Characterization of plastid psbT sense and antisense RNAs

Ouafa Zghidi-Abouzid, Livia Merendino, Frank Buhr, Mustafa Malik Ghulam and Silva Lerbs-Mache*

Laboratoire de Physiologie Cellulaire Végétale, UMR 5168, CNRS/UJF/INRA/CEA, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex, France

Received December 10, 2010; Revised February 23, 2011; Accepted February 24, 2011

ABSTRACT
The plastid psbB operon is composed of the psbB, psbT, psbH, petB and petD genes. The psbN gene is located in the intergenic region between psbT and psbH on the opposite DNA strand. Transcription of psbN is under control of sigma factor 3 (SIG3) and psbN read-through transcription produces antisense RNA to psbT mRNA. To investigate on the question of whether psbT gene expression might be regulated by antisense RNA, we have characterized psbT sense and antisense RNAs. Mapping of 5' and 3'-ends by circular RT–PCR and /or 5'-RACE experiments reveal the existence of two different sense and antisense RNAs each, one limited to psbT RNA and a larger one that covers, in addition, part of the psbB coding region. Sense and antisense RNAs seem to form double-stranded RNA/RNA hybrids as indicated by nuclease digestion experiments followed by RT–PCR amplification to reveal nuclease resistant RNA. Western immunoblotting using antibodies made against PSBT protein and primer extension analysis of different plastid mRNA species and psbT antisense RNA suggest that sequestering of psbT mRNA by hybrid formation results in translational inactivation of the psbT mRNA and provides protection against nucleolytic degradation of mRNA during photo-oxidative stress conditions.

INTRODUCTION
The existence of many non-coding RNAs (ncRNAs) and different regulatory pathways mediated by antisense RNA base-pairing induced mechanisms have been discovered during the last years. Most of this work concerns eukaryotic nucleus-encoded natural antisense transcripts (NATs) and ncRNAs in bacteria (1–3). Much less is known on ncRNAs and regulatory antisense mechanisms in organelles like mitochondria and chloroplasts.

The presence of ncRNAs in chloroplasts and mitochondria of a higher plant has first been demonstrated by a general analysis after cDNA cloning of small (50–500 nt) RNAs in Arabidopsis (4). Later on cloning of cDNAs corresponding to small plastid ncRNAs from tobacco revealed 11 candidates for plastids, 2 of them oriented in antisense direction to known plastid genes (5). These RNAs are very short (comprising between 19 and 53 nt) and most of them are located in intergenic regions. No function has been attributed to them until now and it cannot be excluded that at least some of them are just processing intermediates. A long antisense RNA, complementary to the reading frame of the ndhB gene, has been recently described. The RNA starts within the reading frame of the ndhB gene, the sequence covers two editing sites of the ndhB gene and a group II intron splice acceptor site, but no function has been attributed to this RNA, neither (6). First indication for the function of a plastid natural antisense RNA has very recently been obtained for AS5 by over-expression of the RNA via plastid transformation in tobacco (7). Results indicate a decrease in stability of SS rRNA by AS5 over-expression. However, secondary effects due to plastid transformation cannot be excluded, e.g., the transgene insertion leads to the accumulation of some precursor transcripts.

In the present article, we have characterized plastid psbT sense/antisense RNAs. The psbT gene is part of the psbB operon (composed of psbB, psbT, psbH, petB and petD, 8) where a single gene (psbN) is located between the psbT and psbH genes, but on the opposite strand of the DNA (9). Transcription of the psbN gene is under control of sigma factor 3 (SIG3) and part of the transcription complexes proceed transcription at the end of the psbN gene and produce antisense RNA to the psbT mRNA. Only two genes seem to be specifically transcribed by SIG3-PEP holoenzyme, i.e. psbN and atpH (10). While

*To whom correspondence should be addressed. Tel: +33 (0)4 38 78 05 69; Fax: +33 (0)4 38 78 50 91; Email: silva.lerbs-mache@ujf-grenoble.fr; silva.mache@cea.fr

© The Author(s) 2011. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
psbN is exclusively transcribed by SIG3-PEP, atpH is also transcribed as polycistronic RNA together with all other genes of the large ATPsynthase operon and the lack of SIG3-specific atpH transcription does not change the ATPH protein level (Malik Ghulam, M. et al., unpublished data). A SIG3 knock-out plant provides, therefore, a unique tool to elucidate the function of psbN initiated psbT antisense RNA. The psbT antisense RNA is of particular interest because this antisense RNA is not restricted to the 5' or 3' UTR of the sense mRNA. In bacteria, ncRNAs generally base pair to mRNAs in their 5'- or 3'-UTR and the corresponding formation of RNA duplexes modifies translation efficiency and/or stability of the corresponding RNA. In the case of psbT, the antisense RNA covers the whole psbT coding region, extending at least up to the ATG translation initiation codon, as previously shown by a primer extension (PE) experiment (Figure 4B, left hand side, in 10). Furthermore, a psbB/psbT transcript had been revealed by PE having its 5'-end within the coding region of the psbB gene indicating either degradation of psbB/psbT co-transcripts or the possibility of decoupling of psbT transcription from read-through transcription of the psbB operon (Figure 4B, right hand side, in 10). These peculiarities stimulated us to characterize psbT sense and antisense RNAs in more detail with the hope to get some ideas for an eventual role of psbT antisense RNA in regulation of gene expression.

A mechanism in which entire genes are transcribed in sense and antisense orientation has so far been analysed in nuclear transcription where it was shown that RNA polymerase II complexes collide during transcription and stall each other or that transcription from a strong promoter hinders the transcription from a weaker promoter. This mechanism is known as ‘transcriptional interference’ (11,12). Regarding the high copy number of plastid genomes it is not very likely that such a mechanism exists in plastids. Also, concerning transcription of psbN and psbT, we could not detect changes in psbT transcript levels in sig3 mutants, i.e. the absence of transcription from the psbN promoter does not change the quantity of psbT transcripts (Figures 3B and 4B on the right hand side, in 10). Therefore, if psbT sense/antisense RNA transcription is connected to any kind of regulation, it is probably not a mechanism that implies transcriptional interference.

Two types of RNA polymerase are engaged in the transcription of the plastid genome: the phage-type NEPs (nucleus-encoded plastid RNA polymerases) that are of special importance during early plant development and the eubacterial type PEP (plastid-encoded plastid RNA polymerase) that transcribes preferentially photosynthesis-related genes during later developmental stages (13,14). For correct initiation of transcription PEP needs to associate with transcription initiation factors of the sigma type (15–17) and psbN transcription is specifically controlled by SIG3. As none of the transcripts of the psbB operon changes in a sig3 plant, we can conclude that all these RNAs are made by PEP in association with another sigma factor than SIG3 (10). Taken all together, psbT sense and antisense RNAs should be synthesized differently by different PEP–sigma combinations and it should be more likely that a regulatory mechanism acts via RNA/RNA hybrid formation than via transcriptional interference.

To test this hypothesis, in the present article, we have determined the 5'- and 3'-ends of psbT sense and antisense RNAs in order to characterize possible RNA/RNA hybrids, we have analysed whether RNA/RNA hybrids do exist and we have determined the consequence of the absence of psbT antisense RNA on the PSBT protein level.

MATERIALS AND METHODS

Plant material and RNA isolation

Surface-sterilized Arabidopsis seeds were spread on MS agar plates, kept for 72 h at 4°C in darkness and then transferred into a growth chamber and grown for 6 days at 23°C under 16/8 h light/dark cycle at 110 µmol of photons m⁻² s⁻¹. Total RNA was prepared from seedlings as described in Privat et al. (18). Briefly, frozen material of plants was ground in a mortar and the powder was suspended in three volumes of solution A (10 mM Tris–HCl pH 8; 100 mM NaCl; 1 mM EDTA; 1% SDS). After two phenol–chloroform and one chloroform extractions, RNAs were separated from the bulk of DNA by precipitation in 2 M LiCl overnight and then RNAs were precipitated with ethanol.

Primer extension

The PE experiment has been performed as described in Favory et al. (19) using 10 µg of total RNA. Upon denaturation at 65°C, total RNAs were annealed with 100 nmol of 5’ ³²P-labelled primer and then retro-transcribed in the presence of 100 U of SuperScript II (Invitrogen) at 42°C according to manufacturer’s protocol. RNAs were digested with 10 µg of RNase A. Before cDNAs were purified by phenol/chloroform treatment and ethanol precipitation, a ³²P-labelled PCR product was added to the reaction as loading control. The cDNAs were separated on a 6% polyacrylamide denaturing gel in parallel with the sequencing reaction that had been performed with the same primer. The following primers were used for PE: 5’-ATGGAAACAGCA ACCCTAGTC-3’ (psbN antisense, Primer 9), 5’-CATAT TGCCCCTCTGACAG-3’ (atpI), 5’-TTCATAGTGGCATTGC TACT-3’ (rrn 16S), 5’-GATGTATCTCCTTCTCTCCA GG-3’ (clfP), 5’-GTCCAATAGAAGCAAGC-3’ (atpH), 5’-ATGGAAGCATTGGTTTATAC-3’ (psbT antisense, Primer 1).

5’-RACE

The discrimination between transcription start sites and processing sites of the psbT precursor RNAs was done by RNA Ligase Mediated Rapid Amplification of cDNA Ends [first choice RLM-rapid amplification of cDNA ends (RACE) kit, Ambion] without and with previous tobacco acid pyrophosphatase (TAP) treatment of RNAs. Reactions were performed according to the supplier’s protocol but without removal of free 5’-phosphates.
by Calf Intestine Alkaline Phosphatase. PCR products were analysed on Agarose gels after two successive PCR amplifications using at first two outer primers and the second two inner primers. Primers are as follows: psbT (8) 5'-GAGGGCTGATTACGTATATGAAG-3', psbT (7) 5'-GAAATTTCAGTTGGTCCC-3', psbT (6) 5'-ATC CCTAAAAGTGGATACTAGAG-3', psbT (5) 5'-GAG TCAAAGAACCGGCAACG-3'. The inner and outer adapter primers are those of the RLM-RACE Kit.

Circular RT–PCR

In order to map the extremities of the psbT antisense transcripts, the first choice RLM-RACE kit (Ambion) was used. Total RNAs were first incubated with TAP and then self-ligated. About 400 ng of RNAs were retro-transcribed using Primer (2) 5'-ATGGAACGATT GGTTTATA-3' as gene-specific primer and the SuperScript II enzyme. Subsequent PCR amplification was performed in the presence of primer (3) 5'-ATTCCT AAAATGGATACTAAG-3' and primer (4) 5'-CGGGA ACCAATTTAAAATTCC-3'. Reactions without TAP and without RT reaction were carried out as controls. The produced cDNAs have been cloned into pCR<sup>®</sup>2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) and sequenced.

Antibody production and characterization

Antibodies against the PSBB and PSBH proteins of Photosystem II (PSII) have been obtained from Agrisera. Antibodies against nucleus-encoded plastid ribosomal protein L4 have been described earlier (20). Peptide antibodies against PSBT have been produced by Eurogentec using the following peptide: AIFFREPPKIS TKK.

Protein purification and western blot analysis

A 200 mg of plant material were frozen-grounded and resuspended in 200 µl of protein loading dye. After boiling for 10 min, protein extracts were cleared by full speed centrifugation in a micro-centrifuge. Equal amounts of protein extracts were separated by SDS–PAGE and transferred to nitrocellulose membranes. For immunodetection antibodies were diluted 1 : 1000 (PSBT), 1 : 5000 (PSBH) and 1 : 2000 (PSBB) and revealed by the ECL detection kit (GE Healthcare).

Characterization of double-stranded RNA

Total RNA has been treated with DNase (Ambion) according to the manufacturer’s instructions until no traces of DNA could be detected by PCR analysis. DNA-free total RNA (1 µg) has been digested with Nuclease S1 at different concentrations (28) at room temperature for 30 min. Nuclease S1 was inactivated by addition of EDTA (final concentration 1.6 mM) and incubation at 75°C for 15 min. Reverse transcription of the different mRNAs was carried out using 100 ng RNA from the Nuclease S1 assay, SuperScript II (Invitrogen) and gene-specific primers according to the manufacturer’s protocol. A 1 µl of the cDNA reaction was taken for further PCR amplification using Primer 1 and Primer 5'-GAGGGCTGATTACGTATATGAAG-3' for psbT amplification and Primers 5'-TGATATT ATGGATGACTGGTACC-3' and 5'-TGCAACCTTCTAAATAGGAACTGG-3' for psbD amplification.

RESULTS

Mapping of 3'-ends of psbT antisense transcripts

By using a primer that starts at the ATG translation initiation codon of the psbT gene, we have recently mapped the 5'-end of the psbT antisense transcript by PE. We have shown that the psbT antisense RNA arises from a long PpsbN initiated transcript that is cleaved in the intergenic region between psbT and psbN (10). This cleavage that provides the 5'-end of the psbT antisense RNA is illustrated in Figure 1A. The dotted line represents the PpsbN initiated transcript and the cleavage site is indicated by the vertical open arrow with the scissor (PE, 1).

In order to determine now the 3'-ends(s) of the psbT antisense transcript, we performed circular RT–PCR (cRT–PCR) analyses (21). The principle of the method is schematized on the left hand side in Figure 1B. After joining 5'- and 3'-ends of the RNAs by treatment with RNA ligase, circularized RNAs have been reverse transcribed using Primer 2 and the produced cDNAs have been PCR amplified with Primers 3 and 4. The sequences surrounding the 5' and 3' joining points reveal the 5'- and 3'-ends of the antisense RNAs. The reaction resulted in two different PCR products (Figure 1B, right hand side) indicating the existence of two different antisense RNAs. The sequencing of both PCR products showed that both antisense RNAs have the same 5'-end corresponding to the 5'-end of the psbT antisense RNA as determined previously by PE using Primer 1 (10 and Figure 1A). However, the 3'-ends are different. The 3'-end of the 'a' transcript is situated within the coding region of the psbB gene and the 3'-end of the 'b' transcript is located in the intergenic region between psbB and psbT. The sequences at the 3'-ends of the 'a' and the 'b' antisense RNAs are reported in Figure 1C.

Mapping of 5'-ends of psbT sense transcripts

Our previous PE experiment had revealed two different psbT transcripts, one with a 5'-end located in the intergenic region between psbT and psbB and a longer transcript with a 5'-end positioned within the psbB coding region (10). These transcripts are reported by dotted lines in Figure 2A (Primer 7, PE). From the PE experiment, it was not clear whether these RNAs are made by transcription initiation or by processing of a PpsbB-initiated multicistronic mRNA. To answer this question, we have performed 5'-RACE experiments with and without prior treatment of RNAs with TAP. By using either Primers 7 and 6 or Primers 8 and 6 (for primer localization see Figure 2A), we obtained a single-PCR product, and this product was only obtained after TAP treatment showing that this transcript is made by transcription initiation (Figure 2B). The corresponding cDNA has been cloned and sequenced and the location...
of the transcription start site, in the intergenic region between psbB and psbT, is indicated in Figure 2D as PpsbT.

When compared with our previous result obtained by PE (labelled as 154 RNAs in Figure 4B and C, in 10 and in Figure 2D), we realize a small difference between the 5'-ends as revealed by PE and by 5'-RACE. We interpret this difference by rapid nucleolytic removal of the first 9/10 nt at the 5'-end of the PpsbT-initiated mRNA immediately after synthesis. This is in agreement with the fact that PE experiments always showed several cDNAs of slightly different length, a fact that indicates nucleolytic attack at the 5'-end. An explanation for the sensitivity to nucleolytic digestion of the PpsbT-initiated mRNA at its 5'-end is provided by the secondary structure of this RNA (Supplementary Figure S1). It shows formation of a hairpin at the 5'-end of the mRNA that does not include the nucleotides between positions PpsbT and (154) (Figure 2D). This hairpin structure might stabilize the 5'-end of the psbT mRNA and protect the mRNA from further nuclease digestion. This structure might also explain why we did not detect the shorter transcripts by 5'-RACE. Probably, ligation will not be successful on 5'-ends that are base-paired within a hairpin structure.

For exact mapping of the 5'-end of the longer psbT transcript that is located within the coding region of the psbB gene (Figure 2A, dotted lines, 7 PE), we needed to use an inner primer that is located close to the psbB gene (Primer 5 in Figure 2A). 5'-RACE shows the existence of several RNAs and only one of them is made by transcription initiation (Figure 2C). This transcript is much longer than that expected for the PE product. Cloning of the corresponding cDNA revealed a promoter that is located within the coding region of the psbB gene. The sequence surrounding this transcription start site is shown in Figure 2D. The other cDNAs of the 5'-RACE
experiment that do not result from transcription initiation indicate the existence of several cleavage intermediates of the psbB mRNA. These have not been further analysed. The results obtained here indicate the existence of two different psbB/psbT co-transcripts, one initiating at PpsbB and the other one initiating at PpsbBi (Figure 2A). The length of these two transcripts correspond well to the two RNAs of about 2000 and 1000 bases that have been revealed by Northern hybridization in our previous paper (10).

Mapping of the 3′-end of the psbT sense transcript

In order to get an idea of the 3′-end of the psbT sense transcripts, we next analysed the psbT/psbH intergenic region by PE. This should reveal the 5′-end of the cleavage product of the psbT/psbH co-transcript (Figure 3). We used a primer that was located at the beginning of the psbN gene as indicated in Figure 3A (Primer 9). To test whether the absence of antisense RNA influences the processing in the psbT–psbH intergenic region, we analysed RNA prepared from wild type (WT) the SIG3 knockout mutant (Δ3) in this experiment. We observed three different cDNAs, labelled with (a) and (*) in Figures 3A and B. All three cDNAs are also found in the sig3 plants (compare Lanes 5 and 6 in Figure 3B) showing that the corresponding processing events are not disturbed by the absence of antisense RNA. The 5′-end of the shortest RNA could be localized with the accompanying sequence ladder. It is positioned within a perfect hairpin structure that could form within the intergenic region between psbT and psbN. The exact location in the nucleic acid sequence is reported in Figure 3C. Interestingly, the 5′-end of the psbT antisense RNA that had been determined by cRT–PCR (Figure 1A) and by PE (10) is located in the complementary hairpin (labelled with PE and cRT in Figure 3C). The two longer transcripts (asterisks) have not been observed in all RNA preparations and have not been mapped.

Characterization of sense/antisense double-stranded RNAs

Our mapping of 5′- and 3′-ends of psbT sense and antisense RNAs offers several possibilities for RNA/RNA hybrid formation. The 3′-ends of the sense and antisense RNAs are well defined and seemed to be stable. Equally, the 5′-end of the antisense RNA could be determined with precision. However, the 5′-end(s) of the psbT sense RNAs that comprise part of the psbB coding region are divergent and multiple degradation intermediates seem to exist (Figure 2C). The two most likely possibilities of sense/antisense hybrids are schematized in Figure 4A. We assume that PpsbBi initiated transcripts that are not protected by hybrid formation will be degraded from the 5′-end and this gives rise to the degradation intermediates that we observe in Figure 2C. The complete RNA sequences of the short psbT sense/antisense RNA hybrid are shown in Figure 4B. The estimated location of a chloroplast ribosome on the single-stranded psbT sense mRNA as deduced from the work of Kim and Mullet (22) is indicated by the open circle above the sequence.

It shows that the single-stranded mRNA could be protected from 5′ nucleolytic attack by an initiating ribosome, but only up to the 5′-end that had been revealed by PE (vertical flashes on the sequence). The first 10 nt of the PpsbT-initiated mRNA would not be protected by an initiating ribosome. Thus, in addition to the secondary structure of free psbT mRNA (see Supplementary Figure S1) initiating ribosomes could also protect the 5′-end from degradation, except for the first 10 nt.

Do psbT RNA sense/antisense hybrids exist in vivo?

Having shown the existence of psbT sense and antisense transcripts, it was of interest to know whether RNA/RNA double strands do indeed form in vivo. This question was examined by treating total RNA with the single-strand-specific nucleases Nuclease S1. If sense/antisense RNA hybrids exist the RNAs that are engaged in hybrid structures should be protected from nuclease digestion. In contrast, mRNA that is not protected by antisense RNA should be totally degraded.

Figure 3. Mapping of the 3′-end of the psbT sense transcript. (A) Schematic representation of a part of the psbB operon and the psbN antisense transcript (a) as determined by PE using Primer 9. The horizontal arrow indicates the localization of the primer. (B) PE analysis of psbN antisense RNA performed with Primer 9. The accompanying sequence ladder has been established by using the same primer. The revealed cDNA (a) is schematically demonstrated in (A). (C) Localization of the cleavage sites of psbT sense/psbN antisense (upper hairpin) and psbN sense/psbTantisense RNAs (lower hairpin).
We have analysed \( psbT \) mRNA in WT and \( sig3 \) plants and \( psbD \) mRNA in WT plants taken as control without and after treatment with several concentrations of Nuclease S1 (Figure 4C). The \( psbD \) control mRNA was no more detectable when the nuclease concentration was 0.2 U/\( \mu l \) or higher (Lanes 11–14). In WT plants the \( psbT \) mRNA level diminishes after treatment with 0.2 U/\( \mu l \) of nuclease, but then remains stable even after treatment with a 10× higher nuclease concentration (Lanes 2–5). In contrast, in the \( sig3 \) mutant the \( psbT \) mRNA disappears completely after high nuclease treatment (Lanes 6–10). This result strongly suggests that \( psbT \) sense/antisense hybrids exist in vivo.

**Functional characterization of \( psbT \) sense/antisense hybrids**

The question of the function of the \( psbT \) antisense RNA can best be analysed by using the \( SIG3 \) knockout mutant. In the absence of \( SIG3 \) the \( psbN \) gene is not transcribed and \( psbT \) antisense RNA cannot be produced. In the following, we have analysed the protein levels of PSBT and of the adjacently encoded PSBB and PSBH proteins in WT and \( \Delta SIG3 \) plants (Figure 4D). The amount of the nucleus-encoded plastid ribosomal protein L4 has been analysed as loading control. In the absence of \( psbT \) antisense RNA, we observe a remarkable augmentation of the PSBT protein level. This augmentation is restricted to PSBT. The PSBB and PSBH protein levels are not influenced by the absence of \( psbT \) antisense RNA. This result can be interpreted in that \( psbT \) mRNA that is engaged in a sense/antisense double-stranded RNA hybrid cannot be translated. In the absence of antisense RNA, the quantity of translation competent free single-stranded \( psbT \) RNA increases and more PSBT protein can be produced.
DISCUSSION

We have recently shown that SIG3-PEP-specific transcription initiation at the plastid psbN gene produces antisense RNA to the psbT mRNA (10). In these experiments, antisense RNA was revealed by PE using a primer that corresponds to the 5'-end of the psbT mRNA and the result showed that the antisense RNA covers the entire coding region of the psbT mRNA. In the present article, we have characterized the psbT sense and antisense RNAs in more detail. The determination of RNA 5'- and 3'-ends by either 5'-RACE or cRT–PCR indicates the existence of two different sense and antisense RNAs, one covering only the psbT gene area and the other, longer, one covering in addition a large part of the psbB-coding region (Figures 1–3).

The existence of these four different RNAs offers in principal four different possibilities of RNA/RNA hybrid formation. Only the two more likely to occur in a stable form are schematized in Figure 4A. Hybridization of a short with a long RNA cannot be excluded, but it would leave a large part of the long RNA in single-stranded form, more easily susceptible to nucleolytic degradation. Indeed, a long psbT sense RNA seems to be subject to extensive 5' degradation. This is indicated by the existence of multiple intermediary RNAs as shown in Figure 2C. However, it is not possible to distinguish degradation products of PpssB initiated transcripts from degradation products of PpssBi initiated transcripts. In principle, antisense RNA could also hybridize with PpssB-initiated mRNA. However, in this case, double-strand formation should interfere with psbB mRNA translation and we should have found an increase of the PSBB protein level in the absence of antisense RNA in the western experiment (Figure 4D). As this is not the case, our results rather indicate that all long antisense RNA is sequestered in double strands by hybridization with PpssBi initiated sense transcripts. The absence of antisense RNA could then liberate the PpssBi initiated sense transcripts that might be either degraded or translated from the psbT ATG translation start codon. Another explanation would consist in that the long psbT/psbB antisense RNA is present in a very low amount and its absence does not change the PSBT protein level.

If we regard now, the short sense/antisense RNAs, we see that after formation of the double-strand ribosome association to the sense psbT RNA should be impeded (Figure 4B) and the RNA should not be translatable. Translatable psbT mRNA should be liberated in the absence of antisense RNA. This hypothesis seems to be proven by the analysis of the PSBT protein in WT and sig3 plantlets that shows a considerable increase of the protein in the absence of antisense RNA (Figure 4D). From this result, it becomes clear that differences in the amount of psbT antisense RNA will influence the PSBT protein level.

If we regard now, the short sense/antisense RNAs, we see that after formation of the double-strand ribosome association to the sense psbT RNA should be impeded. Translatable psbT mRNA should be liberated in the absence of antisense RNA. This hypothesis seems to be proven by the analysis of the PSBT protein in WT and sig3 plants that shows a considerable increase of the protein in the absence of antisense RNA (Figure 4D). From this result, it becomes clear that differences in the amount of psbT antisense RNA will influence the PSBT protein level. Translatable psbT mRNA should be liberated in the absence of antisense RNA. This hypothesis seems to be proven by the analysis of the PSBT protein in WT and sig3 plants that shows a considerable increase of the protein in the absence of antisense RNA (Figure 4D). From this result, it becomes clear that differences in the amount of psbT antisense RNA will influence the PSBT protein level.

If we regard now, the short sense/antisense RNAs, we see that after formation of the double-strand ribosome association to the sense psbT RNA should be impeded (Figure 4B) and the RNA should not be translatable. Translatable psbT mRNA should be liberated in the absence of antisense RNA. This hypothesis seems to be proven by the analysis of the PSBT protein in WT and sig3 plants that shows a considerable increase of the protein in the absence of antisense RNA (Figure 4D). From this result, it becomes clear that differences in the amount of psbT antisense RNA will influence the PSBT protein level. Translatable psbT mRNA should be liberated in the absence of antisense RNA. This hypothesis seems to be proven by the analysis of the PSBT protein in WT and sig3 plants that shows a considerable increase of the protein in the absence of antisense RNA (Figure 4D). From this result, it becomes clear that differences in the amount of psbT antisense RNA will influence the PSBT protein level.

To approach this question, we need to consider the production and the function of the psbT protein. Actually, there are three different hypotheses for the function of PSBT. (i) PSBT is required for efficient repair of
photodamaged PSII reaction centres (23), (ii) PSBT plays an important role in dimerization of PSII (24) and (iii) PSBT is required for efficient biogenesis of PSII complexes (25). Interestingly, the crystal structure of PSII shows that PSBT is localized at the PSII dimer interface, consistent with a role in dimerization (26). The kinetics of protein accumulation during greening is different for PSBT when compared with other PSII proteins and the absence of PSBT does not affect the synthesis of other PSII proteins (25). This indicates that PSBT synthesis is differently regulated than other PSII proteins. The mechanisms of this regulation are not known, but it is tempting to speculate that psbT expression is at least partly regulated by an antisense RNA pathway.

In principal, the production of antisense RNA could help to diminish the psbT protein level under some physiological conditions. Our results show that antisense transcription diminishes the level of PSBT protein (Figure 4D). However, diminution of sense RNA availability for translation as prevailing mechanisms of antisense RNA production does not make much sense with regard to the two promoters that are found upstream of the psbT gene (Figure 2). Another possible function of the antisense RNA might be protection of the sense RNA from nucleolytic degradation under some adverse physiological conditions. That psbT sense/antisense RNA hybrids do really exist in vivo is shown in Figure 4C and RNA/RNA hybrids are known to be very stable (27). Furthermore, a protecting effect against mRNA 3′-end degradation by antisense RNA has already been shown in chloroplasts of Chlamydomonas (28). As already mentioned, PSBT is required for repair of photodamaged PSII reaction centres (27). To get a first idea whether mRNA protection might be necessary during photooxidative stress, we have analysed several mRNAs by PE before and after exposure of 7-day old normally grown Arabidopsis plantlets to high light condition (1300 μE) for 4 h (Figure 5). We have analysed atpH (Lanes 1 and 2), 16S (Lanes 3 and 4) and atpH (Lanes 7 and 8) precursor RNAs as examples for PEP transcripts and clp2 (Lanes 3 and 6) precursor RNA as example for a NEP transcript. In all cases, transcript levels diminish strongly after exposure to high light indicating rapid degradation of all these mRNAs under light stress conditions. An exception represents the psbT antisense RNA for which the RNA level remains rather stable (Lanes 9 and 10). If psbT antisense RNA is protected from nucleolytic attack by double-strand formation with psbT sense RNA, the part of the psbT sense RNA that is sequestered in the double strand, should also be protected from degradation. Consequently, the amount of psbT antisense RNAs could determine the amount of psbT sense RNA that is protected during photooxidative stress. Such a mechanism raises the question of how the sense RNA is made available for translation after the stress.

Altogether, our results suggest two possible functions for psbT antisense RNA: inhibition of translation of sense RNA and/or protection of sense RNA from nucleolytic degradation during stress conditions. Both mechanisms might not be mutually exclusive. Although the here presented results do not yet reveal the exact function of the psbT antisense RNA for psbT gene expression they provide solid impact for further analyses.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

The Centre National de la Recherche Scientifique and the French Ministry of Education; Société française d’exportation des ressources éducatives (to M.M.G.); FLORALIS (to F.B.). Funding for open access charge: The Centre National de la Recherche Scientifique.

Conflict of interest statement. None declared.

REFERENCES


