Circadian-regulated expression of a nuclear-encoded plastid σ factor gene (*sigA*) in wheat seedlings

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Abstract The activity of a light-responsive *psbD* promoter in plastids is known to be regulated by a circadian clock. However, the mechanism of the circadian regulation of the *psbD* light-responsive promotor, which is recognized by an *Escherichia coli*-type RNA polymerase, is not yet known. We examined the time course of mRNA accumulation of two *E. coli*-type RNA polymerase subunit genes, *sigA* and *rpoA*, under a continuous light condition after 12 h light/12 h dark entrainment. Accumulation of the *sigA* mRNA was found to be regulated by a circadian clock, while *rpoA* mRNA did not show any significant oscillation throughout the experiment.

Key words: Chloroplast RNA polymerase; Circadian clock; *psbD* light-responsive promotor; *psbD-C* operon; σ Factor

1. Introduction

We previously showed that in wheat chloroplasts, a light-responsive *psbD* promoter (*psbD* LRP) which has been known to be selectively activated by light is also controlled by a circadian clock [1]. This promoter is known to be recognized by an *Escherichia coli*-type RNA polymerase (RNAP) which functions in chloroplasts of higher plants. Including *psbD*, genes encoding photosynthesis-related proteins, such as the main subunits of photosystem I and II complexes, are transcribed by this type of RNAP [2,3]. The *E. coli*-type RNAP core enzyme comprises four subunits encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2* in the plastid genome. These subunits are named α, β, β′ and β″ and considered to be related to those of the eubacterial $\alpha_2\beta\beta'$ RNAP. The promoter recognition by the *E. coli*-type RNAP in plastid probably also requires other subunits, so-called σ factors [4,5]. Several nuclear-encoded σ factor genes have been recently cloned from *Arabidopsis thaliana* [6,7], *Oryza sativa* [8] and *Sinapis alba* [9]. It has been demonstrated that their expression is light-dependent and leaf-specific. In this work, we examined circadian regulation of the mRNA level of a σ factor, SigA, in connection with circadian-controlled plastid gene transcription. For this purpose, we cloned *sigA* from wheat and used it for examining the time course of the *sigA* mRNA level in wheat seedlings under continuous light condition after 6 days of 12 h light/12 h dark entrainment. The results are compared with ones obtained for the *rpoA* encoding the α subunit of the *E. coli*-type RNAP under identical conditions. In contrast to the *rpoA* mRNA, very strong circadian oscillation was observed in the *sigA* mRNA. This persisted for at least 3 days in continuous light with an almost identical peak time and periodicity to those of the oscillation of the mRNA from psbD LRP (*psbD* mRNA) [1], suggesting the possibility that the circadian regulation of the *psbD* LRP activity is under the control of the nuclear-encoded σ factors.

2. Materials and methods

2.1. Plant growth

For circadian experiments, wheat (*Triticum aestivum*) was grown at 25°C under 12 h white light (approximately 5000 lx)/12 h dark cycles (LD). After 6 days, the seedlings were transferred to continuous light (LL). Samples were taken every 3 h for 4 days, starting at the onset of illumination on the last day in LD. The apical 5 cm of the seedlings was cut and frozen rapidly in liquid N_2.

For other experiments, wheat seedlings were grown under LL (approximately 5000 lx) at 25°C. 5 Day old light-grown seedlings (illuminated seedlings) were transferred to the dark and kept there for 24 h (dark-adapted seedlings), then exposed again to light for 2 h (re-illuminated seedlings). Leaves were harvested at each stage of the above growth sequence and frozen rapidly in liquid N_2.

Total cellular RNA extraction was carried out as described previously [1].

2.2. Cloning of a cDNA fragment of sigA from wheat

cDNA fragments were amplified by reverse transcription-PCR against the total cellular RNA prepared from the re-illuminated seedlings with degenerate primers containing deoxyinosine: P1, 5′-GCCTGCAGAYYTIRTICARGRIGG-3′ and P2, 5′-CGCTGCAGSMYTGICKDATCCACCA-3′. These primers were designed on the basis of the conserved amino acid sequences among the principal σ factors of cyanobacteria, *Cyanidium caldarium* [11] and *A. thaliana* EST (GeneBank accession number T88387). An amplified 122 bp fragment of predicted length was cloned into the *Pst*I site of pUC18 and sequenced.

For cDNA library construction, polyA-tailed mRNAs were purified from total cellular RNA of the illuminated seedlings using the mRNA Purification kit (Pharmacia). The cDNAs synthesized with the Time-Saver cDNA Synthesis kit (Pharmacia) were cloned into the λgt10 vector. This cDNA library was screened using plaque hybridization with the ^{32}P-labelled 122 bp probe amplified by PCR. 10 independent positive plaques were obtained from about 200 000 plaques and sequenced with a T7 DNA sequencing kit (Pharmacia) and a ALF-red auto sequencer (Pharmacia).

2.3. Northern hybridization analyses

Hybridization probes were prepared as described below. The 122 bp wheat *sigA* cDNA fragment was amplified by PCR with P1 and P2 primers in the presence of [α-^{32}P]dCTP. The 506 bp DNA fragment corresponding to 896–1401 nucleotide (nt) cDNA of wheat *sigA* (GeneBank accession number AJ132658), the 984 bp DNA fragment corresponding to 67–1050 nt cDNA of wheat *rpoA* (GeneBank acces-

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sion number X15595) and the 1.0 kbp wheat 16s rDNA fragment (+217–+1216) [10] were labelled by Ready To Go DNA labelling beads (Pharmacia) in the presence of [α-^{32}P]dCTP. These DNA fragments were prepared by PCR with the following primers: P3, 5′-TGCATGAGAGGCTGATTGCGATTCG-3′ and P4, 5′-GAAAAACACCTCAGCTCAGTAATCC-3′ for the 506 bp *sigA* fragment; P5, 5′-ATGGTTCGAGAGGTGGTAGCAGG-3′ and P6, 5′-GAGATGCTTCTCTAGAGTGTCCC-3′ for the *rpoA* fragment; P7 5′-AGTAACGCGTAAGAACCTGC-3′ and P8, 5′-TCAGGGTTCCAAACTCATAG-3′ for the 16s rDNA fragment.

The total cellular RNAs (20 or 30 μg per lane) isolated from the illuminated, dark-adapted and re-illuminated seedlings as well as the circadian samples were separated on 1.2% agarose-formaldehyde denaturing gels and transferred to either nitrocellulose (Fig. 2) or nylon (Hybond-N+, Amersham; Fig. 3) membranes. Membranes were prehybridized at 42°C for 2 h in a solution containing 5×SSPE, 59% formamide, 5×Denhardt's solution, 0.5% SDS and denatured salmon sperm DNA (100 μg/ml), then subjected to hybridization in the same solution containing the appropriate probes (2×10^6 cpm per membrane) at 42°C overnight. The membranes were finally washed with 0.2×SSC, 0.1%SDS at 42°C (Fig. 2A) or 56°C (Fig. 3A and C) or with 0.1×SSC, 0.1% SDS at 65°C (Figs. 2B and 3D). Washed membranes were analyzed by either autoradiography (Fig. 2A) or a Bio-image analyzer BAS2000 (Fuji Film, Japan; Figs. 2B and 3).

3. Results

3.1. Cloning of wheat sigA

Very recently, nuclear-encoded chloroplast σ factors have been cloned in higher plants. However, these results were not known at the time that our cloning experiments started. The only information available were the sequences of the σ factors of cyanobacteria, *C. caldarium* [11] and *A. thaliana* EST (GeneBank accession number T88387) which corresponds to *sigA*. We tried to amplify the cDNA fragments by means of reverse transcription-PCR from the re-illuminated wheat seedlings with degenerate primers synthesized on the basis of their conserved amino acid sequences containing the 'rpoD box' [11]. Using the resulting 122 bp cDNA fragment as a screening probe, we cloned a 1573 bp cDNA fragment from the cDNA library constructed against the illuminated wheat seedlings. The amino acid sequence deduced from the largest reading frame which can be aligned in *O. sativa* (Os) SigA, *S. Alba* (Sa) SigA, *A. thaliana* (At) SigA, SigB and SigC is shown in Fig. 1. Although the obtained cDNA was truncated, the deduced amino acid sequence corresponds to the conserved portion (region 1.2–4.2) of other higher plant σ factors (Fig. 1), having a higher similarity to other higher plant SigAs (Os SigA: 75.1%, At SigA: 50.7%, Sa SigA: 52.0%) than At SigB (23.9%) and At SigC (17.4%).

3.2. Light-dependent accumulation of the sigA mRNA

First, we investigated the effect of light on the accumulation of *sigA* mRNA in wheat seedlings by a Northern hybridization analysis. The result shown in Fig. 2A demonstrates that the level of the 2.2 kb *sigA* mRNA is highly light-dependent in wheat as well as in other plants [6,8]. The *sigA* mRNA almost disappeared after 24 h dark adaptation but recovered quickly to the original level after 2 h exposure to light. Accumulation of 16s rRNA (Fig. 2B) and nuclear-encoded 25s rRNA (data not shown) did not change significantly by light.

3.3. Circadian oscillation in the sigA mRNA level

We recently found that the activity of *psbD* LRP is under

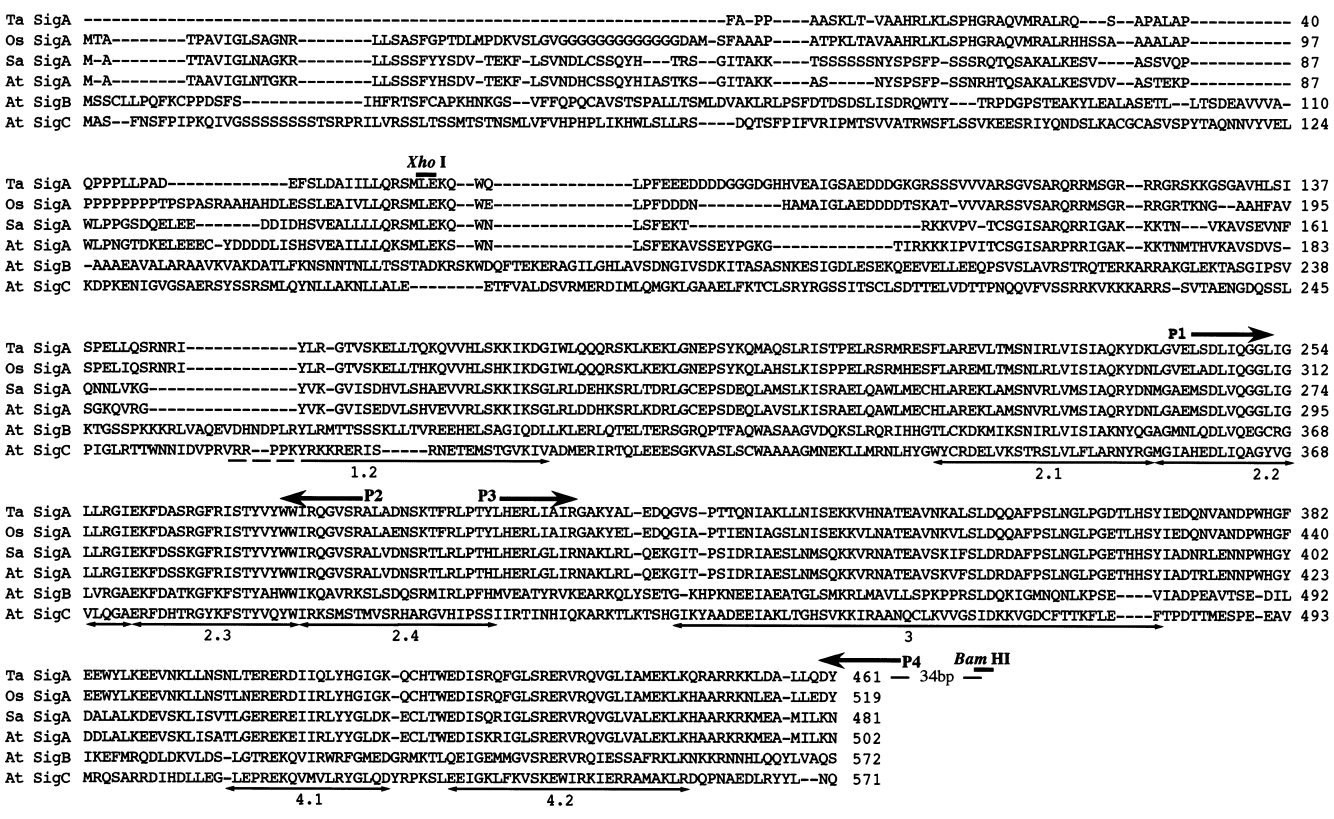

Fig. 1. Alignment of the deduced amino acid sequence of SigA from wheat (Ta: *T. aestivum*) with σ factors in other higher plants (Os: *O. sativa*, Sa: *S. alba*, At: *A. thaliana*). The regions conserved in the σ^{70} family are indicated under the sequences. The bold arrows above the sequence indicate the PCR primers.

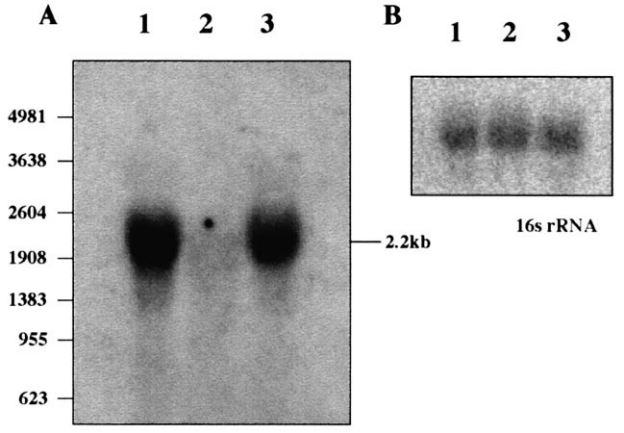

Fig. 2. Light-dependent accumulation of the *sigA* mRNA. Blots of total cellular RNA (30 μg per lane) prepared from illuminated seedlings (lane 1), dark-adapted seedlings (lane 2) and re-illuminated seedlings (lane 3) were hybridized to either a 122 bp *sigA* probe (A) or a 1.0 kbp 16s rRNA probe (B) as described in Section 2. The positions of RNA size markers (RNA Markers, Promega) are indicated in kb. The positions of the *Xho*I and *Bam*HI sites used to prepare the probe for Southern hybridization analyses are also indicated.

regulation of a circadian clock [1]. The *psbD* LRP is known to be recognized by *E. coli*-type RNAP rather than by the nuclear-encoded phage-type RNAP [2]. Although the activity of *psbD* LRP depends on the enhancer sequences located upstream of the −35 element [12,13], the circadian behavior itself does not require the presence of upstream sequences [1]. These facts may suggest that either the activity or the amount of the *E. coli*-type RNAP which is responsible for the *psbD* LRP transcription is controlled by a circadian clock. Here, we examined the possible circadian regulation of the expression of the nuclear-encoded *sigA* and the plastid-encoded *rpoA* genes by Northern hybridization analyses. Fig. 3A shows the result obtained using the 506 bp wheat *sigA* probe. Sampling times are indicated as ZT [14]; ZT0 is dawn at the seventh cycle from imbibition. The lowest level occurred at midnight and its level started to increase before the onset of the light. The *sigA* mRNA level peaked 3 h after onset of the light and declined gradually in the light. Our data clearly showed that the accumulation of wheat *sigA* mRNA oscillates for at least 3 days under LL conditions with a periodicity of ca. 24 h (Fig. 3A, B). The highest levels occurred at ZT3, 30 and 51. The behavior observed was almost identical to that of the *psbD* mRNA [1].

Fig. 3C shows the result of a Northern hybridization analysis of *rpoA*. At least five signals (1.1–4.0 kb) were detected.

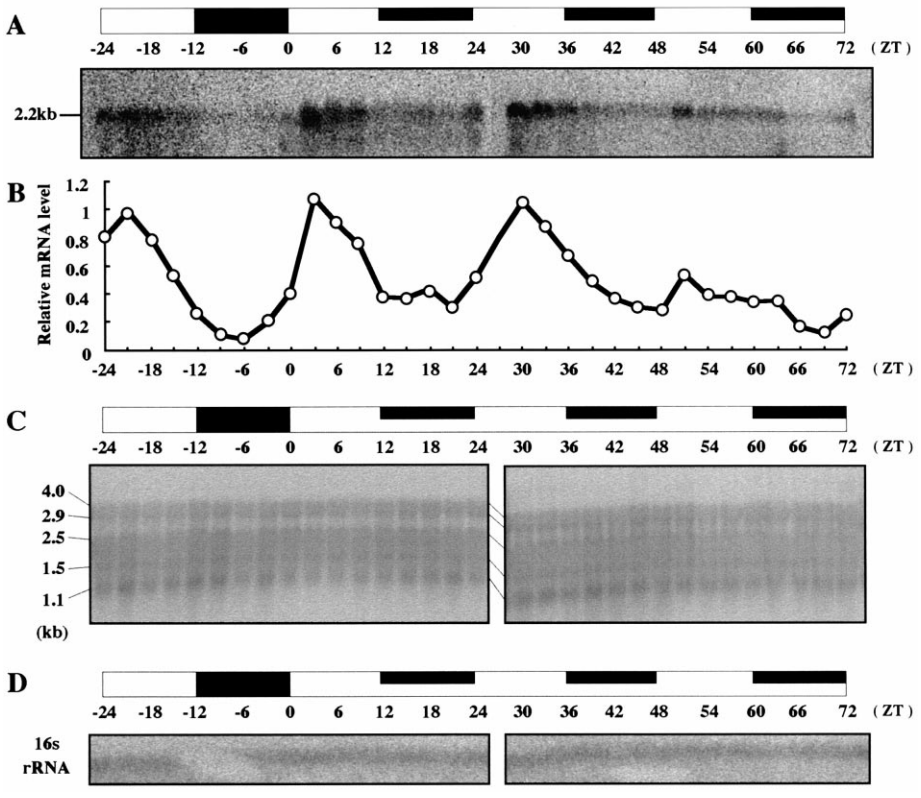

Fig. 3. Rhythmic accumulation of wheat *sigA* mRNA. (A) Northern blot analysis of *sigA* mRNA accumulation with a 506 bp wheat *sigA* probe. The solid and open bars represent dark and light periods, respectively. The inserted dark bars indicate subjective night. Hours before and after the onset of illumination on the first day in LL are indicated as ZT. Each lane contains 30 μg of total cellular RNA. The lane at ZT27 contains no sample. (B) Quantitative presentation of results shown in (A). Since the blots were divided into two membranes, the *sigA* mRNA levels were normalized to the control sample blotted onto both membranes. (C) Northern blot analysis of wheat *rpoA* mRNA accumulation. 20 μg total cellular RNA per lane was used. The estimated sizes of the detected bands were indicated on the left. (D) Northern blot analysis of wheat 16s rRNA. Each lane contains 20 μg total cellular RNA. The lack of the signals around ZT9 is because of the scrape of the membrane.

This reflects the fact that *rpoA* is a part of the large conserved gene cluster *rpl23-rpl2-rps19-rpl22-rps3-rpl16-rps8-infA-rpl36-rps11-rpoA* [15,16]. In barley, a gene-specific probe for *rpoA* was also reported to hybridize into several RNA species ranging from 1.2 to 5.1 kb [17]. Although it may be seen that the intensity of each signal at night periods is slightly higher than at day periods, it is clear that the behavior of *rpoA* mRNA accumulation is completely different from that of *sigA* and *psbD* mRNA. Accumulation of 16s rRNA (Fig. 3D) and nuclear-encoded 25s rRNA (data not shown) did not change significantly.

4. Discussion

We cloned the *sigA* cDNA fragment from wheat. In *Arabidopsis*, the entire *sigA* cDNA sequence was determined and the N-terminal 83 amino acids in the *SigA* open reading frame were shown to be sufficient to function as a transit peptide for import into chloroplasts [7]. Unfortunately, in the wheat *sigA* cDNA fragment obtained in this work, a part of the N-terminal region was truncated. However, the whole amino acid sequence deduced from the fragment was highly similar to SigAs in other plants.

In *Arabidopsis*, each of the three σ factor genes (*sigA*, *sigB* and *sigC*) exists as a single copy [7]. *O. sativa sigA* is also a single copy [8]. In contrast, we detected multiple bands in Southern hybridization analyses with a *Xho*I-*Bam*HI fragment as a probe shown in Fig. 1 (data not shown). This might suggest the existence of a set of multiple copies of *sigA*.

From in vitro transcription analysis, we previously suggested that the RNAP which exists in the tip portion of the re-illuminated wheat seedlings can recognize the TGn motif [18]. In *E. coli*, the TGn motif is recognized by region 2.5 of the σ^{70} in which the glutamate residue at position 458 is supposed to interact with the G-C base pair in the motif and the histidine residue at 455 may participate in the recognition of the motif [19]. The glutamate residue is conserved in all known higher plant SigA and SigB proteins including wheat SigA and the histidine residue is conserved in At SigA, B, Sa SigA. Wheat SigA and Os SigA have the tyrosine residue instead of the histidine residue at the corresponding position. The effect of this substitution should be examined.

The activity of *psbD* LRP which is recognized by *E. coli*-type RNAP is controlled by a circadian clock in wheat [1]. It is generally accepted that circadian clocks are constructed of transcription factors that inhibit their own transcription, forming an autoregulatory feedback circuit [20–22]. However, no proteins were found in the higher plant plastid genome with typical DNA binding motifs [15]. We supposed that the circadian-controlled RNAP activity is regulated by nuclear factor(s) and regarded the σ subunit as a possible candidate. In this work, we found that the *sigA* mRNA level was controlled by the circadian clock. In the case of cyanobacteria, the σ factor named RpoD2 is a component of an output pathway of the circadian clock mediating the circadian expression of a subset of genes [23]. These facts may suggest that SigA mediates the circadian control of RNAP activity, although it should be examined whether the amount of the SigA protein in plastids is controlled by the circadian clock and whether SigA acts as a promoter recognition subunit for the circadian-controlled promoter.

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