunit proteins containing interprotein disulphide bonds. The proteins that are linked by intermolecular disulphide bridges migrate below the diagonal of the gel at the second dimension. The application of diagonal electrophoresis to accomplish search for native protein substrates for holdase activity of AtDeg2 is based on a rationale that (*1*) AtDeg2 holdase activity in high irradiance-stressed WT plants may involve an inhibitory effect on interprotein aggregation of some oxidatively modified chloroplast proteins; (*2*) AtDeg2 lacking its holdase activity in high irradiance-stressed *35S:AtDEG2$^{\Delta PDZ1}$-GFP* transformants may be fully or partially unable to inhibit interprotein aggregation of oxidatively modified chloroplast proteins.

Thus, native substrates for chaperone activity of AtDeg2 were searched for as proteins isolated from chloroplasts of leaves of *35S:AtDEG2$^{\Delta PDZ1}$-GFP* transformants exposed to high irradiance conditions, which migrated under the diagonal of the gel after diagonal electrophoresis and were significantly more abundant than in chloroplasts of high irradiance-exposed WT plants. As shown in Fig. 2*A*, no proteins meeting these criteria were found in chloroplast lysates derived from *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.31* transformants exposed to short-term stress. However, a single protein was detected; it migrated under the diagonal of the gel (at 35 kDa) and was considerably more abundant in chloroplast lysates of the *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants exposed to long-term stress compared to WT plants (306 ± 22% of the value found in lysates of WT plants; Fig. 2*B*). This protein was identified by mass spectrometry as the γ_1 subunit of ATP synthase (score: 1,738; sequence coverage: 42%). Thus, this protein seems to be protected by AtDeg2 acting as a holdase from being aggregated *via* disulphide bridges in response to long-term high irradiance stress. The implicit position of the high irradiance-induced aggregates at the first dimension matched the molecular

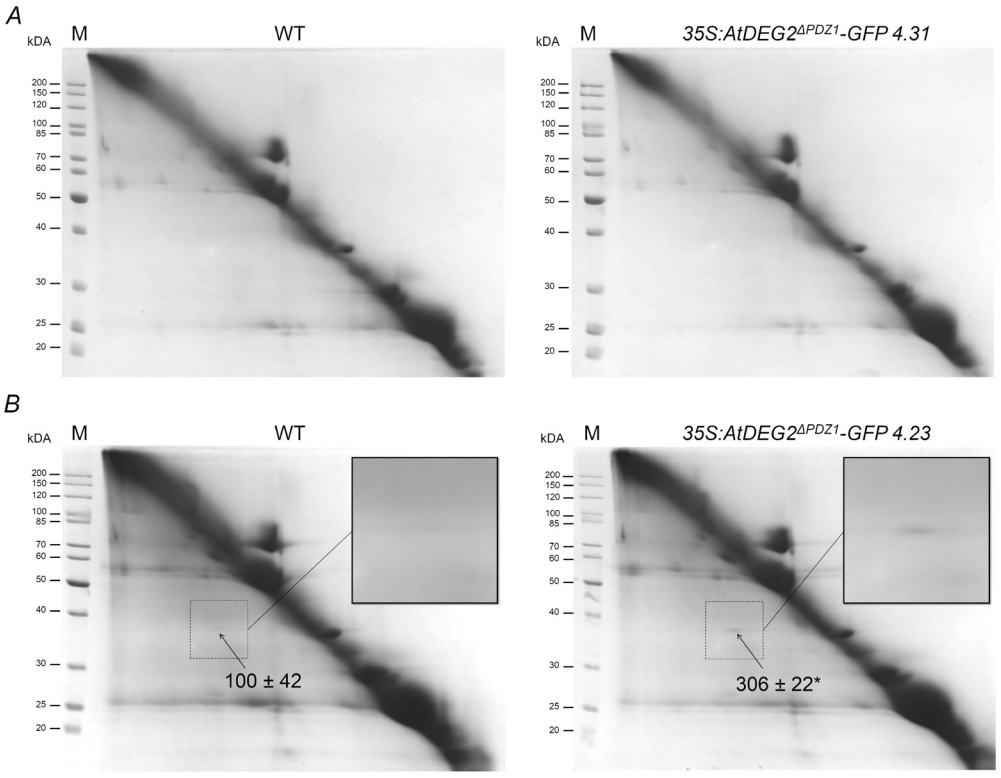

Fig. 2. Diagonal electrophoresis-based analysis of chloroplast proteins isolated from leaves of WT plants as well as *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* and *4.31* transformants. 120 μg of protein of lysates of chloroplasts isolated from leaves of WT plants and *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.31* transformants exposed to short-term high irradiance stress (*A*) or from leaves of WT plants and *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants exposed to long-term high irradiance stress (*B*) was resolved by diagonal electrophoresis and stained according to Blue Silver method. The arrow points to the spot which migrated under the diagonal of the gel and was significantly more abundant in chloroplasts of *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants than in chloroplasts of WT plants (*the asterisk*, $p<0.05$). The region of the gel adjacent to this spot was surrounded by a square and shown in enlarged form in upper right corner of the gel. The indicated signal strength ± SE of the spot in *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants was quantified relative to the value determined for WT plants (100%). Six gels representing two biological replicates were scanned for each of two stresses.

mass of the dimer of γ_1 subunits (*ca* 70 kDa) and no other spot(s) was recorded in between the position of the monomeric γ_1 subunit and the diagonal of the gel, indicating that in the absence of chaperone activity of AtDeg2 γ_1 subunits of ATP synthase tended to homodimerize.

To provide the validation that the γ_1 subunit of ATP synthase is protected by AtDeg2 from being homodimerized *via* disulphide bridges in response to long-term high irradiance stress, we explored the possibility that overaccumulation of this subunit in chloroplast lysates of *35SAtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants occurred rather due to lower efficiency of reduction of disulphide bonds in their chloroplasts *vs*. chloroplasts of WT plants. To prove so, we determined the insulin-targeted disulphide reductase activity of chloroplasts of both genotypes.

Specifically, stroma samples of plants representing both genotypes were analyzed since the reduction of intra- and interprotein disulphide bonds is catalyzed by thiol oxidoreductases (belonging to thioredoxin and glutaredoxin families) which are located in stroma (Rouhier 2010, Nikkanen and Rintamäki 2014). As shown in Fig. 3, stroma samples collected from chloroplasts of WT plants and the transformants exhibited a very similar insulin reduction activity. This finding strongly validates the results of the analyses based on diagonal electrophoresis. Namely, it indicates that the γ_1 subunit of ATP synthase is a substrate for AtDeg2 holdase chaperone activity. AtDeg2 acts in such a way as to protect γ_1 subunit from homodimerization under high irradiance conditions.

Discussion

There are indications that some abiotic stresses known to enhance the production of reactive oxygen species may induce interprotein aggregation *via* disulphide bridges in chloroplasts (Mittler et *al*. 2004, Marín-Navarro and Moreno 2006, Grabsztunowicz *et al*. 2015). No direct data was reported to date demonstrating that this effect can be induced by high irradiance stress as well, even though this stress is also known to stimulate the accumulation of reactive oxygen species (Hideg *et al*. 2002, Grabsztunowicz *et al*. 2015). Our results of the studies based on diagonal electrophoresis allowed us to conclude that γ_1 subunits of ATP synthase could homodimerize in chloroplasts of WT plants grown under high-irradiance conditions and that the homodimers were kept by disulphide bridges (Fig. 2). It was revealed that cysteine sulfenic acid might play an important role as an oxidized sulfur intermediate in disulphide-based dimerization of protein molecules containing free cysteine residues (Rehder and Borges 2010) and the cysteine sulfenic acid-containing non-native protein intermediates may recruit chaperones with holdase activity to inhibit the productive protein dimerization to occur. Our results indicate that in chloroplasts of WT plants exposed to long-term high irradiance stress homodimers of γ_1 subunits of ATP synthase were more than three times less abundant compared to chloroplasts isolated from leaves of the *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants which express – in fusion with GFP – AtDeg2 version lacking both chaperone activities (Fig. 2).

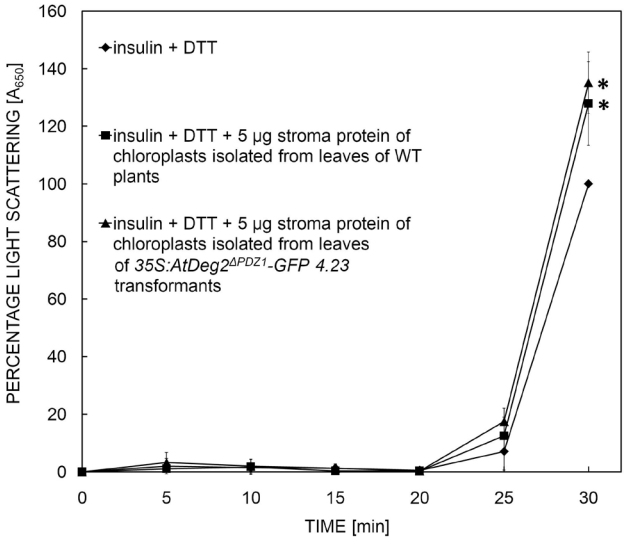

Fig. 3. Disulphide reduction activity in chloroplasts isolated from leaves of WT plants and *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants. The DTT-dependent insulin reduction by stroma samples of chloroplasts isolated from leaves of WT plants and *35S:AtDEG2$^{\Delta PDZ1}$-GFP 4.23* transformants which were exposed to long-term high irradiance stress was recorded as the increase in light absorption at 650 nm due to light scattering by aggregates of free insulin polypeptide chains in the assays containing 0 or 5 µg of the stroma protein. The values A_{650} ± SE indicate the percentages of absorption signal at 650 nm identified at time point 30 min in the assays containing stroma samples relative to value determined in the assay without stroma samples (100%). *The asterisks* indicate the data for which the differences between A_{650} values in assays involving stroma samples and the assay without stroma samples were significant (p<0.05). The absorption values at 650 nm were determined by performing four enzymatic assays representing two biological replicates.

Since AtDeg2 ΔPDZ1 protein was proteolytically active (Jagodzik *et al*. 2018), the overaccumulation of homodimers of γ_1 subunits of ATP synthase in the transformants' chloroplasts could not be ascribed to the reduction in efficiency of hypothetical AtDeg2-dependent degradation of the homodimers. As the total disulphide reductase activity detected in stroma samples of chloroplasts isolated from the leaves of both genotypes was very similar (Fig. 3), the homodimers might be repaired by reduction of disulphide bridges with the same efficiency no matter which genotype is considered. Thus, our data indicate that AtDeg2 inhibits *in vivo* the formation of homodimers of γ_1 subunit of ATP synthase in chloroplasts of *Arabidopsis* plants exposed to long-term high irradiance stress.

The chloroplast ATP synthase (cF_1F_0) uses the proton motive force generated across the thylakoid membrane to drive ATP synthesis from ADP and P_i by rotary catalysis (Junesch and Gräber 1987). It has been known for some time that in plants and green algae chloroplasts, ATP synthase catalytic activity is regulated by thiol modulation of the γ subunit. In the dark, an intrapeptide disulphide bond is formed between two redox-active cysteine residues resulting in ATP synthase inactivation and in the light,

ATP synthase is activated *via* reduction of the disulphide bond catalyzed by reduced thioredoxin-*f* (Arana and Vallejos 1982, Nalin and McCarty 1984). In *A. thaliana*, the classical light/dark redox regulation applies only to γ_1 subunit (Kohzuma *et al*. 2012), one of the two homologues of the regulatory subunit coded by two individual genes located in nuclear genome of this species (Inohara *et al*. 1991). These are Cys199 and Cys205 residues of the γ_1 subunit which are thought to be linked by a disulphide bridge in the dark while Cys89 apparently is not involved (Nalin and McCarty 1984). It may be proposed that exposition to high irradiance results in the formation of non-native interprotein disulphide bridges between those Cys residues which are located in the stroma-exposed shaft of the central rotor of the ATP synthase particle (Cys199, Cys205, and, possibly, Cys89). ATP synthase particles are confined to end grana membranes and stroma lamellae (Daum *et al*. 2010) and AtDeg2 was found to be associated exclusively with the stromal side of non-appressed thylakoid membranes, *i.e*., stroma lamellae, grana margins, and end membranes (Haußühl *et al*. 2001). Since 15% of ATP synthase particles cover stromal side of these membranes in the form of random assemblies of two or more copies (Daum *et al*. 2010), it seems plausible that the monomers included in the assemblies may establish contacts close enough for $\gamma_1 - \gamma_1$ intersubunit non-native disulphide bridges to be formed under oxidizing conditions. A certain population of the sulfenic acid-containing non-native γ_1 intermediates can be recognized and held by AtDeg2 until sulfenic acid becomes reduced by thioredoxin or reacts with GSH to generate a mixed disulfide, later reduced by glutaredoxin (Lo Conte and Carroll 2013). Alternatively, it may be hypothesized that homodimerization of γ_1 subunits *via* disulphide bridges takes place in the stroma during the assembly of CF_1 portion of ATP synthase, before folded γ_1 subunits get associated with $\alpha_3\beta_3$ hexamer to yield an active CF_1 core (Chen and Jagendorf 1994, Mao *et al*. 2015) and that all three Cys residues (Cys89, Cys199, Cys205) could be engaged.

Unexpectedly, no chloroplast proteins were found to be protected by AtDeg2 from being aggregated *via* disuplhide bridges under short-term high irradiance stress. One possible explanation is that AtDeg2 pool in WT plants shifted for 5 h to high irradiance is not large enough to exert a detectable holdase activity. This suggestion is supported by the observation that *AtDEG2* was downregulated about twofold over such treatment (Adamiec *et al*. 2011).

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