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Polyribosome loading of spinach mRNAs for photosystem I subunits is controlled by photosynthetic electron transport

A crucial *cis* element in the spinach *PsaD* gene is located in the 5′-untranslated region

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Summary

In light-, but not in dark-grown spinach seedlings, the mRNAs for the nuclear-encoded photosystem I subunits D, F and L are associated with polyribosomes and this association is prevented by the application of 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU), an inhibitor of the photosynthetic electron transport. To identify the *cis*-elements which are responsible for this regulation, we generated a series of chimeric *PsaD* constructs and tested them in transgenic tobacco. The spinach *PsaD* 5′-untranslated region is sufficient to confer light- and photosynthesis-dependent polyribosome association onto the *uidA* reporter gene, while the tobacco *PsaD* 5′-untranslated region directs constitutive polyribosome association. These results are discussed with regard to signals from photosynthetic electron flow which control processes in the cytoplasm.

Keywords: light regulation, photosystem I, *PsaD*, polyribosome loading, spinach, tobacco.

Introduction

Photosystem I is a multisubunit complex that transfers electrons from plastocyanin to ferredoxin (Chitnis, 2001). It consists of at least 13 different polypeptide species, eight of which are encoded in the nucleus (Herrmann *et al.*, 1991; Scheller *et al.*, 1997). Expression of these genes, as well as of genes which code for the associated polypeptides ferredoxin and plastocyanin, is highly regulated by light and signals from the plastids, and exhibits several unusual features.

As reported for *4CL-1* from parsley (Douglas *et al.*, 1991) and the γ-glutamylcysteine synthase from *Arabidopsis* (Xiang and Oliver, 1998), expression of photosynthetic genes is also regulated at the post-transcriptional level via *cis*-elements in the RNAs. For instance, light-regulated expression of the pea and *Arabidopsis* ferredoxin genes, *Fed-1* and *FedA*, respectively, requires sequences downstream of their transcription start sites (Bovy *et al.*, 1995; Dickey *et al.*, 1992; Elliott *et al.*, 1989; Gallo-Meagher *et al.*, 1992). Detailed analyses revealed that light modulation of the *Fed-1* mRNA abundance requires an open reading frame (Dickey *et al.*, 1994), and that an essential element is located in the 5′-untranslated region (UTR, Dickey *et al.*, 1998). Light-stimulated expression of *Fed-1* correlates with an increased polyribosome association of the message (Dickey *et al.*, 1998) and is dependent on photosynthetic electron transport (Petracek *et al.*, 1997, 1998). The light-responsive element is responsible for a decline in translation efficiency and the *Fed-1* message dissociates rapidly from the polyribosomes after transfer of the plants from light to the dark (Hansen *et al.*, 2001). This mechanism enables the plant to respond rapidly to environmental light changes such as shading. The high translational activity of the *Fed-1* message in light is mediated by the heat-shock protein HSP101 which binds to the light-regulatory element

of the RNA (Ling *et al.*, 2000). *Fed-1* transcripts are stable in light and degraded in the dark, and nonsense codons introduced into the coding region trigger a decay pathway (Petracek *et al.*, 2000).

Similar to the transcriptional control exerted via *cis*-elements in the promoter, light-regulated expression of the pea plastocyanin gene, *PetE*, is mediated by elements located within the transcribed region of the gene (Helliwell *et al.*, 1997). Additionally, sequences surrounding the translation initiation codon increase the translation efficiency of a reporter gene (Helliwell and Gray, 1995). The correct 5′-end of the transcript and the coding region is necessary for *PetE* regulation by light and signals from the plastids, and this regulation includes post-transcriptional events.

Regulated expression of the spinach *PsaD* gene (for the subunit II of photosystem I reaction center) also requires sequences located in the transcribed region and within an intron (Bolle *et al.*, 1996a). Again, as described for many other *cis*-elements either operating at the transcriptional or post-transcriptional level, the same sequences which mediate light-responsiveness also respond to signals from the plastids (Bolle *et al.*, 1996a; Hahn and Kück, 1999; Kusnetsov *et al.*, 1996, 1999; Puente *et al.*, 1996). The most striking example is a single nucleotide exchange in the spinach *AtpC* promoter, which uncouples *AtpC* gene expression from both regulatory pathways (Kusnetsov *et al.*, 1999).

In this study, we demonstrate that light-regulated photosystem I gene expression also occurs at the level of translation by triggering polyribosome loading of the messages, and that this effect is dependent on or mediated by the photosynthetic electron flow. Furthermore, by comparing the expression of chimeric reporter gene constructs with the 5′-untranslated regions (UTR) from the spinach and tobacco *PsaD* genes in transgenic tobacco seedlings, we demonstrate the important role of the 5′-UTR for polyribosome loading. The spinach *PsaD* UTR directs *uidA* messages to polyribosomes only in light, but not in darkness and this effect is inhibited if the electron transport in the thylakoid membrane is limited by 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU) treatments. Apparently, light- and plastid-derived signals operate via the same *cis*-elements of a responsive RNA segment.

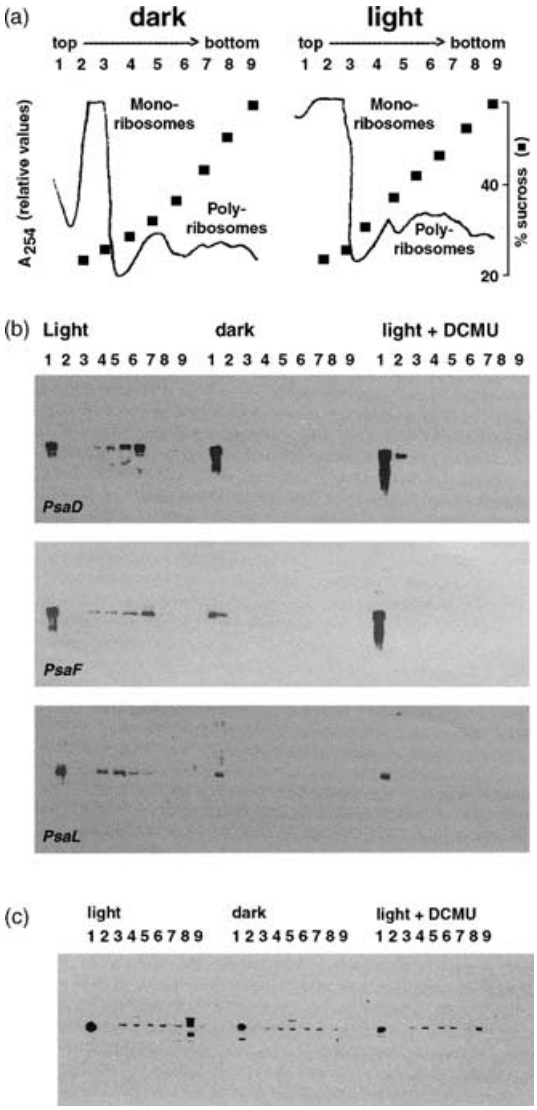

Figure 1. Polyribosome profiles for the spinach *PsaD*, *PsaF* and *PsaL* messages in etiolated or light-grown spinach seedlings.
(a) Absorption (A254) and sucrose concentration profiles after sucrose gradient centrifugation of a polysomal fraction prepared from 7-day-old etiolated and light-grown spinach seedlings. Approximately 100 μl fractions were collected from the gradients and used for the measurements.
(b) Polyribosome profile of the spinach *PsaD*, *PsaF* and *PsaL* messages in spinach seedlings, which were kept in light or in darkness for 7 days (light, darkness). Light + DCMU: 12 h before harvest the seedlings were sprayed with DCMU (10 μM). After sucrose gradient centrifugations and RNA extraction from the individual fractions 1 (top) to 9 (bottom), Northern hybridization was performed with the respective cDNAs. Representative of three independent experiments.
(c) As control, the polyribosome profile of the tubulin gene is given.

Results

Figure 1 demonstrates polyribosome loading of the *PsaD*, *PsaF* and *PsaL* messages (for the subunits D, F and L of the photosystem I reaction center) in light- and dark-grown spinach seedlings and in light-grown seedlings which were treated with DCMU for 12 h. Crude ribosomal fractions were layered on the top of a sucrose gradient. After centrifugation, the monoribosomes remained on the top of the gradient in the region of less than 20% sucrose, while polyribosomes were preferentially found at sucrose concentrations >35%, as measured by UV tracing at 254 nm (cf. Dickey *et al.*, 1998) and sucrose refractometry (Figure 1a). Figure 1b demonstrates that the spinach messages are associated with polyribosomes in light-grown, but not etiolated seedlings, and that polyribosome association in light is prevented by the application of DCMU at

concentrations which limit photosynthetic electron flow (Table 1). We monitored the inhibition of the electron transport by measuring the *in vivo* chlorophyll *a* fluorescence parameters with a pulse amplitude modulated fluorometer (cf. Experimental procedures). Detailed dose–response analyses revealed that 10 μM DCMU limits electron transport to an extent which is comparable to the level in seedlings grown under photosystem I light (Pfannschmidt *et al.*, 2001). Thus, we conclude that polyribosome loading of the investigated messages is dependent on the photosynthetic electron flow.

To determine the *cis*-elements which mediate this regulation, we analyzed the *PsaD* gene and various constructs derived from this gene in transgenic tobacco seedlings. Initially, polyribosome loading was investigated in etiolated and light-grown tobacco seedlings. We have previously demonstrated that a 6-kbp spinach DNA fragment with the promoter and coding region of *PsaD* is highly expressed in transgenic tobacco seedlings, but fails to respond to light and signals from the plastids (Bolle *et al.*, 1996a; Flieger *et al.*, 1994). Figure 2 demonstrates that similar to the regulation in spinach, the spinach *PsaD* message in transgenic tobacco seedlings becomes associated with polyribosomes in a light-dependent manner. These transgenic tobacco plants were used as a test system to identify sequences in the spinach *PsaD* message which are responsible for this regulation. A series of new constructs were generated and introduced into tobacco via *Agrobacterium tumefaciens* (Figure 3, detailed description of the constructs is given in Experimental procedures). We noticed that in light-, but not in dark-acclimated seedlings, the *uid*A mRNA with the complete spinach *PsaD* UTR was also associated with polyribosomes (Figure 4a). Further analysis showed that polyribosome association requires the first 30 bases of the UTR (Figure 4b). The results were the same when transcription was driven by the 35S RNA CaMV promoter (Figure 4c,d). Furthermore, two lines of evidence confirmed that light-dependent polyribosome association was specific for the spinach *PsaD* UTR: first, *uidA* messages without the spinach *PsaD* UTR, expressed either from the bacterial 35S RNA CaMV or the spinach *PsaD* promoter, did not become associated with polyribosomes (Figure 4e,f) and second, *uidA* messages with the tobacco *PsaD* UTR were constitutively associated with polyribosomes in etiolated and light-acclimated seedlings (Figure 4g; cf. Discussion).

Furthermore, the spinach 6-kbp *PsaD* fragment was modified by site-directed mutagenesis to give rise to mutated mRNAs in which the AUG start codon (Figure 4h), the nucleotides −3 to −1 (Figure 4i) or −6 to −4 (Figure 4j) relative to the translation start codon were changed. When

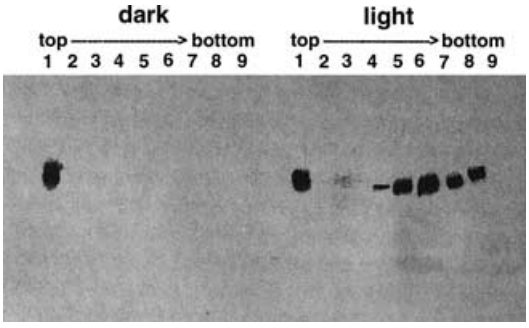

Figure 2. The spinach *PsaD* mRNA is associated with polyribosomes in light-, but not in dark-grown tobacco seedlings. Polyribosome profile of the spinach *PsaD* mRNA in dark- and light-grown transgenic tobacco seedlings, which harbor a 6-kbp spinach *PsaD* fragment (cf. Bolle *et al.*, 1996a). The profiles are representative of three independent experiments.

Spinach *PsaD*: *PsaD*-pro.-TCTACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATCAAATT**ATG**GCCAT-*PsaD*-gene

Const. A: *PsaD*-pro.-TCTACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATCAAATT**ATG**GCCATgggtggtcagtcccttatgtta-*uidA*-gene

Const. B: *PsaD*-pro.-TCTACA----------ACAACAACAATCAAATT**ATG**GCCATgggtggtcagtcccttatgtta-*uidA*-gene

Const. C: 35 S-pro.-AGGACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATCAAATT**ATG**GCCATgggtggtcagtcccttatgtta-*uidA*-gene

Const. D: 35 S-pro.-AGGACA----------ACAACAACAATCAAATT**ATG**GCCATgggtggtcagtcccttatgtta-*uidA*-gene

Const. E: 35 S-pro.-AGGACA----------tctagaggatcccgggtggtcagtcccttatgtta-*uidA*-gene

Const. F: *PsaD*-pro.-TCTACA----------gggtggtcagtcccttatgtta-*uidA*-gene

Const. G: to*PSAD*-pro.-TTCACTTCTCTCAATCCAACTTTTCTAgatccccgggtggtcagtcccttatgtta-*uidA*-gene

Const. H: *PsaD*-pro.-TCTACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATCAAATT**TAC**GCCAT-*PsaD*-gene

Const. I: *PsaD*-pro.-TCTACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATCAAGCC**ATG**GCCAT-*PsaD*-gene

Const. J: *PsaD*-pro.-TCTACACTCCCTCAATCAACCACCAATCCAAACACAACAACAACAATAGGATT**ATG**GCCAT-*PsaD*-gene

Figure 3. *PsaD*-derived constructs which were introduced into tobacco via *Agrobacterium tumefaciens*. Only the region between the transcription start site (A) and the ATG codon (ATG) is presented. The promoter regions derive either from the spinach *PsaD* gene (*PsaD*), the 35S RNA CaMV gene (35S CaMV) or the tobacco *PsaD* gene (to *PsaD*), the coding regions from the spinach *PsaD* gene (*PsaD*) or the bacterial β-glucuronidase gene (*uidA*).

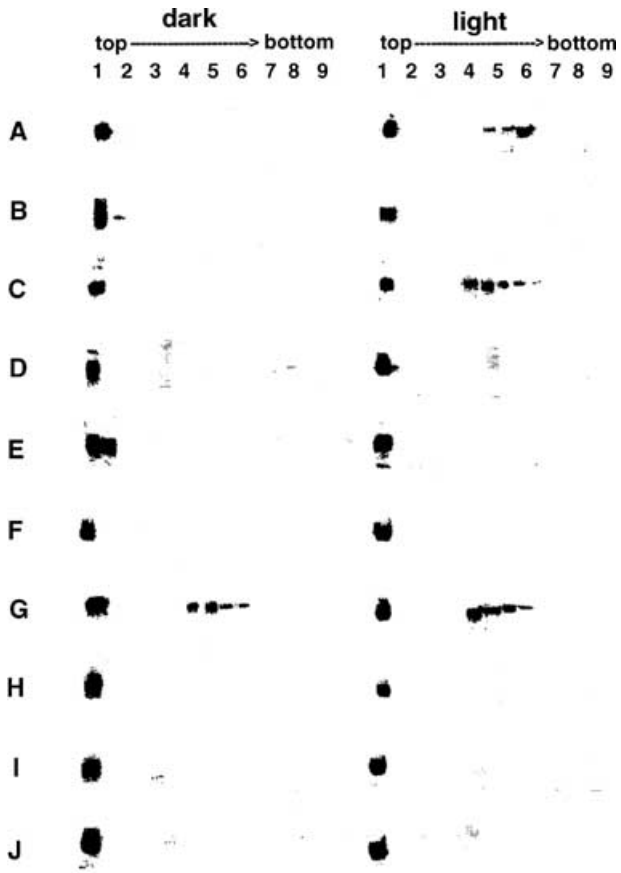

Figure 4. Polyribosome profiles of various transgene mRNAs in tobacco seedlings which were either kept in darkness or white light for 12 days. The following hybridization probes were used: spinach *PsaD*, lanes A–D, H–J; *uidA*, lanes E–G. The messages derive from constructs which are shown in Figure 3. The letters on the left correspond to the constructs shown in Figure 3. Each panel is representative of three independent experiments.

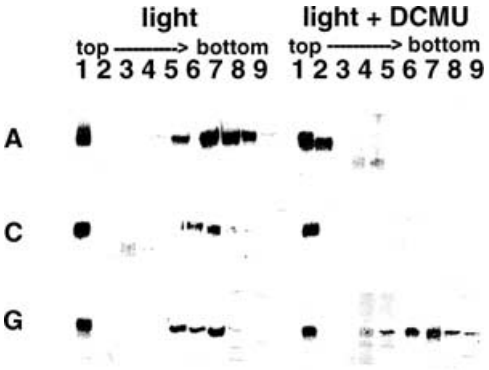

Figure 5. Polyribosome profile of the spinach *PsaD* or bacterial *uidA* messages in transgenic tobacco seedlings which were kept in light for 12 days (left panel). Right panels: 12 h before harvest, the seedlings were sprayed with DCMU (10 μM). The messages derive from constructs shown in Figure 3, letters on the left refer to identical constructs in both figures. Each panel is representative of three independent experiments.

compared to the results obtained for the constructs A, C and G, it becomes obvious that little, if any, polyribosome association can be detected for messages deriving from these constructs, both in light- and dark-grown tobacco seedlings (Figure 4h–j). Thus, the region −6/+3 relative to the translation start site appears to be required for translation of the *PsaD* mRNA.

Figure 1 demonstrated that light-mediated polyribosome association of *PsaD* in spinach is prevented by DCMU. To test this phenomena for the three constructs, for which polyribosome association has been observed (constructs A, C and G; cf. Figures 3 and 4), we kept these transgenic seedlings in white light for 12 days. Twelve hours before the assay, they were either mock-treated (Figure 5; light) or treated with DCMU (Figure 5; light + DCMU). Application of DCMU completely blocked polyribosome association of the spinach *PsaD* UTR::*uid*A messages and after 12 h, no more transcripts could be detected in the polyribosomal fraction. The same phenomenon was observed irrespective of whether transcription was driven by the spinach *PsaD* or the bacterial 35S RNA CaMV promoter (Figure 5, constructs A and C). Furthermore, polyribosome association of the message with the tobacco *PsaD* UTR was not inhibited by the DCMU treatment (compare Figure 5, constructs A and C versus G). This suggests that the light response of the spinach *PsaD* gene in tobacco is coupled to photosynthetic electron flow, that at least one responsive *cis*-element is located in the spinach *PsaD* UTR, and that this effect is specific since it cannot be replaced by the tobacco *PsaD* UTR. Furthermore, this study provides additional evidence that the signals from light and the plastids operate via the same *cis*-element(s) even if they are operative at post-transcriptional levels in the cytoplasm.

To check the velocity of signal transduction from photosynthetic electron flow to polyribosomes in the cytoplasm, the amount of polyribosome-asociated *PsaD* messages in spinach (Figures 6 and 7) and of the messages from the constructs C and G in tobacco (cf. Figure 3) were analyzed kinetically. Ten minutes after the application of DCMU, the *PsaD* message in spinach was still associated with polyribosomes, while after 60 min, no more polyribosome association could be detected (Figure 6). Taking into consideration that DCMU-mediated limitation in the electron flow requires approximately 5–10 min after the application to the seedlings (Pfannschmidt *et al.*, 2001), the transduction of the signal from the thylakoid membranes to the cytosolic ribosomes must be fast. In spite of the large error bars, it is obvious that the response of the *uidA* messages from construct C in tobacco to the DCMU treatment occurs quickly and is similar to the response in spinach, while no effect was observed for the messages from construct G (Figure 7). This confirms that crucial *cis*-elements for the rapid response of the spinach *PsaD* gene to photosynthetic electron flow are located in its 5′-UTR.

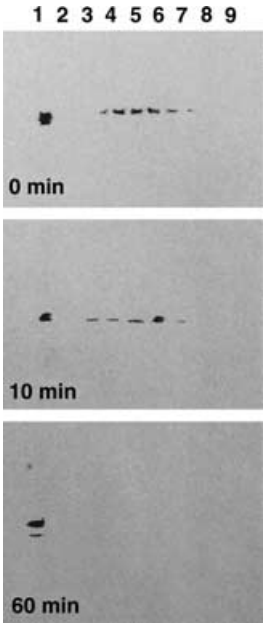

Figure 6. Polyribosome profile of the *PsaD* message in spinach seedlings which were kept in light for 7 days (0 min). 10 min, 60 min: the polyribosome profile 10 or 60 min after the application of DCMU. After sucrose gradient centrifugations and RNA extractions from the individual fractions 1 (top) to 9 (bottom), Northern hybridization was performed with the spinach *PsaD* cDNA. Representative of three independent experiments.

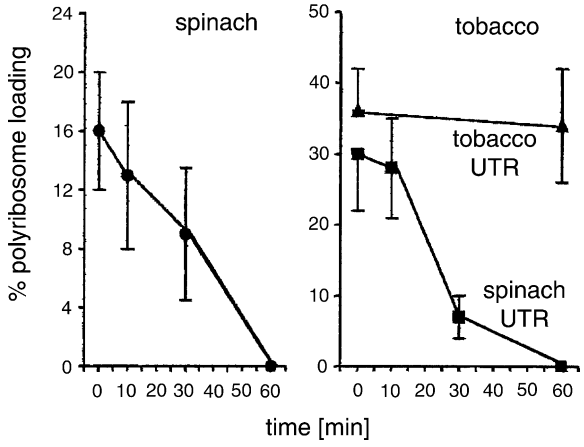

Figure 7. Kinetics of polyribosome association in light-grown seedlings 0, 10, 30 and 60 min after the application of DCMU.
(left) *PsaD* message in 7-day-old spinach seedlings.
(right) *PsaD* UTRs from either spinach (spinach UTR, i.e. construct C in Figure 3) or tobacco (tobacco UTR, i.e. construct G from Figure 3) fused to the β-glucuronidase gene in 12-day-old tobacco seedlings. After sucrose gradient centrifugation and RNA extraction from the individual fractions 1 (top) to 9 (bottom), Northern hybridization was performed with the spinach *PsaD* cDNA (left) or an *uidA*-specific gene fragment (right). Error bars represent SEs, based on three independent experiments.

Table 1 Photosynthetic efficiency of tobacco seedlings after DCMU treatment

	Control	10 μM DCMU
F_v/F_m	0.8035 ± 0.005	0.7345 ± 0.007
qP	0.8270 ± 0.02	0.544 ± 0.02
qN	0.156 ± 0.01	0.1955 ± 0.00
ϕ PSII	0.6415 ± 0020	0.375 ± 0.01

Growth of tobacco seedlings, DCMU application and the photosynthetic parameters (left) are described in Experimental procedures. Values represent means of three measurements each out of two independent experiments ($n = 6$; SEs are given).

Discussion

Our data indicate that the 5′-UTR of the spinach *PsaD* mRNA is sufficient to confer polyribosome loading onto the *uidA* message and that this regulation is dependent on photosynthetic electron flow (Figures 5–7). Furthermore, kinetic investigations suggest that the endogenous *PsaD* message in spinach and the transgene-derived message in tobacco respond with similar rapidity to DCMU treatment (Figures 6 and 7). Deletions of nucleotides at the 5′-end of the spinach *PsaD* message and mutations in the 3′-end of its UTR prevent polyribosome loading and/or translation (Figure 4). In contrast to previous reports for pea *Fed1* and *PetE*, the UTR of the spinach *PsaD* appears to be sufficient for such regulation, although it is unclear whether additional sequences within the coding region also contribute to this scenario. We have previously demonstrated that intron sequences are involved in light- and plastid-dependent expression of the spinach *PsaD* gene (Bolle *et al.*, 1996a); however, several approaches to demonstrate their involvement in polyribosome loading have failed so far, mainly because the intron appears to be only functional in its proper *PsaD* environment. We are currently performing linker-scanning experiments to clarify this question.

An unexpected result was that the *PsaD* genes from spinach and tobacco differ (Figures 4, 5 and 7). Unusual regulation and elements for both genes have been reported earlier (Bolle *et al.*, 1996a,b; Flieger *et al.*, 1994; Nakamura and Obokata, 1995; Yamamoto *et al.*, 1995a,b, 1997). The tobacco UTR, for instance, functions as a translational enhancer (Yamamoto *et al.*, 1995b), while the spinach UTR does not. The spinach gene contains an intron which is involved in plastid- and light-dependent expression (Bolle *et al.*, 1996a), while the tobacco gene lacks an intron, and regulated expression is mainly mediated via *cis*-elements in the promoter (Yamamoto *et al.*, 1997). These differences prompted us to compare both genes in greater detail. First, we demonstrate that UTRs from both genes direct polyribosome association of the *uidA* message (Figure 4). Polyribosome association directed by the

tobacco UTR is not regulated by light, consistent with previous observations that this UTR operates as a general translational enhancer (Yamamoto *et al*., 1995b). In contrast, the spinach *PsaD* UTR responds to light and limitations of the photosynthetic electron flow (Figures 4 and 5). Again, this is consistent with the hypothesis that terminal effectors of the light and plastid-dependent signal pathways operate via the same *cis* element(s). Comparable results have been reported for the pea *Fed-1* gene: constructs in which the *Fed-1* UTR was replaced by the Ω UTR resulted in loss of light regulation as well as increased, unregulated polyribosome loading (Dickey *et al*., 1998). The authors propose that light regulation of *Fed-1* might be caused by a differential translation of the message in light and darkness because of different polyribosome loading: free messages could be more susceptible to degradation than polyribosome-associated messages. This allows a fast and efficient acclimation of photosynthesis gene regulation to changing environmental conditions. The results obtained in this study show that it is likely that the tobacco *PsaD* UTR has a similar function as the Ω UTR. A rapid change in the distribution of mRNAs between smaller and larger polysomes has also been observed for many stress conditions: stress protein mRNAs accumulate in fractions with larger polysomes, while other mRNAs disappear from these fractions. Thus, the translation efficiency of individual mRNAs can be acclimated to environmental changes.

Interestingly, crucial *cis*-elements of the pea and spinach *PetE* genes are also located in different regions and operate at transcriptional and post-transcriptional levels. All *cis*-elements for the light- and plastid-dependent expression of the spinach *PetE* gene are located in the promoter region −259/−79, which is sufficient to activate a heterologous TATA box (Lübberstedt *et al*., 1994), while the UTR of the spinach *PetE* gene influences *uidA* gene expression only quantitatively (Bolle *et al*., 1996b). Conversely, the pea *PetE* UTR is essential for full light response (Helliwell and Gray, 1995), since gene fusion containing the pea *PetE* promoter fused to the *uidA* gene failed to direct proper light regulation in transgenic tobacco plants, while the complete *PetE* did (Pwee and Gray, 1993). Since gene constructs producing transcripts containing 14 bases of the 35S RNA CaMV UTR at the 5′-end of the *PetE* mRNA did not respond properly to light (Helliwell and Gray, 1995; Helliwell *et al*., 1997), the correct 5′-end of the *PetE* mRNA appears to be necessary for full light regulation. Furthermore, in addition to the correct 5′-end of the UTR, sequences within the coding region of the pea *PetE* gene are involved in regulated expression. They operate post-transcriptionally and respond to both light and plastid-derived signals.

A large number of studies have demonstrated that polyribosome association is crucial for the stability of an mRNA and that this is one target site for regulation (cf. Abler and Green, 1996; Sullivan and Green, 1993). In some cases, *cis*-elements in 5′-UTRs were defined or postulated which are involved in these responses (Bolle *et al*., 1994, 1996b; Caspar and Quail, 1993; Dickey *et al*., 1998; Yamamoto *et al*., 1995b); however, no coherent picture is available at present (cf. Dickey *et al*., 1998). Search for putative *cis*-elements in the spinach *PsaD* UTR which might be involved in regulation and which exhibit sequence similarities to elements known from other messages was unsuccessful. We are currently performing linker scanning experiments to define the crucial region in more detail. Alternatively, a secondary structure could be formed, which is recognized by regulatory proteins. An example for such regulation is provided by the γ-glutamylcysteine synthase mRNA, which is efficiently translated in response to cellular redox signals. A region in the UTR which is recognized by a protein has the potential to form a stem loop, and binding of a factor to the UTR is redox sensitive. However, secondary structure prediction programs with the spinach *PsaD* UTR sequence did not lead to any conclusive result.

The light response of the spinach *PsaD* message is mediated by the photosynthetic electron flow. The tobacco construct can be considered as a control, since messages from this construct did not respond to the limitation of electron flow (Figures 4 and 7). How this signal can be transduced from the plastids to the cytoplasm is unclear at present; however, this regulatory network provides another example for extraplastidic processes which are controlled by events initiating in the plastids. Recent studies with the mutants *gun5* and *laf6* suggest that chlorophyll precursors and their transport abilities accross the chloroplast envelope can influence the transcription of nuclear genes for plastid proteins (Mochizuki *et al*., 2001; Møller *et al*., 2001), a hypothesis which has been suggested earlier (Johanningmeier and Howell, 1984; Kropat *et al*., 1997). It is conceivable that cytoplasmic events are controlled by the same signaling systems.

Polyribosome loading triggered by photosynthesis has also been described for the *Fed-1* gene (Petracek *et al*., 1997). It remains to be determined whether other photosynthesis mRNAs are also regulated by this mechanism. Petracek *et al*. (1997) analyzed light-regulated polyribosome association of messages for the light-harvesting chlorophyll *a/b*-binding protein and found that, similar to *Fed-1*, these transcripts also responded to the photosynthetic electron flow. This is consistent with previous reports by Escoubas *et al*. (1995). More recently, studies with transgenic plants harboring promoter::*uid*A gene fusions in combination with light sources preferentially exciting either photosystem I or II (Pfannschmidt *et al*., 1999) provided evidence that transcription of several nuclear-encoded genes for photosynthesis or photosynthesis-related proteins including photosystem I genes is controlled by redox signals from the plastids (Pfannschmidt *et al*., 2001;

Sherameti *et al.*, 2002). Nevertheless, many questions remain to be answered. Besides the signal transduction process in our system, the origin of this signal also is unclear.

Experimental procedures

Plant growth

Transgenic tobacco seedlings were either kept in darkness or white light for 12 days on half-strength Murashige and Skoog (1962) medium in a temperature-controlled growth chamber (22°C). Plant material was then harvested and immediately frozen in liquid nitrogen. Spinach (*Spinacea oleracea*, var. Monatol) seeds were germinated on vermiculite in white light at 20–22°C. After transfer to darkness or white light for 7 days, they were used for experimentation.

Transgenic plants and gene constructs

Transgenic lines with the 6-kbp spinach *PsaD* fragment have been described by Flieger *et al.* (1994) and Bolle *et al.* (1996a). Additional constructs introduced into tobacco are schematically shown in Figure 3. Transgenic tobacco lines with the constructs (A) and (C) (Bolle *et al.*, 1996a; Flieger *et al.*, 1994) (E) (Jefferson *et al.*, 1987) and (G) (Yamamoto *et al.*, 1997) have also been described. In brief, construct (A) contains a *PsaD* fragment with 1802 bp upstream of the transcription start site, plus the 50 bp-long UTR and additional five nucleotides of the coding region, fused to the *uidA* gene (Flieger *et al.*, 1994). In construct (C), the sequences upstream of the transcription start site of *PsaD* were replaced by the 35S RNA CaMV promoter (Bolle *et al.*, 1996a). The constructs (B), (D) and (F) were generated by PCR. Starting from the constructs (A) and (C), the respective DNA fragments for constructs (B) and (D) were amplified by PCR, the amplification products were ligated together and cloned into the pGEM-T Easy vector (Promega, Madison, USA). The construct (B) differs from (A) in that it lacks the *PsaD* UTR region +4/+32 relative to the transcription starts site of *PsaD* (i.e. the *PsaD* fragment −1802/+3 was fused to a fragment which contains the *PsaD* coding region plus 17 nucleotides upstream of the ATG codon). In construct (D), the latter fragment was fused to the 35S RNA CaMV promoter segment −1500/+3, and for construct (D) the *PsaD* segment −1802/+3 was fused to the *uidA* gene. pGEM-T Easy plasmids were completely sequenced from both sites to confirm the right orientation and junction of the two combined DNA fragments. The tobacco *PsaDb* construct (G) was generated by inserting the *PsaDb* promoter sequence plus 23 bp for the UTR into the *Pst*I/*Bam*HI site of pBI101. The resulting mRNA contains the full-length *PsaDb* UTR followed by the pBI221 sequence downstream of the *Bam*HI site including the *uidA* gene (Yamamoto *et al.*, 1997). Site directed mutageneses for the constructs (H)–(J) were performed with an *Eco*RV fragment of the spinach *PsaD* gene (Bolle *et al.*, 1996a; Flieger *et al.*, 1994) as described by Mikaelian and Sergeant (1992). The *Eco*RV fragment contains the entire sequence for the transit peptide plus 50 nucleotides upstream of the codon for initiator methionine. After site-directed mutagenesis, the *Eco*RV wild-type fragment of the 6 kbp-long spinach *PsaD* fragment (cf. Figure 1; cf. Bolle *et al.*, 1996a; Flieger *et al.*, 1994) was replaced by the mutant fragments.

Polyribosome analysis, hybridization conditions

Polyribosome analysis was performed essentially as described by Davies and Abe (1995) and modified by Dickey *et al.* (1998). Sucrose gradients (20–60%) were prepared in Beckman Ultra Clear SW 40 Tl tubes (10 ml each) and kept at 4°C. Samples of 0.2 g of plant material was frozen in liquid nitrogen and homogenized with a Micro-Dismembrator S (Braun Biotech International). After transfer to Eppendorf tubes, 1.25 ml of buffer U (200 mM Tris–HCl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 100 g ml^{-1} heparin, 2% polyethylene 10 tridecyl ether, 1% deoxycholic acid) was added. The cell debris was removed by centrifugation (15 000 × *g*; 10 min, 4°C) and 1 ml of the supernatant was loaded on the sucrose gradients, centrifuged in a SW 40 Tl swinging bucket rotor (Beckman) at 72 000 ***g*** for 3 h at 4°C. The gradients were fractionated by puncturing the bottom of the tube with a needle. 1.2 ml fractions of the gradient were collected in an equal volume of phenol/chloroform, the proteins were extracted, and the RNA was precipitated with ethanol and dried. The RNA was re-dissolved in 100 µl loading buffer (18% deionized formamid, 2 mM EDTA, pH 7.5, 0.02% Bromphenol blue, 0.002% Xylencyanol) and 30 µl was separated on a MOPS gel (20 mM MOPS, pH 7.0, 5 mM NaAc, 1 mM EDTA) before transfer of the RNA to nylon membranes. Hybridization under stringent conditions (Sambrook *et al.*, 1989) was performed with gene-specific fragments: an *Eco*RV fragment of spinach *PsaD* which contains the entire sequence for the transit peptide plus 50 nucleotides upstream of the ATG initiator methionine, and a *Sma*I/*Sac*I fragment of the *uidA* gene isolated from pBI101.2 (Jefferson *et al.*, 1987). The hybridization signals were detected by a phosphorimager (Molecular Dynamics, Model 802). For the Northern analysis shown in Figure 1, homologous gene-specific cDNA fragments from spinach were used.

Chlorophyll fluorescence measurements

In vivo Chl *a* fluorescence parameters were measured with a pulse amplitude modulated fluorometer (PAM 101/103, Heinz Walz, Effeltrich, Germany). Tobacco seedlings were set together as a dense population on a Petri dish prior to measurements. Fluorescence parameter were determined as described earlier (Pfannschmidt *et al.*, 2001). The optimal quantum yield of photosystem II was calculated as F_v/F_m. Fluorescence quenching parameter qP (photochemical quenching) was calculated as $qP = (F_{m'} - F_s)/(F_{m'} - F_o)$ and qN as $1 - (F_{m'} - F_{o'})/(F_m - F_o)$ (Schreiber *et al.*, 1986). The effective quantum yield of PSII (ϕ PSII) was calculated as ϕ $PSII = (F_{m'} - F_s)/F_{m'}$ (Genty *et al.*, 1989).

3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU) treatments

The electron transport inhibitor DCMU (Sigma) was applied to the plants grown in Petri dishes by spraying 0.5 ml of a 10 µM solution on the leaves using a 10-ml fine-sprayer. Control seedlings were treated with the solvent without inhibitors. Wild-type seedlings were illuminated with white light until Chl fluorescence measurements were performed. A stock solution of DCMU was 10 mM in 50% ethanol. The inhibitor solution was prepared by dilution of the stock solution with sterile water (tobacco) directly before use (cf. Pfannschimdt *et al.*, 1999, 2001). For the experiments with spinach seeds, non-sterile conditions were used. After RNA extraction, hybridizations were performed with spinach cDNAs for *PsaD*, *PsaF* and *PsaL* (Herrmann *et al.*, 1991). Quantification of the data presented in Figure 7 was performed such that the total intensity of the

hybridization signals of one sucrose gradient was taken as 100% and the percentage of signals detectable at sucrose concentrations >35% was considered as polyribosome-associated mRNAs. Error bars in Figure 7 refer to three independent experiments.

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