Organ-specific effects of dark treatment on photosynthesis and the expression of photosynthesis-related genes

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Abstract

The effects of two-day dark treatment, applied to whole plants or to individual organs, on the photosynthetic apparatus in cotyledons and first rosette leaves of young *Arabidopsis thaliana* plants were studied. Darkness affected the individually darkened pair of cotyledons as well as the cotyledons of whole darkened plants (DP) in a similar manner as revealed by the significant decrease in the actual yield of photosystem 2 electron transport and the down-regulation of the *psaB* and *rbcL* transcript levels. However, cotyledons and rosette leaves responded differently to darkness with respect to the non-photochemical quenching (NPQ) and the non-regulated energy dissipation (Φ_{NO}), indicating different capacity for photoprotection depending on the type of the applied dark treatment. Besides, the expression of the genes for the two plastid proteases FtsH5 and Deg1 involved in D_1 protein degradation was inhibited in both leaf organs, suggesting that these proteases function mainly under irradiance. Upon re-irradiation, dark-treated cotyledons recovered from the applied stress and during further senescence the changes in the photosynthetic parameters and the mRNA levels of *psaB*, *rbcL* and *SAG12* were similar as in the control plants. However, in the course of recovery typical chloroplast senescence symptoms were observed only in individually darkened leaves while re-irradiated DP leaves maintained high photosynthetic capacity.

Additional key words: cotyledons, photosystem 2, plastid protease, rosette leaves, senescence.

Introduction

The photosynthetic apparatus is highly dynamic and able to respond to environmental stresses, including changes in the quality and quantity of incident radiation (Szabo *et al.* 2005, Chowdhury *et al.* 2009), mineral starvation (Dannehl *et al.* 1996), drought and extreme temperatures (Smart 1994, Humbeck *et al.* 2007, Zhang *et al.* 2009). The transfer of individual intact or detached leaves, as well as whole plants, to darkness is a widely used experimental approach to study the molecular basis of the stress response and the adaptive mechanisms allowing plants to survive the adverse conditions (Biswal and Biswal 1984, Oh *et al.* 1996, Weaver *et al.* 1998). Darkness has been implicated in provoking a decline in total chlorophyll and protein contents, photosystem 2 (PS 2) activity and transcript levels of photosynthesis-related genes (Kleber-Janke and Krupinska 1997, Lu and Zhang 1998, Lin and Wu 2004). Moreover, transcripts of senescence-associated genes (*SAG*s), which normally appear during natural (age-mediated) senescence, have also been detected in dark-treated leaves (Azumi and Watanabe 1991, Weaver *et al.* 1998). On the other hand, genes induced during dark treatment are not necessarily expressed during natural senescence, suggesting that these two senescence processes are not identical (Becker and Apel 1993). As revealed by microarray analysis, the number of genes which are up-regulated during natural senescence is higher compared with those which are down-regulated, whereas in individually darkened intact or detached leaves the fractions are similar or opposite (Van der Graaff *et al.* 2006). In addition, only half of the genes up-regulated during developmental senescence are also strongly enhanced in leaves of darkened plants

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Abbreviations: DP - whole plants transferred to darkness; IDC - individually darkened cotyledons; IDL - individually darkened first rosette leaves; NPQ - non-photochemical quenching; PS - photosystem; Φ_{NO} - quantum yield of the non-regulated energy dissipation; Φ_{PS2} - actual quantum yield of the PS 2 electron transport in the light-adapted state.
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(Lin and Wu 2004, Buchanan-Wollaston *et al.* 2005). Nowadays, it is considered that the triggering signal in dark-induced senescence is the rapid reduction in sugar contents leading to stimulation in the lipid degradation (Buchanan-Wollaston *et al.* 2005). During natural senescence, photosynthetic rate gradually declines, but sugar synthesis is still taking place, thus providing enough energy for realization of the cellular processes.

The ability of darkness to induce senescence depends on the level at which it is perceived (whole plant or individual leaf organ) and the leaf age at the time of treatment (Weaver and Amasino 2001). It was shown that darkening of whole *Arabidopsis* plants provoked retention in leaf development including an inhibition of senescence-specific degradation of total protein and chlorophyll. As a result, the leaves of dark-stressed plants showed delayed senescence compared with controls. On the other hand, individually darkened leaves senesced faster than the controls and the observed changes were similar to those induced at the final stages of leaf development (Weaver and Amasino 2001). Keech *et al.* (2007) observed that the photosynthetic capacity of whole darkened *Arabidopsis* plants was maintained, while the mitochondrial respiration decreased. This result underlies the mechanism of delayed senescence in whole darkened plants observed earlier by Weaver and Amasino (2001). By contrast, in individually darkened leaves a rapid decline in photosynthetic rate was found while maintaining high mitochondrial respiration rate. The changes provoked by the two dark treatments were accompanied with an unequal degradation of chloroplasts and mitochondria in the senescing leaves (Keech *et al.* 2007). In addition, senescence progression is faster in older leaves, thus indicating an age-mediated component of this stress response (Weaver and Amasino 2001).

It has been suggested previously that in contrast to differentiated leaves, cotyledons of darkened plants exhibit typical senescence symptoms and in this case senescence is not reversed upon re-irradiation, but rather accelerated (Weaver and Amasino 2001). Epigeal cotyledons possess a specific anatomical structure and they are the first photosynthesizing organs in plant ontogenesis (Tsukaya *et al.* 1994). The major physiological function of cotyledons is to ensure the development of the growing seedling until differentiation of photosynthetically efficient leaves. The specific function of these leaf organs when compared with true leaves implies the involvement of different regulatory mechanisms mediating the dark stress response. Therefore, it is worth studying the differential responses of cotyledons and true leaves to darkness applied to whole plants or to individual organs. In our recent investigations with zucchini cotyledons we have shown that short-term darkening (24 h) of whole plants resulted in a reduced rate of overall chloroplast transcription and a decrease in the content of biologically active cytokinins, especially *trans*-zeatin (Ananieva *et al.* 2007). Like in many other monocarpic plants, the longevity of zucchini leaf organs is controlled by the developing reproductive structures. However, the senescence mechanisms found in zucchini plants might not be the same as in *Arabidopsis* where leaf senescence does not appear to be under correlative control, but rather determined by the life span of the whole plant (Lim *et al.* 2007).

In the present study, the stress responses of the photosynthetic apparatus in the cotyledons and first two rosette leaves of two-day darkened *Arabidopsis* plants were compared to those observed in individually darkened intact organs. In order to study the changes in the photosynthetic performance total chlorophyll content and chlorophyll *a* fluorescence parameters were measured. The role of darkness as a senescence-promoting factor was examined by following the expression of three key plastid-encoded photosynthetic genes (*psbA*, *psaB* and *rbcL*) which are regarded as senescence-downregulated genes and one nuclear-encoded gene (*SAG12*) which is strongly upregulated in the course of natural senescence. In addition, we studied in more details the impact of short-term dark treatment on the expression of the two plastid-localized proteases FtsH5 and Deg1 which are supposed to be involved in degradation of the PS 2 core protein D_1 (Adam *et al.* 2006, Kapri-Pardes *et al.* 2007).

Materials and methods

Arabidopsis thaliana L. (ecotype Columbia) sterilized seeds were sown on Petri dishes with Murashige and Skoog (MS) medium containing 0.8 % agar and 3 % sucrose. The Petri dishes were kept for 2 d at 4 °C in the dark to synchronize germination and after that transferred for 7 d into a greenhouse under a 16-h photoperiod (irradiance of 300 μmol m^{-2} s^{-1}), 60 % humidity and day/night temperatures of 23/20 °C. Further the seedlings were grown on a *Vermiculite*/soil mixture (1:5) under the same conditions. Plants were darkened by covering the trays with cardboard boxes in the same greenhouse. Individual darkening was performed by wrapping the pair of cotyledons or one of the first true leaves in aluminum-foil bags. Dark treatment was applied for 2 d starting from day 6 after planting followed by return to normal light regime. Samples were collected between 09:00 and 11:00.

Cotyledons and first rosette leaves (the first or the second differentiated leaf) were frozen in liquid nitrogen, ground in 80 % (v/v) acetone and transferred to an ultrasonic bath in a cold room at 4 °C for 20 min. After centrifugation, the pigment content of the supernatant was determined spectrophotometrically at 664 nm (chlorophyll *a*) and 647 nm (chlorophyll *b*) according to Ziegler and Egle (1965). Results represent the means of 4 different experiments.

Chlorophyll fluorescence was analyzed using a pulse-modulated imaging fluorometer (*Imaging PAM Chlorophyll Fluorometer*, *Walz*, Effeltrich, Germany). After recording the minimal (F_0) fluorescence, cotyledons and first rosette leaves of dark-adapted plants were exposed to a saturating light flash for measuring the maximal (F_m) fluorescence. After 60 s of dark adaptation the leaf organs were irradiated with actinic radiation (290 μmol m^{-2} s^{-1}) and saturating flashes were applied every 20 s. Following 515 s of irradiation, the maximum fluorescence of light-adapted cotyledons and leaves (F'_m) and the steady-state fluorescence (F_S) were registered. The actual quantum yield of the PS 2 electron transport in the light-adapted state $\Phi_{PS2} = (F'_m-F_S)/F'_m$ and the non-photochemical quenching $NPQ = (F_m-F'_m)/F'_m$ were calculated based on the above parameters. The quantum yield of the non-regulated energy dissipation, $\Phi_{NO,}$ were calculated according to Kramer *et al.* (2004).

Total RNA was extracted from fresh cotyledons or first true leaves (50 - 100 mg) using *TRIzol* reagent (*Invitrogen*, Darmstadt, Germany). Briefly, the leaf tissue was ground with liquid nitrogen and incubated for 10 min with *TRIzol* in a water bath preheated to 60 °C. After centrifugation for discarding the cell debris, deproteinization was carried out by using chloroform/ isoamyl alcohol mixture (24:1). RNA was precipitated with isopropanol, washed with 70 % ethanol, resuspended in diethylpyrocarbonate-treated water and kept at -80 °C. The purity and the quantity of the isolated RNA were determined spectrophotometrically using *ND-1000* (*NanoDrop Products*, Wilmington, USA). Sample collection for RNA extraction was done by day 8 (immediately after ceasing of the dark treatment), day 14 (6 d of recovery, when massive yellowing of the control cotyledons occurred) and day 21 (13 d of recovery, when massive yellowing of the first rosette leaves of control plants occurred).

Total RNA was treated with RNase-free DNase I (*Fermentas*, St. Leon-Rot, Germany) and first-strand cDNA was synthesized using *Superscript III* reverse transcriptase (*Invitrogen*) and hexa-random primers. The cDNA-containing solution was purified with PCR purification kit (*Qiagen*, Hilden, Germany) and used as a template for quantitative real-time PCR with the help of *iCycler* (*BioRad*, Munich, Germany). The PCR reactions based on *Platinum® SYBR® Green qPCR Supermix-UDG* (*Invitrogen*) were carried out in a total volume of 0.02 cm^3 with fluorescein as a calibration dye. Primer pairs were chosen to yield amplification products between 110 and 150 bp (final concentration of each primer in the reaction mixture 0.3 μM). The primer sequences and their localization are listed in Table 1. The cDNA-dilution series of 1/256, 1/1024 and 1/4096 were used for each of the chloroplast-encoded genes (*rbcL*, *psbA* and *psaB*), while these for the nuclear-encoded genes (*SAG12*, *FtsH5* and *Deg1*) were undiluted cDNAs, 1/4 and 1/16. To account for the variability in cDNA concentrations, the results were normalized to the level of *18S rRNA* cDNA as an internal standard. To determine the relative expression rate of the target gene versus the reference gene (*18S rRNA*), a relative expression software tool (*REST©*) for group-wise comparison and statistical analysis of relative expression results in real-time PCR was used (Pfaffl *et al.* 2002). Results were obtained using cDNA preparations from 3 different experiments, each of them being analyzed twice.

Table 1. Primers designed for quantitative real-time PCR.

Primer	Sequence	Gene localization
At*rbcL*fw	TTACAAAGGACGATGCTACCAC	chloroplast
At*rbcL*rev	TGAACCCAAATACATTACCCACAA	chloroplast
At*psbA*fw	CAAGCCGCTAAGAAGAAATGTAA	chloroplast
At*psbA*rev	CAGAAAATGAATCTGCTAATGAAG	chloroplast
At*psaB*fw	TAAACTTTGCGTTTGTGGATGGA	chloroplast
At*psaB*rev	TTTATGCTCAAAACCCCGACTC	chloroplast
At*SAG12*fw	GCTTTGCCGGTTTCTGTTG	nucleus
At*SAG12*rev	CAACACAAATAAAGAAAGGGAAAC	nucleus
At*FtsH5*fw	GGCTCACCTTTTTCGCTCCTA	nucleus
At*FtsH5*rev	TGCTCCGGTTGTCACATTCTC	nucleus
At*Deg1*fw	CATGTTATTCGTGGTGCTTCTG	nucleus
At*Deg1*rev	GTATAGGTCTTAATTTGTTTTTCGG	nucleus
At*18SrRNA*fw	GCATTTGCCAAGGATGTTTTC	nucleus
At*18SrRNA*rev	GCGGAGTCCTATAAGCAAC	nucleus

Results

The main goal of this work was to study the effect of darkness alone as a senescence-promoting factor. In order to eliminate the interference of age as a strong inducer of natural senescence, we used only young cotyledons and

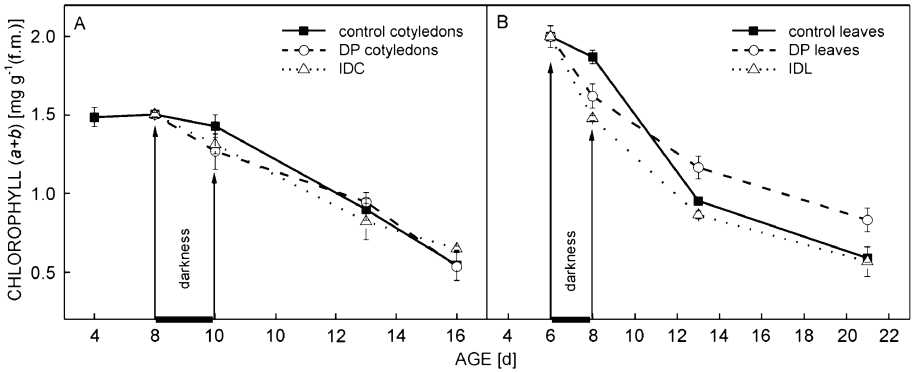

Fig. 1. Chlorophyll (*a*+*b*) content in control and dark-treated cotyledons (*A*) and rosette leaves (*B*) in the course of ontogenesis. Chlorophyll was determined in whole darkened plants (DP) or in individually darkened cotyledons (IDC) and leaves (IDL). Means ± SE of 4 independent experiments.

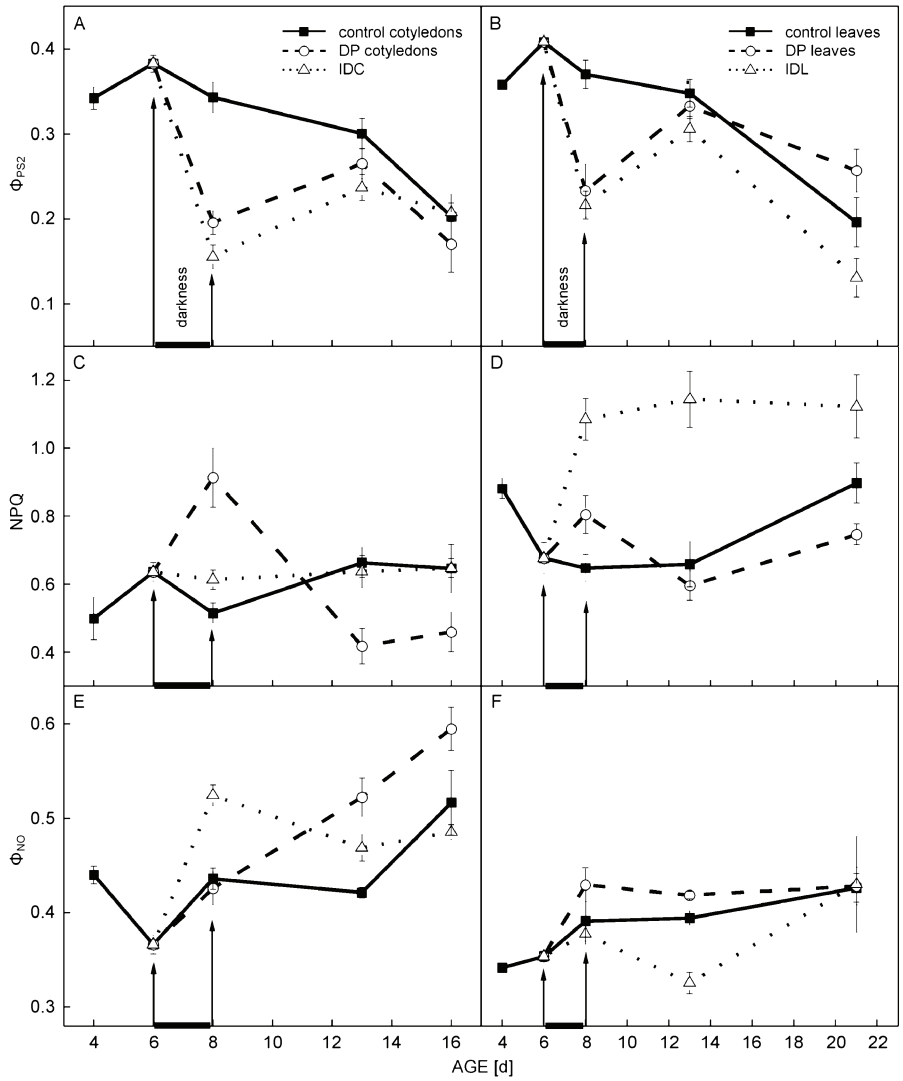

Fig. 2. Changes in chlorophyll fluorescence parameters. The actual quantum yield of PS 2 electron transport in the light adapted state (Φ_{PS2}) in control and dark-treated cotyledons (*A*) and rosette leaves (*B*). The non-photochemical quenching (NPQ) in control and dark-treated cotyledons (*C*) and rosette leaves (*D*). The non-regulated energy dissipation (Φ_{NO}) in control and dark-treated cotyledons (*E*) and rosette leaves (*F*). After the initial saturating light flash the leaf organs were incubated for 60 s in darkness and then under actinic light (290 μmol m^{-2} s^{-1}) for 515 s. Means ± SE of 5 independent measurements.

rosette leaves. On day 6 after planting the cotyledons and first two differentiated leaves were well developed while the third and the forth leaf had just emerged. At this age both cotyledons and first rosette leaves were at their maximum of chlorophyll accumulation (Fig. 1) and effective PS 2 quantum yield (Fig. 2*A,B*). Two days later (8 d after planting) control plants had four developed rosette leaves and two more at their onset of development. Furthermore, no detectable levels of *SAG12* mRNA as a specific molecular marker of natural senescence were registered by quantitative real-time PCR in both cotyledons and leaves of 8-d-old control plants (data not shown). Thus, day 6 after transfer of the seedlings to soil was chosen as the start of the two-day dark treatment.

The most striking effect of darkness on day 8 after planting was observed in the case of DP where the growth stage was the same as in 6-d-old control seedlings at the beginning of darkening. In controls as well as in plants with individually darkened leaf organs both the 5th and the 6th differentiated leaves were visible. A slight inhibition (about 10 %) in total chlorophyll content (Fig. 1*A*) together with a significant decrease in the actual yield of the PS 2 electron transport, Φ_{PS2} (Fig. 2*A*) were registered in darkened cotyledons regardless of the type of darkening. In DP cotyledons, the drop in Φ_{PS2} was accompanied by 1.8-fold increase in the non-photochemical quenching, NPQ (Fig. 2*C*), which reflects mainly the thermal dissipation of the excess light energy *via* the xanthophyll cycle in PS 2 antenna complexes (Horton *et al.* 1994). It must be noted that the analysis of NPQ was made based on similar variable to maximum fluorescence ratio, F_v/F_m, in the different treatments. In contrast to NPQ, the levels of the non-regulated dissipation Φ_{NO} which represents the basal intrinsic non-photochemical loss of the light energy in PS 2 (Kramer *et al.* 2004) remained similar to that of the controls (Fig. 2*E*). Unlike DP cotyledons, in IDC the decay in Φ_{PS2} was accompanied by an increase in Φ_{NO} (Fig. 2*E*) without considerable stimulation in NPQ (Fig. 2*C*).

Furthermore, the transcript levels of three key chloroplast-encoded genes were studied *via* quantitative real-time PCR. Two of them (*psaB* and *psbA*) encode core proteins in PS 1 and PS 2 that span the thylakoid membrane while the third one (*rbcL*) codes for the large subunit of Rubisco which is a typical stroma-localized protein. Two-day dark treatment resulted in a significant decrease in the amount of *psaB* and *rbcL* mRNAs in the stressed cotyledons (Fig. 3*B,C*). In the present work, only changes in the gene expression by more than two times were considered as physiologically significant and were further analyzed. The levels of mRNA in controls were always considered as 1 at the different time points. The levels of *psaB* were about 5-fold lower compared with the controls regardless of the type of darkening (Fig. 3*B*). The *rbcL* mRNA accumulation was affected to a greater

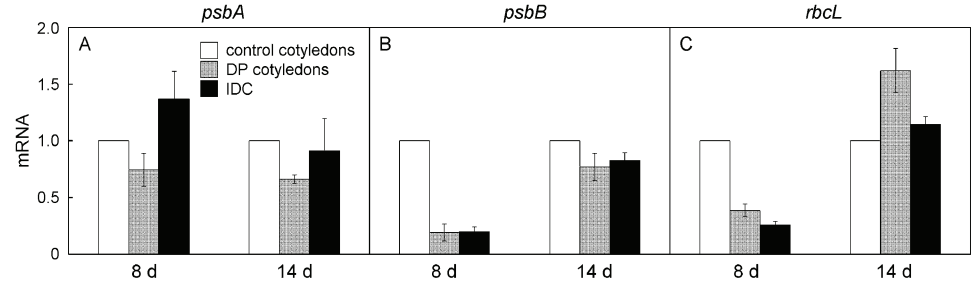

Fig. 3. Quantitative real-time PCR analysis of the mRNA of *psbA* (*A*), *psaB* (*B*) and *rbcL* (*C*) in cotyledons of control, whole darkened plants (DP) or individually darkened cotyledons (IDC). Total RNA was extracted from cotyledons following 2-d dark treatment (8 d after planting) and on day 14 when massive yellowing of the cotyledons in control plants was observed. In each case the transcript levels of the three chloroplast-encoded genes were normalized to the level of *18SrRNA* as a reference gene. The control values were always set as 1 at the different time points. Means ± SE of 3 independent measurements.

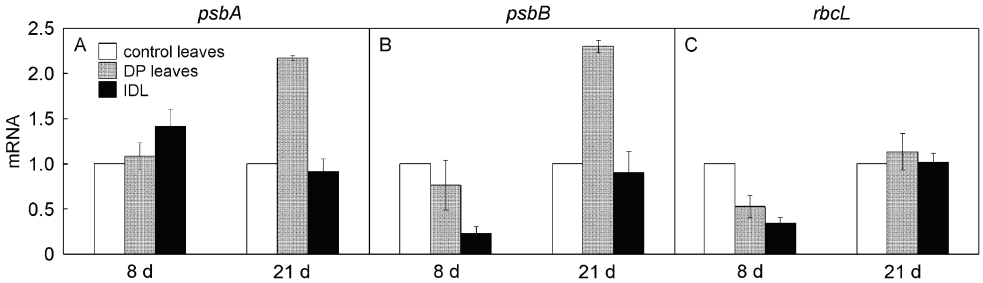

Fig. 4. Quantitative real-time PCR analysis of the mRNA of *psbA* (*A*), *psaB* (*B*) and *rbcL* (*C*) in rosette leaves of control, whole darkened plants (DP) or individually darkened true leaf (IDL). Total RNA from leaves was extracted following 2-d dark treatment (8 d after planting) and on day 21 when massive yellowing of the first rosette leaves from the control plants was observed. In each case the transcript levels of the three chloroplast-encoded genes were normalized to the level of *18SrRNA* as a reference gene. The control values were always set as 1 at the different time points. Means ± SE of 3 independent measurements.

extent in IDC (3.9-fold inhibition) when compared with DP cotyledons (2.6-fold inhibition) (Fig. 3*C*). Concerning the effect of darkness on the *psbA* transcript levels, no significant changes were registered in IDC and DP cotyledons, thus suggesting the existence of specific regulation of the D_1 transcript accumulation (Fig. 3*A*).

In contrast to cotyledons, the response of photosynthetic apparatus of the first true rosette leaves to the applied dark stress was found to be strongly dependent on the irradiance of the rest of the plant. It should be emphasized that all measurements on the individually darkened leaves were compared with those done on the first and the second true leaves of normally irradiated control plants. Thus, we excluded the possibility for incorrect reading of the control values while using the other leaf from the same plant as a reference control because of the potential influence of the darkened leaf organ on the physiological performance of the opposite one. A decrease in the chlorophyll amount was detected in IDL and DP-leaves after two days of dark incubation (Fig. 1*B*). The individual darkening provoked a 1.7-fold increase in the non-photochemical quenching NPQ, while the measured stimulation in DP leaves reached only 24 % (Fig. 2*D*). In contrast to cotyledons, darkness did not cause any significant changes in Φ_{NO} neither in DP leaves, nor in IDL (Fig. 2*F*). Meanwhile, Φ_{PS2} was inhibited to a similar extent (about 40 %) regardless of the type of darkening (Fig. 2*B*).

The expression levels of the aforementioned three chloroplast-encoded genes in the first two rosette leaves under dark treatment were also investigated. The mRNA levels of *psaB* and *rbcL* in IDL were significantly decreased (4.4 and 2.9-fold, respectively; Fig. 4*B*,*C*). On the other hand, a slight inhibition in the expression of *rbcL* and almost the same transcript levels of *psaB* as in the controls were registered in the leaves of DP, thus suggesting higher resistance to the applied stress compared with individual darkening. Similar to dark-stressed cotyledons, the *psbA* transcript levels in DP leaves and IDL remained unaffected (Fig. 4*A*).

Further on, we studied the capability of cotyledons and true leaves to recover upon re-irradiation. As revealed by the dynamics in total chlorophyll content (Fig. 1), the lowest pigment levels in control cotyledons were observed after day 13, while the minimum chlorophyll content in the first and second differentiated leaves was registered after day 20. By the time the control cotyledons were yellowing, chlorophyll content and Φ_{PS2} of the cotyledons recovering from the dark stress were very similar to the control values (Figs. 1*A*, 2*A*). Soon after reaching high values on day 8, NPQ in DP cotyledons rapidly declined and on day 16 the measured values were lower than those of the controls (Fig. 2*C*). However, Φ_{NO} in DP cotyledons was slightly higher when compared to the controls and IDC (Fig. 2*E*). Furthermore, the expression levels of the three photosynthesis-related genes and *SAG12* in the recovering cotyledons were also close to the control values (Figs. 3, 5). Therefore, dark-stressed *Arabidopsis* cotyledons did not demonstrate accelerated senescence and they died together with the controls regardless of the type of darkening.

After the 13-d recovery, the individually darkened leaves showed lower photosynthetic performance than the controls as judged by the low Φ_{PS2} and the high NPQ, the latter being significantly elevated during the whole period under investigation (Fig. 2*B*,*D*). Concerning chlorophyll accumulation, no significant differences were observed when compared with the controls, which started to yellow at the same time (Fig. 1*B*). The mRNA levels of *psaB, psbA* and *rbcL* were also similar to the controls showing natural senescence symptoms (Fig. 4). The accumulation of *SAG12* transcripts as an estimation of senescence progression, however, was 3-fold higher (Fig. 5). On the contrary, upon recovery the first rosette leaves of DP preserved their pressure potential and exhibited higher chlorophyll content (about 40 % more than in yellowing control leaves of the same age; Fig. 1*B*) and the PS 2 activity (30 % stimulation of Φ_{PS2} and about 17 % lower NPQ; Fig. 2*B*,*D*). These changes were not accompanied by respective alterations in Φ_{NO} which preserved similar values in control and dark-treated leaves (Fig. 2*F*). The *SAG12* mRNAcontent in recovered DP leaves was undetectable (Fig. 5). The transcript levels of the core proteins of the two photosystems exceeded the respective values in the control leaves by more than 2-fold (Fig. 4*A*,*B*). However, such a stimulatory effect was not registered in the *rbcL* levels which were almost equal in all variants (Fig. 4*C*). Hence, different mechanisms of regulation of the expression levels of the thylakoid and stromal proteins could exist in recovering DP leaves.

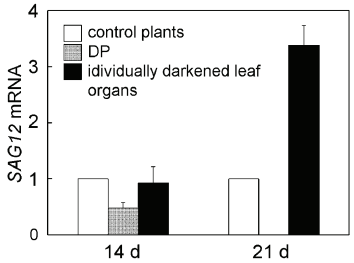

Fig. 5. Accumulation of *SAG12* transcripts in cotyledons and first rosette leaves recovering from the dark stress. The total RNA extraction was performed by the time the control cotyledons and leaves were senescent (day 14 and 21, respectively). In each case the controls were set as 1 and the *SAG12* transcript levels were normalized to the level of *18SrRNA* as a reference gene. Means ± SE, $n = 3$.

One possible mechanism of action of the dark stress is related to the intensification of the degradation processes due to raised proteolytic activity. In order to test the possibility for stimulated expression of genes encoding components of the protein degradation machinery in plastids, the transcript levels of two chloroplast proteases, FtsH5 and Deg1, supposed to be responsible for

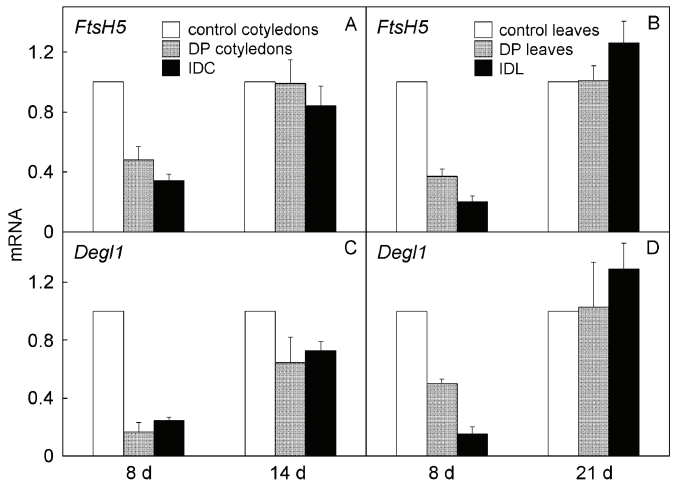

Fig. 6. Changes in the transcription of the two plastid proteases which are supposed to be involved in D_1-protein degradation. Upper panel: relative amount of *FtsH5* mRNA in dark-treated cotyledons (*A*) and rosette leaves (*B*). Lower panel: relative amount of *Deg1* mRNA in dark-treated cotyledons (*C*) and rosette leaves (*D*). For real-time PCR analysis, total RNA was extracted from cotyledons and leaves following 2-d dark treatment as well as at the stage of massive yellowing of the control leaf organs. In each case the transcript levels of the two genes were normalized to the level of *18SrRNA* used as a reference gene. The control values were always set as 1 at the different time points. Means ± SE, $n = 3$.

D_1-degradation (Kapri-Pardes *et al.* 2007) were investigated. The analysis of the relative expression rates showed that, instead of stimulation, two-day dark treatment led to a significant decrease in the *FtsH5* and *Deg1* mRNA levels in both cotyledons and rosette leaves (Fig. 6). In cotyledons, the inhibitory effect of darkness was more pronounced in the case of *Deg1* (about a 5-fold decrease; Fig. 6*C*), although *FtsH5* levels were also reduced (Fig. 6*A*). Regarding the first true leaves, the transcript levels of the two plastid proteases were less affected in DP, while individual darkening provoked a significant inhibition in the *FtsH5* and *Deg1* mRNA accumulation (5-fold and 6-fold, respectively) (Fig. 6*B*,*D*).

Six days upon recovery from both dark treatments the mRNA levels of the two proteases registered in dark-treated cotyledons did not differ significantly from the control senescent cotyledons (Fig. 6*A*,*C*). Surprisingly, the same was true not only for IDL, but also for the DP leaves (Fig. 6*B*,*D*) which showed remarkable differences when compared to control leaves with respect to all other parameters studied. It should be noted that the mRNA levels of the two proteases changed in a synchronous manner, thus suggesting possible interdependence in the expression of FtsH5 and Deg1 at transcriptional level.

Discussion

In the present work, we studied the effect of darkness by using young cotyledons or leaves where natural senescence had not been induced yet. As suggested earlier, the response to darkness varies depending on age and plant organ at which it is perceived (Weaver and Amasino 2001). That is why the effect observed in cotyledons and leaves of whole darkened plants was compared to that in individually darkened intact leaf organs.

Two-day dark treatment caused disturbance in the photosynthetic performance of the cotyledons and the first two rosette leaves to a similar extent as revealed by the changes in Φ_{PS2} (Fig. 2*A*). The inhibition of Φ_{PS2} in the dark reflects a respective decrease in the rate of photosynthetic CO_2 assimilation which is linearly related to the PS 2 electron transport (Genty *et al.* 1989, Rolfe and Scholes 1995). However, the type of darkening apparently influenced differently response of NPQ where a strong stimulation in DP cotyledons and IDL and weaker effect on DP leaves were registered (Fig. 2*C*,*D*). According to the model of Kramer *et al.* (2004) the energy absorbed by PS 2 is dissipated in three fundamentally different directions – photochemical utilization, regulated heat dissipation (ΔpH-dependent quenching, photoinhibition, *etc*.) and non-regulated energy dissipation (other non-photochemical losses). In DP cotyledons, the specific increase in NPQ as a photoprotective mechanism in conditions of lowered quantum efficiency of PS 2 photochemistry (Chowdhury *et al.* 2009) was not accompanied by rise in the levels of non-regulated basal dissipation Φ_{NO} (Fig. 2*A*,*C*,*E*). Thus, DP cotyledons were able to alleviate to a great extent the effect of photodamage after ceasing of the dark stress by converting the absorbed light energy into harmless heat. Meanwhile, in IDC darkness led to an increase in Φ_{NO} without significant rise in the photoprotective thermal dissipation NPQ. Changes in Φ_{NO} have been also observed in drought-stressed cotton leaves which have

been associated with constitutive alterations in PS 2 upstream of Q_A (Massacci *et al.* 2008). However, this parameter seems to be insensitive to high irradiance, in contrast to NPQ which is rapidly stimulated with increasing irradiance (Kramer *et al.* 2004). Unlike cotyledons, Φ_{NO} remained unaffected in the first two rosette leaves following both dark treatments (Fig. 2*F*). Therefore, the effect of darkness on leaves comprised changes in the proportion of the two remaining processes for dissipation of the absorbed light energy – photochemistry and regulated dissipation. The similar drop in Φ_{PS2} in the leaves following both dark treatments was accompanied by an increase in the photoprotective thermal dissipation NPQ (Fig. 2*B*,*D*). NPQ was remarkably higher in IDL in comparison with DP leaves, thus indicating higher sensitivity of IDL to the dark stress.

In order to analyze the effects of dark treatment on the expression of the key photosynthesis-related genes we measured the transcript levels of *psbA*, *psaB* and *rbcL* in the differently treated cotyledons and first rosette leaves. We found a clear down-regulation of *psaB* and *rbcL* after dark treatment regardless of the type of darkening (Figs. 3, 4). It has been shown that the decline in the *psaB* and *rbcL* transcript levels is at least in part due to a decrease in the rate of transcription as revealed by *in organello* run-on transcription assays (Krause *et al.* 1998). The inhibitory effect of darkness on the amount of *psaB* and *rbcL* transcripts in IDC and IDL as well as in DP cotyledons was very similar. However, the inhibition in DP leaves seemed to be lower which, together with the weaker effect on the non-photochemical quenching, revealed their specific response to darkness.

In contrast to *psaB* and *rbcL*, no effect on the mRNA levels of *psbA* (coding for the D_1 protein) was detected in the cotyledons and first rosette leaves following both dark treatments (Fig. 3*A*, 4*A*). High stability of the *psbA* transcript levels during short-term dark stress has also been observed in primary leaves of barley (Krause *et al.* 1998). Recently, it has been demonstrated that the *psbA* transcription does not exhibit a strong response even after treatment of detached leaves with exogenous cytokinins, which normally leads to overall transcription stimulation in chloroplasts (Zubo *et al.* 2008). It has been demonstrated that the elevated *psbA* and *psbD* mRNA levels in mature chloroplasts are due primarily to selective stabilization of the mRNA molecules which is consistent with the higher needs for D_1 and D_2 in the photosynthesizing organs (Baumgartner *et al.* 1993). Among all thylakoid proteins, the *psbA*-encoded D_1 protein has the highest turnover rate and its metabolism is subjected to photoregulation with degradation coupled to photosynthetic electron transport (Mattoo *et al.* 1984).

Since the D_1 protein synthesis and degradation are tightly interconnected, the rate of removal of photodamaged D_1 protein from the PS 2 complex may adjust the *psbA* gene transcription (Kettunen *et al.* 1997). Recent investigations on artificially induced senescence due to elevated sugar levels in girdled leaves revealed the stimulation in the expression of many chloroplast proteases under stress conditions (Parrott *et al.* 2007). Here we studied in more details the impact of short-term dark stress on the changes in the transcript levels of two plastid-localized proteases. The serine protease Deg1 has been proposed to play a role in PS 2 repair through degrading photodamaged D_1 in combination with the metalloprotease FtsH, although genetic data confirming this role are lacking (Sakamoto 2006). The expression of FtsH and Deg isomers at transcriptional level seems to be stimulated by abiotic stresses, such as high irradiance (Sinvany-Villalobo *et al.* 2004). However, we found a drastic decrease in *FtsH5* and *Deg1* transcript levels in both cotyledons and leaves after two-day dark treatment (Fig. 6). The observed decline in the expression of the two plastid proteases at transcriptional level could be explained in the light of the model for their involvement in the PS 2 repair cycle (Adam and Clarke 2002). In particular, the absence of photoinhibition during the dark period determines the limitation of the photoprotection mechanisms, part of which is the removal of oxidatively damaged copies of the D_1 protein. In addition, the synchronous manner of inhibition in the mRNA levels of the two proteases (Fig. 6) is consistent with the concept that their expression could be coordinately regulated (Sakamoto 2006, Kapri-Pardes *et al.* 2007).

Several days after re-irradiation, chlorophyll content and Φ_{PS2} in DP cotyledons and IDC reached the control values which were apparently lower than those in non-senescent young cotyledons (Fig. 1*A*, 2*A*). The observed down-regulation of PS 2 photochemistry in the course of senescence could be an adaptive mechanism for adjustment of ATP and NADPH levels to the weaker intensity of the Calvin cycle reactions (Lu and Zhang 1998). The mRNA levels of the senescence-downregulated genes *rbcL, psaB* and *psbA* as well as *SAG12*, which is a typical senescence-associated gene, were also similar to those in the control yellow cotyledons (Figs. 3, 5). Hence, dark-treated *Arabidopsis* cotyledons were able to recover from the applied stress irrespective of the type of darkening and further on, they senesced simultaneously with the controls. This result is in contrast with previous data according to which senescence induced in darkened cotyledons was not reversed by re-irradiation, but rather accelerated (Weaver and Amasino 2001). This discrepancy could be due to the differences in the radiation regime that obviously influences the response of cotyledons to darkness. In the case of accelerated senescence shown by Weaver and Amasino (2001) *Arabidopsis* seedlings were grown under continuous irradiance which normally led to a shortened vegetative phase and faster flowering as compared with plants grown under a photoperiod used in the present work.

In contrast to cotyledons, the type of darkening influenced strongly the capability of the first rosette leaves to recover from the dark stress. As revealed by the changes in the functional parameters and the qRT-PCR analysis, senescence progression in DP leaves was

apparently delayed which is in accordance with previous observations (Weaver and Amasino 2001). On the other hand, typical senescence symptoms were observed in IDL several days upon re-irradiation and the leaves died prematurely or simultaneously with the controls. Recent experiments with *Arabidopsis* leaves have also shown differences between IDL and DP leaves in their responses to darkness (Keech *et al.* 2007). In the above study, a substantial loss of photosynthetic capacity in IDL was observed throughout the whole 6-d dark period while the slight inhibition found in DP leaves after two days in darkness was not followed by a further decrease until the end of the treatment. This finding highlights the hypothesis for the metabolism of the DP leaves which enters a "stand-by mode" in order to maintain functional photosynthetic machinery (Keech *et al.* 2007). In the present work, the only photosynthetic parameter which remained constant in the course of leaf senescence was Φ_{NO} (Fig. 2*F*). By contrast, Φ_{NO} tended to be higher in the course of cotyledon senescence (Fig. 2*E*). The different dynamics of Φ_{NO} in cotyledons and first rosette leaves during natural senescence and dark stress is likely to reflect specificities in the functional organization of the photosynthetic apparatus in both organs. Interestingly, during the recovery period the transcript levels of *FtsH5* and *Deg1* in senescent IDL and non-senescent DP leaves were very similar (Fig. 6*B*,*D*). Little is known about the involvement of the FtsH and Deg protease families in the thylakoid protein degradation during senescence. For instance, *in vitro* studies have revealed FtsH6-dependent degradation of LHC 2 apoproteins in senescing *Arabidopsis* leaves, but the impact of senescence on the *FtsH6* expression *in vivo* remains obscure (Zelisko *et al.* 2005). In our case, the lack of stimulation of the *FtsH5* and *Deg1* mRNA levels in senescent IDL which is normally expected in the course of massive degradation of D_1 and other thylakoid proteins in the gerontoplast, does not necessarily mean lack of changes in the amount of the corresponding proteins or their enzymatic activities. Therefore, further experiments at the protein level could clarify the role of FtsH5 and Deg1 in senescent tissues.

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