Darkness Affects Differentially the Expression of Plastid-Encoded Genes and Delays the Senescence-Induced Down-Regulation of Chloroplast Transcription in Cotyledons of *Cucurbita pepo* L. (Zucchini)

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In contrast to differentiated leaves, the regulatory mechanisms of chloroplast gene expression in darkened cotyledons have not been elucidated. Although some results have been reported indicating accelerated senescence in *Arabidopsis* upon reillumination, the capacity of cotyledons to recover after dark stress remains unclear. We analysed the effect of two-days dark stress, applied locally or at the whole-plant level, on plastid gene expression in zucchini cotyledons. Our results showed that in the dark the overall chloroplast transcription rate was much more inhibited than the nuclear run-on transcription. While the activities of the plastid-encoded RNA polymerase (PEP) and nuclear RNA polymerase II were strongly reduced, the activities of the nuclear-encoded plastid RNA polymerase (NEP) and nuclear RNA polymerase I were less affected. During recovery upon reillumination, chloroplast transcription in the cotyledons was strongly stimulated (3-fold) compared with the naturally senescing controls, suggesting delayed senescence. Northern blot and dot blot analyses of the expression of key chloroplast-encoded photosynthetic genes showed that in contrast to *psbA*, which remained almost unaffected, both the transcription rate and mRNA content of *psaB* and *rbcL* were substantially decreased.

**Key words:** Cotyledon Senescence, Dark Stress, NEP, PEP

Introduction

Darkness can affect the senescence progression in plants by modulating the photosynthetic efficiency, the generation of reactive oxygen species, as well as by activation of different signalling cascades (Lers, 2007). One of the earliest targets of dark-induced senescence is the chloroplast where a number of ultrastructural and functional alterations cause a rapid drop in the photosynthetic activity (Nooden *et al.*, 1997; Krupinska and Humbeck, 2004). Chloroplast senescence includes intensive degradation of pigments, membrane lipids, nucleic acids, and stroma-localized proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The degradation of thylakoid proteins from photosystem II (PSII), photosystem I (PSI), and the light-harvesting complexes (LHCs) of both photosystems is accelerated at later stages of leaf senescence compared to the stromal proteins (Humbeck and Krupinska, 2003).

It has been shown that darkness can induce two opposite senescence-related responses depending on the level at which it is perceived (whole plant or individual leaf organ) as revealed by experiments with *Arabidopsis thaliana* rosette leaves (Weaver and Amasino, 2001). One response represents the locally induced promotion of senescence when dark treatment is applied to individual leaves. In these leaves, a rapid decline in photosynthetic activity has been found while high mitochondrial respiration is maintained associated with the
rapid degradation of cellular components and consequent nutrient remobilization (Keech et al., 2007). On the other hand, senescence is repressed when darkness is applied at the whole-plant level due to preserved photosynthetic capacity as well as retention in leaf development including an inhibition of the senescence-specific degradation of total protein and chlorophyll (Weaver and Amasino, 2001; Keech et al., 2007). Thus, the metabolism in the leaves of whole darkened plants enters a “stand-by-mode”, which may be a strategy to maintain the chloroplast intactness and the photosynthetic machinery, thus allowing the leaf to resume photosynthesis upon reillumination.

Concerning the regulation of the photosynthetic activity in the course of natural senescence, organ-specific differences between cotyledons and true leaves have been reported (La Rocca et al., 1996). However, much less is known about the response of cotyledons to darkness. It has been suggested that in contrast to differentiated leaves, cotyledons of whole darkened Arabidopsis plants exhibit typical senescence symptoms, and in this case senescence is not reversed upon reillumination, but rather accelerated (Weaver and Amasino, 2001). In our recent investigations with zucchini cotyledons, we found a lack of significant changes in the pigment content and the activities of PSII and PSI after two-day dark treatment of whole plants, as well as delayed chloroplast senescence in the subsequent recovery period upon reillumination (Mishev et al., 2009). Our results suggested also higher resistance of zucchini cotyledons to the applied dark stress as compared to the primary leaves with respect to the photosynthetic parameters. In the present study, we extend our previous investigations on dark-induced cotyledon senescence in the subsequent recovery period upon reillumination (Mishev et al., 2009). The experimental scheme is presented in Fig. 1. Dark treatment was applied for 2 d at the age of 14 d either to individual intact cotyledons (a single or both cotyledons from the pair) using paper mittens or at the whole-plant level. At the end of the dark treatment (in the morning of day 16 after seed germination) plants or individually treated cotyledons were returned to normal light regime.

RNA extraction and blotting

Total RNA was extracted from about 100 mg fresh cotyledons using TRIzol reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer’s protocol. The RNA concentration was determined spectrophotometrically using NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). The RNA samples (10 μg each) were electrophoretically fractionated on 1% (w/v) formaldehyde-agarose gels and transferred onto Hybond™-N+ membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Hybridization DNA probes were 32P-labelled by random priming using Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany) in the presence of 50 μCi [α-32P]dCTP (3000 Ci mmol–1; Izotop, Budapest, Hungary). Membrane hybridization with the 32P-labelled DNA probes was carried out according to Sambrook and Russell (2001). Membranes were subsequently exposed to an X-ray film (Hyperfilm™ MP; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for hybridization signal visualization. Signal intensities were quantified using the ImageJ software (http://rsb.info.nih.gov/ij/), and the results from three independent experiments were averaged.

Material and Methods

Growth conditions and treatments

Seeds of Cucurbita pepo L. (zucchini) were germinated on moistened filter paper in the dark at 28 °C for 96 h. The 4-day-old etiolated seedlings were grown further on a nutrient solution in a growth chamber at a photon flux density of 100 μmol m–2 s–1, (26 ± 2) °C, and a 12 h/12 h day/night cycle.
Nuclei isolation, nuclear run-on transcription assays, and α-amanitin inhibition

The isolation of nuclei from zucchini cotyledons was performed as previously described (Ananiev et al., 1987). The DNA content in the nuclei suspensions was determined according to Burton (1956). The run-on transcription assays were carried out at 24 °C for 10 min in the presence of 5 μCi [α-32P]UTP (3000 Ci mmol–1; Izotop) according to Ananiev et al. (1987). The reaction was stopped by spotting aliquots onto Whatman DE-81 filters premoistened with 0.5 M EDTA which were then processed according to Hallick et al. (1976). Total [32P]UMP incorporation into plastid RNA was measured in a Beckman liquid scintillation counter.

Blotting of chloroplast genes and hybridization with in organello synthesized plastid RNA

For estimation of the transcription rates of individual plastid genes, run-on transcription assays were carried out with 106 chloroplasts in the presence of 100 μCi [α-32P]UTP (3000 Ci mmol–1) according to the protocol of Deng and Gruissem (1995). In vitro synthesized RNA was deproteinized, precipitated with isopropanol, and resuspended in diethylypyrocarbonate-treated water.

Dot-blot hybridization assays were performed with the same zucchini chloroplast DNA fragments as used as probes for Northern blot analysis. The recombinant plasmid DNA clones containing the plastid gene fragments were denatured and dotted onto Hybond™-N+ membranes in decreasing series of 400 and 100 ng DNA for each gene with the help of a Bio-Dot microfiltration apparatus (Bio-Rad, Munich, Germany). Membrane hybridization with 32P-labelled plastid RNA probes was carried out according to Deng and Gruissem (1995). Membranes were exposed to an X-ray film (Hyperfilm™ MP) for 10 d.
Results

Dark-induced changes in the overall chloroplast transcription rate

It is well known that transcription rate and mRNA stability in plants are highly dynamic in the course of natural and stress-induced senescence (Krupinska and Humbeck, 2004; Baginsky et al., 2007). In order to assess the changes in chloroplast transcription after dark stress, we carried out in organello run-on transcription, thus eliminating the influence of mRNA stability on transcript content. Our results showed that darkening of whole plants (DP) for two days led to a decrease of the total chloroplast RNA polymerase activity in the cotyledons by 45%, while the decrease in the first true leaf was much more pronounced and reached almost 80% (Fig. 2A). Individual darkening of the cotyledon pair (individually darkened cotyledons, IDC) resulted in a stronger decrease (by 70%) in the overall plastid transcription rate when compared to DP cotyledons, thus demonstrating the role of the light status of the rest of the plant in the dark stress response. Furthermore, we studied the differential effect of darkness on the activity of the two main plastid RNA polymerases, nuclear-encoded (NEP) and plastid-encoded (PEP), using tagetitoxin. Tagetitoxin is a powerful inhibitor of all prokaryotic RNA polymerases including PEP, but does not affect the three plant nuclear RNA polymerases and the NEP (Mathews and Durbin, 1990). Our previous results with tagetitoxin had shown an inhibition of the overall plastid transcription by about 87% in chloroplasts isolated from juvenile 8-day-old cotyledons which reflected the portion of the PEP-initiated transcription (Mishev et al., 2006). Two-days dark treatment of whole plants caused a 53% decrease in the PEP activity (Fig. 3B) in comparison with the PEP activity in control cotyledons (Fig. 3A). However, the activity of NEP remained unchanged when compared to control cotyledons.

In contrast to senescing control cotyledons which showed a sharp decline in total plastid RNA polymerase activity, a 3-fold higher chloro-
plast transcription rate was observed in the reilluminated DP cotyledons (Fig. 2B). Besides, the analysis of tagetitoxin inhibition revealed that the PEP activity in cotyledons of 27-day-old plants after an 11-days recovery period stayed close to the values measured in 16-day-old DP cotyledons (Fig. 3C).

**Effect of darkness on the nuclear transcription rate**

Our experiments with isolated nuclei showed that, compared to chloroplast transcription, short-term dark treatment of intact zucchini cotyledons did not affect significantly the overall nuclear RNA polymerase activity. While nuclear RNA synthesis in IDC was slightly decreased (by about 20%), it remained almost unaffected in DP cotyledons (Fig. 4). To further characterize the differential effect of darkness on nuclear RNA polymerases, run-on transcription assays in the presence of α-amanitin were carried out, thus allowing direct estimation of the activity of the drug-resistant RNA polymerase I. Two-days dark treatment did not reduce the RNA polymerase I activity in both IDC and DP cotyledons (Fig. 4). By contrast, the α-amanitin-sensitive RNA polymerase II activity was strongly decreased, the reduction reaching 60% and 80% in DP cotyledons and IDC, respectively.

**Differential regulation of the photosynthetic gene expression in dark-stressed cotyledons**

Next, we studied the changes in the rate of transcription and the mRNA content of some key photosynthetic plastid-encoded genes. Generally, an increase in the mRNA level could result from its enhanced synthesis or retarded breakdown, and vice versa a decline in the mRNA level could be due to suppression of its synthesis or breakdown acceleration. In particular, we were interested in the expression of *psbA* (coding for the D1 protein of the PSII reaction centre), *psaB* (coding for the PSI apoprotein PsaB), *rbcL* (coding for the large subunit of Rubisco which is a stroma-localized protein), and *rrn16* (coding for 16S rRNA which is a typical housekeeping gene). The hybridization assays were performed using DNA fragments derived from the plastome of *Cucurbita pepo* which has not been sequenced so far. The alignment of the amplified zucchini DNA fragments showed high levels of homology (97% for *psbA*, 98% for *psaB* and *rbcL*, 100% for *rrn16*, data not shown) to the corresponding regions of the *Cucumis sativus* plastid genome which was recently published (Kim et al., 2006).

Northern blot analysis of the studied chloroplast genes in dark-stressed cotyledons revealed significant differences in the transcript levels of *psbA* and *psaB*. The mRNA content of *psbA* was slightly decreased in IDC and remained almost unaffected in DP cotyledons (Fig. 5). On the other hand, the *psaB* transcript levels substantially
declined in the dark, especially in IDC where the reduction reached about 70% based on the densitometric analysis (data not shown). In addition, a substantial decrease (about 5-fold) in the \textit{rbcL} gene expression was found compared to cotyledons of 16-day-old control plants. As expected, the high levels of chloroplast 16S rRNA remained unaffected by the applied stress, regardless of the type of darkening (Fig. 5).

The transcriptional regulation of chloroplast gene expression was studied by dot-blot hybridization of zucchini plastid DNA probes with \textit{de novo} synthesized plastid RNA. Our results showed that the transcription of \textit{psaB} and \textit{rbcL} was strongly inhibited by the dark treatment (Fig. 6). The decline in the transcription rates of these two genes was very similar in IDC and DP cotyledons and reached about 50% of the control (densitometric data). In contrast to \textit{psaB} and \textit{rbcL}, the \textit{psbA} gene transcription was decreased to a lesser extent (by 35%) in IDC and almost unaffected in DP cotyledons (Fig. 6). Concerning the \textit{rrn16} gene, the rate of transcription was lower compared to the photosynthetic genes not only in the darkened cotyledons, but also in the controls.

**Discussion**

While chloroplast gene expression in dark-treated differentiated leaves has been extensively studied, the changes in cotyledon chloroplast transcription are still poorly analysed. It is well known that chloroplast RNA synthesis is regulated mainly at the level of plastid RNA polymerases, the activities of which change depending on the developmental stage and growth conditions (Mache and Lerbs-Mache, 2001). One possible impact of short-term dark stress on the chloroplast transcription could be due to the unequal sensitivity of the two classes of plastid RNA polymerases, NEP and PEP, to darkness. Changes in the ratio between the NEP and PEP activities in the dark were reported for barley foliage leaves (Krause \textit{et al.}, 1998), but the role of the two chloroplast RNA polymerases in cotyledons remained unclear. In the present study, we found a decrease in the PEP activity in zucchini cotyledons after 2-days dark treatment of whole plants, while the NEP activity remained unchanged (Fig. 3). Concerning the overall chloroplast RNA polymerase activity, we observed for the first time higher sensitivity in IDC in comparison with DP cotyledons (Fig. 2A). Moreover, the comparative analysis of the overall transcription rate in nuclei and plastids revealed a lesser impact of darkness on the nuclear RNA polymerase activity affecting mainly RNA polymerase II (Fig. 4). Therefore, darkness affects primarily the expression of nuclear protein-coding genes and does not substantially affect rRNA gene transcription. Besides, similar to the activities of PSI and PSII (Mishev \textit{et al.}, 2009), the plastid transcription rate in DP cotyledons recovering under photoperiod was apparently higher than that in naturally senescing cotyledons (Fig. 2B). This finding definitively indicates delayed chloroplast senescence in DP cotyledons upon reillumination. Previous results with \textit{Arabidopsis} have shown delayed senescence only in rosette leaves, but not in cotyledons which did not recover from the applied dark treatment (Weaver and Amasino, 2001). The different capacity of cotyledons of \textit{Arabidopsis} and \textit{C. pepo} to recover after the applied dark stress could be attributed to the different mechanisms of senescence in these two plant species. The senescence of individual leaves in \textit{Arabidopsis} depends on plant longevity (Lim \textit{et al.}, 2007), whereas in other monocarpic plants, including \textit{C. pepo}, senescence progression is controlled by the appearance of the generative organs. Besides, our results indicated that zucchini cotyledons were more resistant to the applied dark stress compared to true leaves (Fig. 2A). This organ-specific response could be due to the dual function of cotyledons, being reserve storage organs during the early period of
germination and photosynthesizing organs at later stages of ontogenesis (La Rocca et al., 1996).

It has been recently suggested that chloroplasts possess a complex enzymatic machinery for light-dependent regulation of the plastid mRNA stability (Baginsky and Gruissem, 2002; Baginsky et al., 2007). Proteomic analysis has revealed that the dark-induced RNA degradation pathway involves enzymatic activities differing from those that direct RNA processing and stabilization in the light (Baginsky et al., 2007). The degradation of mRNA coding for proteins involved in photosynthesis which are not needed in conditions of light deprivation is likely to fill up the nucleotide pool for ensuring other chloroplast metabolic activities. In our study, we analysed the expression of four key plastid-encoded genes in dark-treated cotyledons by comparing the changes in the rate of their transcription and the levels of mRNA accumulation which allows distinguishing the role of mRNA stability as an essential factor in the post-transcriptional regulation (Rapp et al., 1992).

Among the most intensively transcribed chloroplast genes is rbcL which, similar to psbA, can be transcribed only from PEP promoters (Courtois et al., 2007). Darkness led to a drastic decrease in the rate of transcription and the mRNA levels of the rbcL gene in cotyledons (Figs. 5 and 6). Therefore, the decline in the rbcL mRNA content after two days in the dark is at least in part due to delayed transcription. A similar inhibition of the rbcL gene expression has been observed in primary foliage leaves of barley plants darkened for two days (Krause et al., 1998). However, experiments with tobacco plants revealed a dark-induced reduction in the rate of rbcL gene transcription without any changes in the steady-state mRNA levels, thus suggesting an increased mRNA stability due to stabilization of the mRNA molecule via its 5’-UTR (Shiina et al., 1998). Similar to rbcL, the substantial decrease in the mRNA levels of psaB in dark-treated zucchini cotyledons (Figs. 5 and 6) was at least partially due to a reduced rate of transcription, thus demonstrating the significance of the light-mediated regulation of psaB expression.

In dark-stressed cotyledons, neither the transcription rate, nor the mRNA levels of the gene encoding the D1 protein, psbA, were considerably affected, in contrast to the above two genes (Figs. 5 and 6). Recently, it has been found that psbA transcription is very stable and remains unchanged even after strong stimulation of plastid RNA synthesis in detached barley leaves after cytokinin treatment (Zubo et al., 2008). In normally illuminated plants, the regulation of the psbA gene expression mainly occurs at the post-transcriptional level, since chloroplasts possess a stable pool of psbA mRNA molecules (Kettunen et al., 1997). During leaf development psbA and psbD transcript levels are differentially elevated in mature chloroplasts relative to other plastid mRNAs which is consistent with maintaining a high capacity to synthesize the D1 and D2 proteins (Baumgartner et al., 1993). Among the chloroplast proteins, the D1 protein has the highest turnover rate in order to ensure rapid replacement of D2 copies damaged as a consequence of PSII photochemistry (Kettunen et al., 1997). Moreover, the transcription of psbA in mature plastids of 16-day-old zucchini cotyledons was much more intensive than that of the rrm16 housekeeping gene which contrasted with the observed substantial accumulation of 16S rRNA at this growth stage (Figs. 5 and 6). The latter finding reflects the high stability and long half life of the 16S rRNA molecules as components of the stable chloroplast ribosomes (Rapp et al., 1992).

In conclusion, we report for the first time that short-term dark stress of cotyledons causes a strong reduction of chloroplast transcription due to decreased PEP activity, while the overall nuclear transcription was found to be less sensitive. In addition, in contrast to previously reported data, the senescence of darkened cotyledons was delayed after reillumination.


Baumgartner B. J., Rapp J. C., and Mullet J. E. (1993), Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (Hordeum vulgare) chloroplast development –


