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1. 題目　Studies of Molecular Mechanism of Light Regulatory Transcription of Chloroplast Genes
葉緑体遺伝子における光応答転写の分子機構の研究

2. 公表の方法・時期

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コムギ*psb*D/C遺伝子クラスターにおける光誘導転写調節因子の探索
Research in Photosynthesis, 1992, Vol III, 259-262.

*In vitro* analysis of light-induced transcription in wheat *psb*D/C gene cluster using plastid extracts from dark-grown and short-term illuminated seedlings.
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Plant Physiology, 1994, 104, 1259-1267.

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コムギ葉緑体*psb*D/C遺伝子クラスターの光応答プロモーターにおけるシス配列及びトランス因子
Photosynthesis, 1995, Vol III, 591-594.

Light-dependent alternation of chloroplast RNA polymerase in recognition of core promoter structure: a key event of light responsive behavior of the *psb*D promoter.
葉緑体RNAポリメラーゼの光に応答したコアプロモーターの認識の変換：*psb*Dプロモーターにおける光応答の主要機構
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博士論文

# Studies of Molecular Mechanism of Light Regulatory Transcription of Chloroplast Genes

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②

博士論文

# Studies of Molecular Mechanism of Light Regulatory Transcription of Chloroplast Genes

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# Abbreviations

| | |
|---|---|
| DCMU | 3-(3,4-Dichlorophenyl)-1,1-dimethylurea |
| DTT | 1,4-Dithiothreitol |
| EDTA | *di*-Sodium dihydrogen ethylenediamine tetraacetate dihydrate |
| HEPES | [2-4-(2-Hydroxyethyl)-1-piperazinyl] ethane sulfonic acid |
| NADP | Nicotin amide adenine dinucleotide phosphate |
| LRP | light responsive promoter |
| PCR | polymerase chain reaction |
| PIPES | Piperazine-1.4-bis(2-ethane sulfonic acid) |
| PMSF | Phenylmethylsulfonyl fluoride |
| PSII | Photosystem II |
| RT-PCR | Reverse Transcription polymerase chain reaction |
| SDS | Sodium dodecyl sulfate |
| Tricine | N-[tris(hydroxymethyl)methyl]glycine |
| Tris | Tris(hydroxymethyl)aminomethane |

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# 1. Introduction

One of the most distinctive features of plants is the existence of chloroplasts which are the site of photosynthesis. Chloroplasts have four multisubunit complexes in thylakoid membranes involved in photosynthetic energy conversion, i.e., photosystem I (PSI), photosystem II (PSII), a cytochrome *b6/f* complex, and an ATP synthetase complex (Fig.1-1). The PSI, PSII and cytochrome *b6/f* complex participate in the light-driven electron transfer from water to $NADP^+$, which produces molecular oxygen and NADPH. At the same time, the electron transport-chain pumps $H^+$ in the lumen of the chloroplast to establish the gradient of electrochemical potential of $H^+$ across the thylakoid membranes, which acts as the proton-motive force to drive the synthesis of ATP in the stroma *via* the ATP synthase. NADPH and ATP are consumed in the course of carbon-fixation cycle in the stroma where $CO_2$ is converted to organic compounds.

Among those four complexes, PSI and PSII have chlorophyll dimers in which absorbed light energy initiate a series of redox reactions that result in the production of the chemical energy. PSII complex, consisting of a reaction center core, a water-splitting apparatus and a light-harvesting antennae complex, functions as a water/plastoquinone oxidoreductase and contains about 20 different polypeptides as well as bound cofactors such as chlorophylls, carotenoids, quinones and hemes. The number of PSII complexes in a chloroplast varies drastically depending on various environmental factors such as light, salt and temperature etc.. Among them, light is one of the most critical factor involved in the regulation of turnover of PSII complexes. When PSII is exposed to light with intensity higher than that required for saturating photosynthetic electron flow, PSII core proteins, i.e., D1, D2, CP43 and CP47 were degraded by high oxidizing potentials

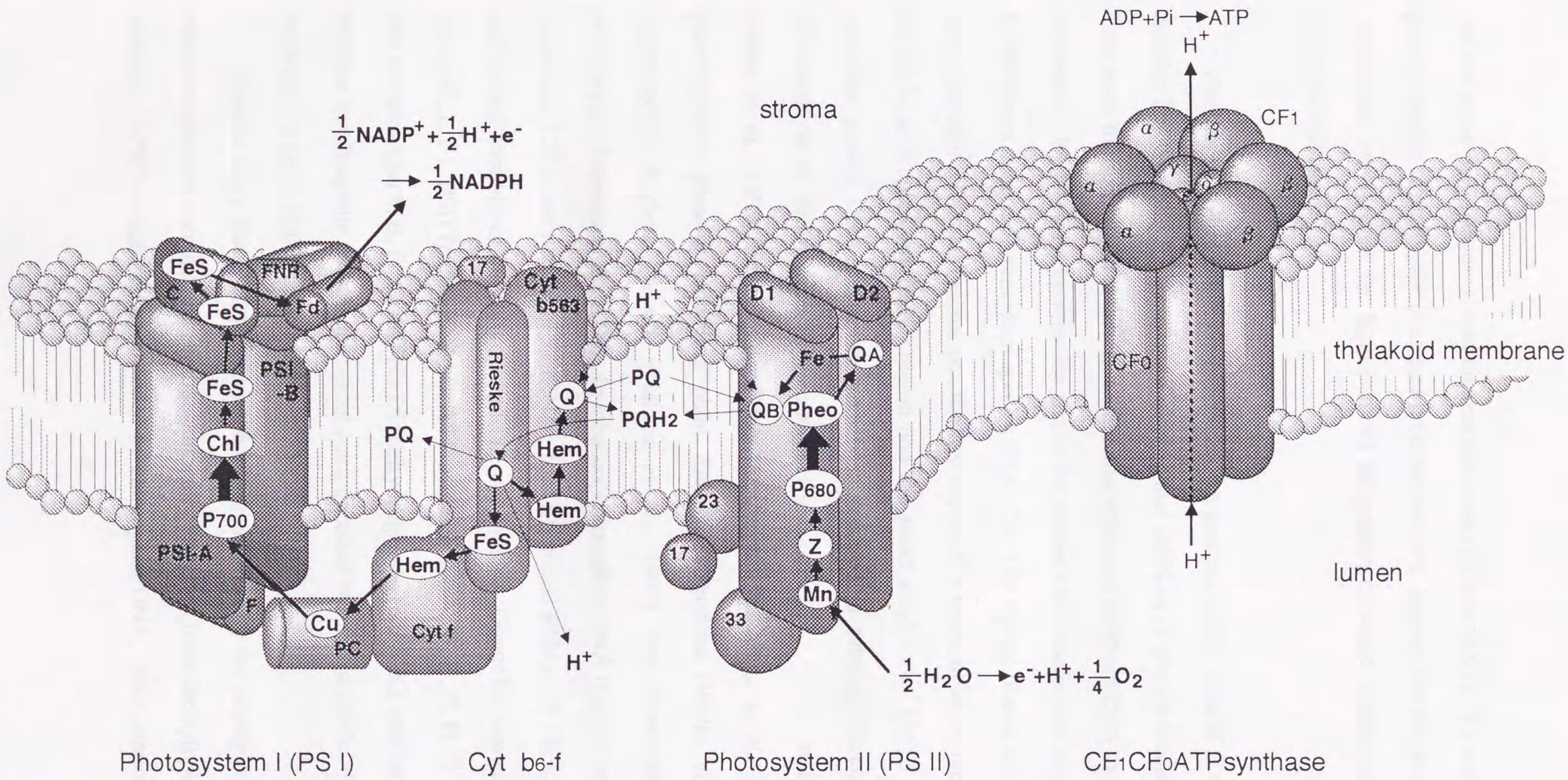



Fig.1-1 The Schematic drawing of phtosynthetic electron transport complexes and the electron flow.
Chloroplast contains the thylakoid, the inner envelope and the outer envelope menbranes enclosing three aqueous compatments, i.e., the thylakoid lumen, the stroma and the intermembrane space. Four multisubunit complexes involved in the photosynthtic electron transport are embedded in the thylakoid membrane.

and active oxygen species around the reaction center (Meris, 1991). To avoid the loss of photosynthetic capacity, the damaged proteins turnover rapidly, but the gene expression mechanism involved in the light-induced biogenesis of those proteins has not been analyzed well as yet.

Plastids in higher plants contain their own genome in the form of double-stranded circular DNA coding for the rRNAs, tRNAs and mRNAs of plastid-localized proteins. They have transcription-translation machineries which are different from those of nucleo-cytoplasm. With few exceptions, the size of the plastid DNA molecules ranges from 120 to 180kbp in many different plant species (Fig.1-2). The typical plastid DNA molecules have four characteristic regions, i.e., the two copies of a large, inverted repeat(IR), split by the large single-copy (LSC) region and the small single-copy (SSC) region. The complete plastid DNA sequences have been reported in tobacco *Nicotiana tabacum* (Shinozaki *et al.*,1986), rice *Oryza sativa* (Hiratsuka *et al.*, 1989), maize *Zea mays* (Maier *et al.*, 1995), black pine *Pinus thunbergii* (Wakasugi *et al.*, 1994), non-photosynthetic parasitic flowering plant *Epifagus virginiana* (Wolfe *et al.*, 1992), euglenophyta *Euglena gracillis* (Hallick *et al.*, 1993) and liverwort *Marchantia polymorpha* (Ohyama *et al.*, 1986). In tobacco, chloroplast DNA is predicted to code for more than 120 genes including 31 tRNAs, four ribosomal RNAs, 19 ribosomal proteins and several subunits of the chloroplast RNA polymerase. Genes for components of PSI (*psa*A-C, I, J ), PSII (*psb*A-F, H-N), ATP synthase (*atp*A, B, E, F, H, I), cytochrome *b6/f* complex (*pet*A, B, D, G) and NADH dehydrogenase (*ndh*A-K), and large subunit of ribulose bisphosphate carboxylase (*rbc*L) are encoded by plastid DNA. More than 40 unidentified genes (ORFs) also exist.

Despite more than 120 plastid proteins are encoded by the organella genome, the others are nuclear-encoded and posttranslationally imported from the cytoplasm. Several subunits of PSII complexes are encoded by chloroplast DNA, while others are encoded

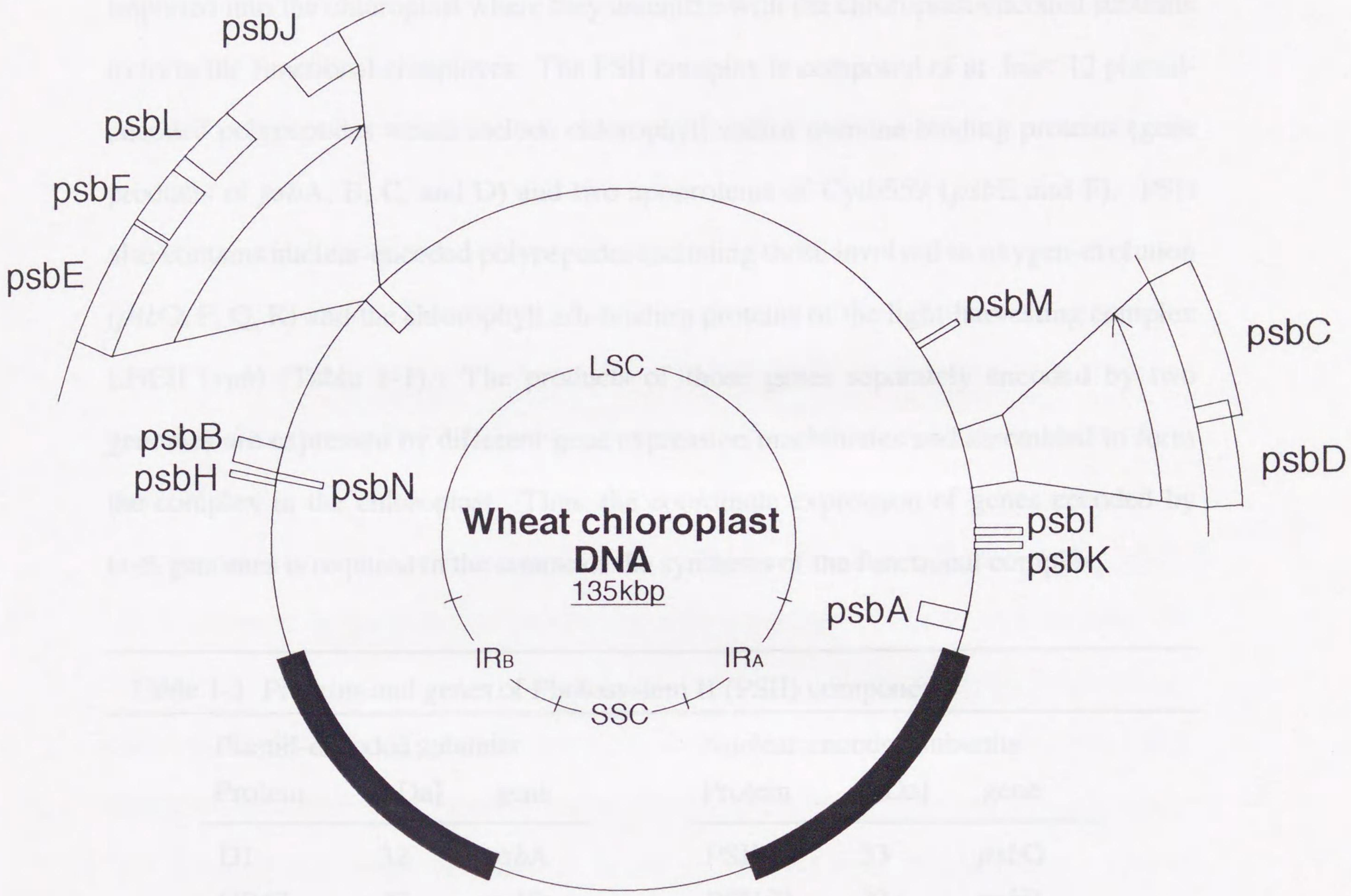



Fig.1-2 Genetical map of PSII genes on wheat chloroplast gemome.

Genes outside of the circle are encoded by A-strand and transcribed counterclockwise direction, and those inside are encoded by B-strand and transcribed clockwise. IR indicates the inverted-repeat.

by the nuclear genome and are translated as precursors on cytosolic ribosomes and imported into the chloroplast where they assemble with the chloroplast-encoded subunits to form the functional complexes. The PSII complex is composed of at least 12 plastid-encoded polypeptides which include chlorophyll and/or quinone-binding proteins (gene products of *psb*A, B, C, and D) and two apoproteins of Cytb559 (*psb*E and F). PSII also contains nuclear-encoded polypeptides including those involved in oxygen-evolution (*psb*O, P, Q, R) and the chlorophyll a/b-binding proteins of the light-harvesting complex LHCII (*cab*) (Table 1-1). The products of those genes separately encoded by two genomes are expressed by different gene expression machineries and assembled to form the complex in the chloroplast. Thus, the coordinate expression of genes encoded by both genomes is required in the course of the synthesis of the functional complex.

Table 1-1 Proteins and genes of Photosystem II (PSII) components

| Plastid-encoded subunits | | | Nuclear-encoded subunits | | |
|---|---|---|---|---|---|
| Protein | [kDa] | gene | Protein | [kDa] | gene |
| D1 | 32 | *psb*A | PSII-O | 33 | *psb*O |
| CP47 | 47 | *psb*B | PSII-P | 23 | *psb*P |
| CP43 | 43 | *psb*C | PSII-Q | 16 | *psb*Q |
| D2 | 33 | *psb*D | PSII-R | 10 | *psb*R |
| cyt *b559* α | 9 | *psb*E | PSII-S | 22 | *psb*S |
| cyt *b559* β | 4 | *psb*F | PSII-T | 4 | *psb*T |
| PSII-H | 10 | *psb*H | PSII-W | 6 | *psb*W |
| PSII-I | 5 | *psb*I | | | |
| PSII-J [a] | 4 | *psb*J | LHCII | | *cab* |
| PSII-K | 3.5 | *psb*K | | | |
| PSII-L | 4 | *psb*L | | | |
| PSII-M | 4 | *psb*M | | | |
| PSII-N | 5 | *psb*N | | | |

[a] The *psb*J designation is reserved for an open reading frame cotranscribed with *psb*E,F and L. Association of the *psb*J gene product with PSII is not yet certain in higher plant.

As concerns to the gene expression regulated by light in higher plants, it has been known that light is absorbed by at least three photoreceptors, i.e., phytochrome, holochrome and cryptochrome.

Phytochrome is a chromoprotein consisting of a linear tetrapyrrole chromophore covalently linked to a 120kDa polypeptide (Rudiger and Scheer, 1983), and it takes two photointerconvertible forms: Pr and Pfr with an absorption maximum near 667 and 730nm, respectively. Phytochrome has been shown to regulate the expression of a number of nuclear genes involved in photosynthesis and $CO_2$ fixation, such as genes encoding the chlorophyll a/b-binding protein of the light-harvesting complex (*cab*) (Mosinger *et al.*, 1988), the small subunit of ribulose bisphosphate carboxylase (*rbc*S) (Thompson *et al.*, 1983), and the apoprotein of phytochrome itself (Colbert *et al.*, 1985). The expression of a number of plastid-encoded genes such as those encoding the D1 subunit of PSII complex (*psb*A) and the large subunit of ribulose bisphosphate carboxylase (*rbc*L) has been shown to be regulated by phytochrome as well (Link, 1982; Zhu *et al.*, 1985).

Protochlorophyllide holochrome is the second red light photoreceptor. This is reduced to chlorophyllide on exposure to light and yields chlorophyll a (Castelfranco and Beale, 1983). Holochrome is also involved in the control of chlorophyll biosynthesis and in the accumulation of the chlorophyll a apoproteins (Klein *et al.*, 1988).

Cryptochrome is a specific photoreceptor for the UV/blue light region of the spectrum. The nature of cryptochrome is unknown, but it controls a variety of developmental processes including stomatal opening (Assmann, 1988), inhibition of stem elongation (Shinkle and Jones, 1988) and chloroplast differentiation (Richter *et al.*, 1987). In addition, blue light affects the expression of the nuclear-encoded genes such as *cab* (Mars and Kaufman, 1989) and *rbc*S (Fluhr and Chua, 1986), and also chloroplast-encoded *psb*D gene (Gamble and Mullet, 1989). The *psb*D encodes D2 protein which is one of the most important subunit of PSII complex.

It is predicted that those photoreceptors exist in the cytoplasm or in the plasma membrane, and that the light responsive expression of plastid genes is mediated by the signals from those receptors. However, a signal transduction pathway involved in the light responsive expression has not been understand well as yet.

Regulation of plastid gene expression was shown at various levels, ranging from DNA modification, transcription, RNA maturation, translation, to posttranslational modifications. The importance of post transcriptional regulation, such as regulation in the stability of mRNA (Gruissem *et al.*, 1988), and translational regulation (Klein & Mullet, 1987) has been emphasized in the plastid gene expression during the conversion from an etioplast to a chloroplast. On the other hand, strong evidence has supported the involvement of transcriptional regulation during plastid differentiation in particular genes and plants. In monocotyledonous plant *Sorghum*, light induced an enhancement in overall transcription activity (Schrubar *et al.*, 1990). Transcription activity of *psb*A was found to be differentially enhanced during light-induced chloroplast biogenesis in wheat (Kawaguchi *et al.*, 1992) and barley (Klein & Mullet, 1990).

In wheat, chloroplast-encoded PSII genes during light-induced greening processes have been classified into three groups in the accumulation pattern of transcripts of those genes (Kawaguchi *et al.*, 1992). i) The accumulation is increased intensively in the light-induced greening process (*psb*A). ii) The amount of mRNA accumulation is decreased by light or not changed depending on the light and growth levels (*psb*E-F-L-J, *psb*M, *psb*N). iii) Transcripts were varied both quantitatively and qualitatively by light (*psb*K-I-D-C, *psb*B-H).

The *psb*A gene classified into the first group is monocistronically transcribed. The amount of *psb*A mRNA is very little in 5-day dark-grown seedlings, and it was increased gradually to about 5-fold over 72h illumination. Transcriptional activity also increased, but it has the peak after 6h illumination and then decreased to a quite low level within

10h. However, these phenomena are observed specifically in early developmental seedlings. In mature plants, the apparent transcription activity is independent of the light condition (Satoh *et al.*, 1997). Nevertheless, at least the translation rate of psbA was reversibly accelerated by light. These facts indicate that in mature plants light-dependent turnover of D1 protein is regulated at the level of translation. Genes classified to the second group may be also mainly regulated on posttranscriptional level, since the accumulation pattern of transcripts of those genes is irrelevant to the light. While, genes classified into the third group have been suggested to be regulated on transcriptional level. The *psb*D gene forms an operon (*psb*D/C operon) together with other genes involving *psb*C gene which encodes a PSII subunit protein CP43. Four distinct promoters present in the operon in wheat (Wada *et al.*, 1994, see Fig.2-1). Among them, the third promoter from the 5' end of the operon is a unique light-responsive promoter (termed LRP by Christopher *et al.*, 1992). It was revealed that the change in the transcription rate from the *psb*D LRP substantially regulates the synthesis of D2 protein (Satoh *et al.*, 1997). This thesis aims to reveal the molecular mechanism of transcriptional regulation involved in the light-responsive plastid gene expression.

Typical plastid promoters contain hexanucleotide sequences resembling bacterial '-10' (TATAAT) and '-35' (TTGACA) motifs (Hanley-Bowdoin and Chua, 1987) (Table 1-2), indicating that they are structurally and functionally similar to *Escherichia coli* $\sigma^{70}$ type promoters (Reznikoff *et al.*, 1985). The importance of *E. coli*-like promoter elements for transcriptional initiation of plastid genes has been shown by mutational analyses *in vitro* (Link, 1984; Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985a; Gruissem and Zurawski, 1985b). The *psb*A gene is transcribed from a promoter which contains bacterial type '-35' and '-10' motifs. 90-bp region in the upstream region of *psb*D LRP is conserved among several monocotyledonous and dicotyledonous plants (Christopher *et al.*, 1992), but the putative '-35' motif of *psb*D LRP exhibits only

minimal sequence similarity to the bacterial consensus motif. It was recently proposed from *in vivo* (Allison and Maliga, 1995) and *in vitro* (Kim and Mullet, 1995; To *et al.*, 1996; Satoh *et al.*, 1997) analyses that transcription from *psb*D site is controlled *via* upstream *cis* elements and specific proteins which recognize these sequences. However, it was also demonstrated that these upstream sequences act as enhancers, but are not responsible for the light-dependent on/off switch of the *psb*D transcription (Satoh *et al.*, 1997). Thus, the region which is responsible for the light-switching on psbD LRP might be located in the core promoter region which is the contact site of RNA polymerase.

In bacteria, the σ subunit (σ factor) of RNA polymerase promotes accurate initiation of transcription by recognizing '-10' and '-35' promoter elements. The core enzyme ($\alpha_2\beta\beta'$) can initiate nonspecific transcription, whereas specific initiation of transcription by the holoenzyme ($\alpha_2\beta\beta'\sigma$) has been attributed to the ability of σ to increase the affinity of holoenzyme for DNA sequences containing promoters. It has been shown in bacteria that multiple σ factors which recognize different promoter sequences control the differential gene expression (Helman and Chamberlin, 1988; Errington, 1993).

Table 1-2 Core promoter structure of plastid-encoded genes

| gene | '-35'element | spacer length (nt) | '-10'element |
|---|---|---|---|
| *psb*A | TTGACA | 18 | TATACT |
| *psb*B | TTGCGT | 18 | TAGAAT |
| *psb*C | TTGCTT | 18 | TACATT |
| *psb*D | TTTAAT | 15 | TATTCT |
| *psb*E | TTGCTG | 18 | TATACT |
| *rbc*L | TTGCGC | 18 | TACAAT |
| *atp*B2 | TTGACA | 18 | TAGTAT |
| bacterial consensus | TTGACA | 17 | TATAAT |

Bacterial type chloroplast RNA polymerases have been isolated as a multi-subunit enzyme from a number of plant species; maize (Hu and Bogorad, 1990), pea ( Rajasekher *et al.*, 1991), spinach (Larbs *et al.*, 1985), and mustard (Pfannschmidt and Link, 1994) (Table 1-3). Protein sequencing of highly purified polypeptides from maize has provided evidence that the genes homologous to those of α, β, and β' subunits of *E. coli* core RNA polymerase, *rpo*A, *rpo*B and *rpo*C, respectively, are encoded by the plastid genome (Hu and Bogorad, 1990). In most plastid genomes, *rpo*C is split into *rpo*C1 and *rpo*C2 encoding β' and β" subunits, respectively which are homologous to the amino- and carboxyl- terminal portions of the *rpo*C gene product in bacteria. Although genes for core subunits of chloroplast RNA polymerase are encoded by plastid genome, *rpo*D genes for σ factors have not been found in the plastid genome, suggesting that plastid σ factors are encoded by the nuclear genome. In chloroplasts and etioplasts of mustard, three types of σ-like factors (SLFs) have been found (Tiller *et al.*, 1991; Tiller and Link, 1993a), but genes of σ factors have not been cloned from higher plants as yet.

In this work, at first, a plastid *in vitro* transcription system which reflects characteristic features of the light-regulated transcription observed *in vivo* was constructed from the extract of wheat plastids, to elucidate molecular mechanisms responsible for light-responsive transcription. By using this system, we found that the extract prepared from plastids of light-illuminated seedlings contains the light-regulatory factor(s) which activates specifically transcription from *psb*D LRP. These results are presented in section 2.

Next, systematic mutational analysis of psbA promoter by means of *in vitro* transcription was carried out on behalf of comparison the core promoter structure of psbA with that of psbD LRP. The properties of chloroplast RNA polymerases which recognize core promoter regions of psbA and psbD LRP were elucidated. These results are shown

in section 3.

In section 4, the results of cloning and sequencing of cDNA of a chloroplast RNA polymerase σ factor from wheat seedlings are presented. Cloned σ factor was expressed only under illumination.

Results in this work suggest the contribution of light-induced σ factor(s) to the recognition of the *psb*D LRP by chloroplast RNA polymerase. The light dependent on/off switching on psbD LRP seems to be contributed by alternation of σ factor(s). In section 5, a model for the molecular mechanism of light regulatory transcription of *psb*D LRP is proposed. This is the first model concerning light-responsive transcription of chloroplast DNA in mature plants.

Table 1-3 Compositions of highly purified chloroplast RNA polymerases

| Maize | Pea | Spinach | Mustard chloroplast | Mustard etioplast |
|---|---|---|---|---|
| 180 kDa (β"subunit) | | | | 154 kDa |
| | 150 kDa | 150 kDa (β"subunit) | | |
| | | 145 kDa | 141 kDa | |
| 120 kDa (β subunit) | 130 kDa | | | 120 kDa |
| | 115 kDa | 120 kDa (β subunit) | | |
| | 110 kDa | | 110 kDa | |
| | 95 kDa | 102 kDa | 107 kDa | |
| | | | | 93 kDa |
| 85 kDa (β' subunit) | 85 kDa | 78 kDa (β'subunit) | 78 kDa | 85 kDa |
| | 75 kDa | 75 kDa | 72 kDa | 72 kDa |
| | | 64 kDa | 70 kDa | |
| | | 58 kDa | | 61 kDa |
| | 48 kDa | 48 kDa | 48 kDa | 42 kDa |
| 38 kDa (α subunit) | 39 kDa | 38 kDa (α subunit) | 36 kDa | 38 kDa |
| | | 34 kDa | 35 kDa | 33 kDa |
| | | 27 kDa | 29 kDa | |
| | | 17 kDa | 16 kDa | |
| | | | 13 kDa | |

# 2. *In vitro* Analysis of Light-Induced Transcription in Wheat *psb*D/C Gene Cluster Using Plastid Extracts

## 2.1 Introduction

Transcriptional activities (Klein and Mullet, 1990; Sexton *et al*., 1990a; Klein, 1991; Schrubar *et al*., 1991; Kawaguchi *et al*., 1992; Baumgartner *et al*., 1993) and levels of the transcripts of some chloroplast genes (Rodermel and Bogorad, 1985; Klein and Mullet, 1987; Gamble *et al*., 1988; Kawaguchi *et al*., 1992; Baumgartner *et al*., 1993) are affected by light. For example, run-on transcription experiments with wheat and barley have shown that transcription of *psb*A is differentially enhanced by illumination (Klein and Mullet, 1990; Kawaguchi *et al*., 1992). Some particular transcripts from the *psb*D/C operon have also been observed to be light-induced among several transcripts within the operon during light-induced greening of dark-grown seedlings of several monocot and dicot plant species (Christopher *et al*., 1992; Kawaguchi *et al*., 1992). Similar observations have been made with the *pet*E operon of maize (Haley and Bogorad, 1990) and *psb*B operon of wheat (Kawaguchi *et al*., 1992). Thus, although post-transcriptional control may be of importance in the light-dependent regulation of plastid gene expression, it is also evident that some chloroplast genes are regulated at the level of transcription.

Studies on the transcriptional control of nuclear genome expression have been greatly advanced by the isolation of various transcriptional regulatory factors from cell lysates (Gilmartin *et al*., 1990; Thompson and White, 1991). Concerning organelle gene expression, it has been revealed by using plastid extracts that the chloroplast transcription apparatus has many features in common with that of prokaryotes, such as the use of prokaryotic-type promoter elements (Link, 1984; Bradley and Gatenby, 1985; Gruissem

and Zurawski, 1985a; Gruissem and Zurawski, 1985b; Igoli and Kossel, 1992). However, there is only limited information about the light-regulatory mechanisms of plastid gene transcription.

As illustrated in Fig.2-1, the wheat *psb*D/C gene cluster, which is differentially expressed *in vivo* during light-induced plastid development (Christopher *et al.*, 1992; Kawaguchi *et al.*, 1992), has four distinct promoters (Kawaguchi et al., 1994). Three of these operate in the dark, but the other one (D/C-3 as *psb*D LRP; Light-Responsive Promoter, in Fig.2-1) responds to light, producing two light-induced transcripts. Similar results were previously obtained in barley (Sexton *et al.*, 1990a). This light responsive promoter was also recently reported to be conserved in chloroplast DNAs from several diverse higher plant species, including wheat (Christopher *et al.*, 1992). To elucidate molecular mechanisms responsible for light-induced transcription from *psb*D LRP, we have isolated transcriptionally active plastid extracts from dark-grown and short-term illuminated wheat seedlings. The extract prepared from 4-h-illuminated seedlings was capable of transcribing *in vitro* from the light-responsive *psb*D site (D/C-3), but the extract from dark-grown seedlings was not. In this section, I present evidence suggesting that certain light regulatory component(s) which selectively activates *psb*D LRP is produced or converted into an active form during 4-h-illumination of dark-grown seedlings.

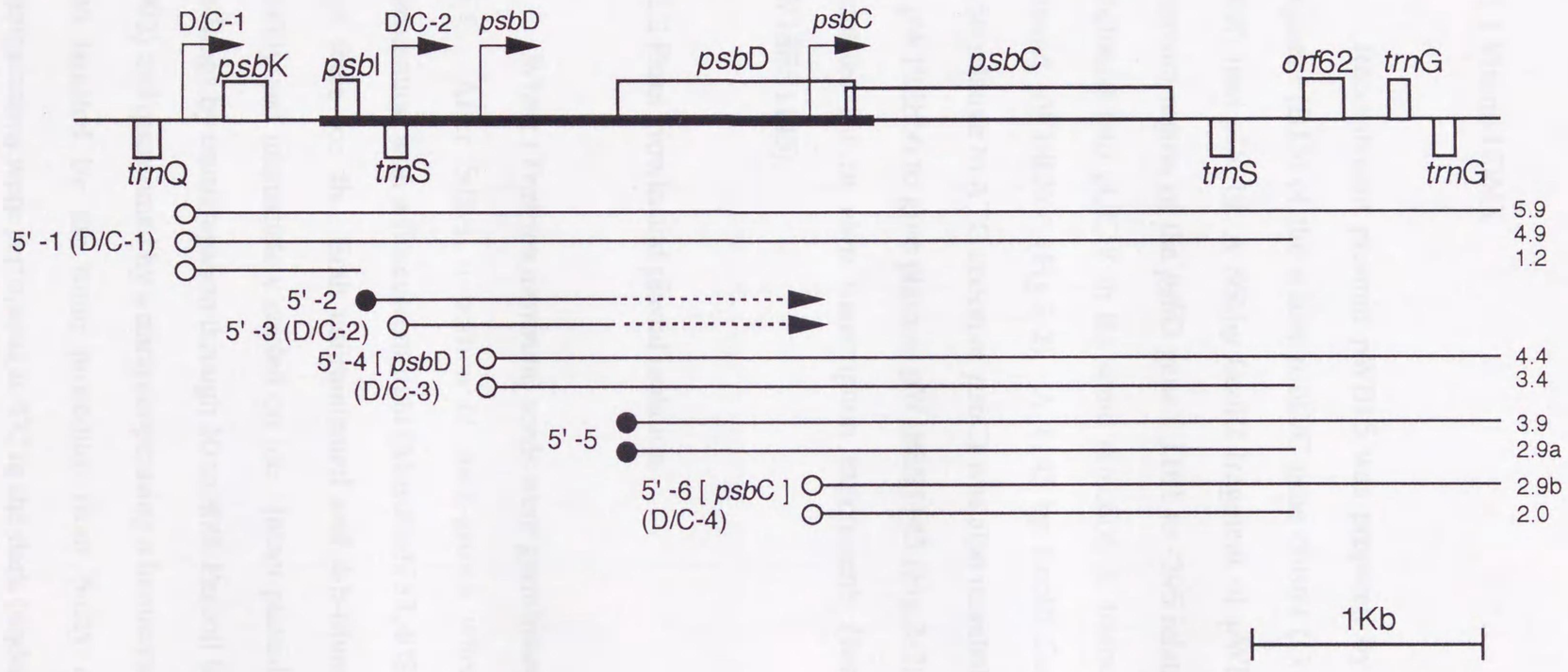



Fig.2-1 Gene (upper) and transcript (lower) map of the wheat *psb*D/C gene cluster.
Gene are designated by open boxes. *psb*I, *trn*S, *psb*D, and a part of *psb*C are positioned based on the wheat chloroplast DNA sequence (bold line). Other genes in the map are positioned based on the chloroplast genome map of rice (Hiratsuka *et al.*, 1989). Solid lines represent the transcripts that have been visualized by nothern hybridization analysis (Kawaguchi *et al.*, 1994). The site of the 5'-end and the length of each transcript were determined by combination on the results of nothern hybridization with seven different probes and primer extension using six probes as primers. The 3'-end of each transcript was not determined, but estimated from the site of the 5'-end and the length of each transcript. S1 nuclease protection assay gave signals at the positions designated as 5'-2 and 5'-3, but the transcript mapping to these ends was not detected in nothern hybridization analysis. Open circles (○) represent transcriptional initiation sites determined by *in vitro* capping assay (Kawaguchi *et al.*, 1994). Solid circles (●) indicate putative processing sites. Numbers to the left and to the right of each transcript indicate the name of 5'-end and size (in kb) of the transcript, respectively. Four transcription sites are designated as D/C-1, D/C-3, *psb*D(D/C-3) and *psb*C(D/C-4).

## 2.2 Materials and Methods

### 2.2.1 Plasmid DNA

Recombinant plasmid pWB15 was prepared by inserting a 3.3kb BamHI DNA fragment (B15) of the wheat *psb*D/C gene cluster (Courtice *et al.*, 1985; Howe *et al.*, 1988) into pUC19. A 866bp EcoRI fragment of pWB15, containing a portion of the upstream region of the *psb*D gene (-1161 to -295 relative to ATG codon of *psb*D) was subcloned into pUC18 in the same direction as transcription of the *lac*Z gene to give plasmid pW18E866 (Fig.2-2). A 1145 bp EcoRI-SacI fragment of pWB15 (-989 to +156 relative to ATG codon of *psb*C) was also inserted into pUC18 in the same manner as pW18E866 to give plasmid pW18ES1145 (Fig.2-2). These constructs were used as templates for *in vitro* transcription experiments (template pW18E866 and template pW18ES1145).

### 2.2.2 Plant growth and plastid isolation

Wheat (*Triticum aestivum*) seeds were germinated and grown in a dark chamber at 25°C. After 5days, a portion of dark-grown wheat seedlings was transferred to illumination with a fluorescent light (Mitsubishi FL40SD/38) at 200 moles $m^{-2}$ $sec^{-1}$ and kept there for 4h. Both unilluminated and 4-h-illuminated seedlings were harvested quickly and immediately cooled on ice. Intact plastids were isolated from both of the seedlings by centrifugation through 10 to 80% Percoll linear gradients (Kawaguchi *et al.*, 1992) and quantitated by a microscope using a hemacytometer. Mature chloroplasts were also isolated by the same procedure from 5-day old light-grown seedlings. All manipulations were performed at 4°C in the dark (under green dim light) for dark-grown seedlings or under room light for 4-h-illuminated and light-grown seedlings.

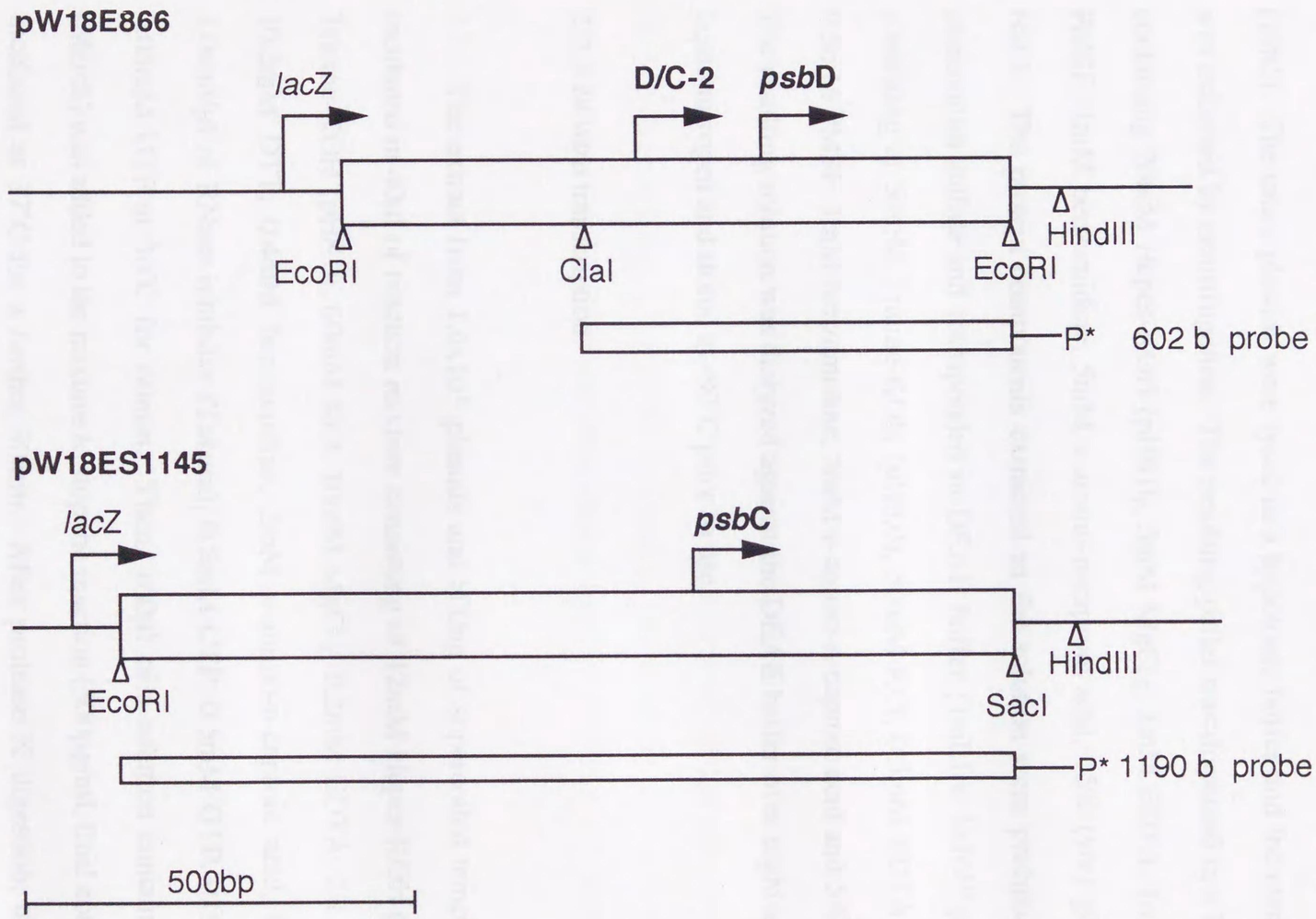



Fig.2-2 Schematic representations of the truncated *psb*D and *psb*C constructs used for analysis of *in vitro* transcription.

The 886-bp EcoRI fragment of wheat chloroplast DNA containing putative promoter regions of the D/C-2 and *psb*D (D/C-3) sites was inserted into pUC18 to give pW18E866. The 1145-bp EcoRI-SacI fragment containing the *psb*C (D/C-4) site was inserted into pUC18 to give pW18ES1145. The arrows show the direction and sites of transcription initiation. The boxed regions indicate wheat chloroplast DNA sequences and the single lines indicate vecter sequences. The 602-base ClaI-HindIII probe and 1190-base EcoRI-HindIII probe used for S1 protection analysis of the *in vitro* synthesized RNAs are shown below the figures of their respective plasmids.

### 2.2.3 Preparation of high-salt extracts from plastids

Transcriptionally active plastid extracts were prepared according to Orozco *et al.* (1985). The intact plastids were lysed in a hypotonic buffer and the membrane fraction was collected by centrifugation. The resulting pellet was dispersed in a high-salt buffer containing 20mM Hepes-KOH (pH8.0), 5mM $MgCl_2$, 1mM EDTA, 1mM DTT, 1mM PMSF, 1mM benzamidine, 5mM ε-amino-n-caproic acid, 15% (v/v) glycerol and 1M NaCl. The protein components extracted in the solution were precipitated with 50% ammonium sulfate and resuspended in DEAE buffer (1ml for $1x10^{10}$ plastids extract) consisting of 50mM Tricine-KOH (pH8.0), 50mM KCl, 0.5mM EDTA, 0.5mM DTT, 0.5mM PMSF, 1mM benzamidine, 5mM ε-amino-n-caproic acid and 5% (v/v) glycerol. The resulting solution was dialyzed against the DEAE buffer over night at 4°C, frozen in liquid nitrogen and stored at -80°C prior to use.

### 2.2.4 *In vitro* transcription

The extract from $1.6x10^8$ plastids and 800ng of supercoiled template DNA were incubated in 40μl of reaction mixture consisting of 12mM Hepes-KOH (pH8.0), 20mM Tricine-KOH (pH8.0), 60mM KCl, 10mM $MgCl_2$, 0.2mM EDTA, 2% (v/v) glycerol, 10.2mM DTT, 0.4mM benzamidine, 2mM ε-amino-n-caproic acid, 0.2mM PMSF, 1.0unit/μl of RNase inhibitor (Takara), 0.5mM CTP, 0.5mM GTP, 0.05mM ATP and 0.05mM UTP at 30°C for 60min. Then, 160μl of a solution containing protease K (Merck) was added to the mixture to stop the reaction (500μg/ml, final concentration) and incubated at 37°C for a further 30min. After protease K digestion, the solution was subjected to phenol-chloroform extraction and ethanol precipitation. The RNAs were purified from the nucleic acid mixture by treatment with RQ-1 DNase (Promega), followed by phenol-chloroform extraction and ethanol precipitation.

### 2.2.5 *In vitro* RNA processing

As shown in Fig.2-3, a 866 bp EcoRI restriction DNA fragment spanning three 5'-ends of *psb*D/C [5'-2, 5'-3 (D/C-2) and 5'-4 (*psb*D)] was cloned into the polylinker region of pSP73 plasmid vector (pW73E866). The plasmids were cleaved at a vector restriction site (EcoRV or NdeI) located downstream of the insert fragment, and submitted to transcription (Fig.2-3). The transcription was carried out using the T7 RNA polymerase promoter located upstream of the insert in the linearized plasmid in the presence of [$\alpha$-$^{32}$P]GTP. After the DNA templates were digested by treatment with RQ-1 DNase, the *in vitro* synthesized RNAs (2,000 cpm/reaction) were incubated in the plastid extract prepared from 4-h-illuminated seedlings at 30°C for 60min under the same conditions as the *in vitro* transcription experiments were carried out except that template DNA was excluded. Following incubation, the resulting RNAs were isolated and analyzed by electrophoresis and autoradiography.

### 2.2.6 S1 nuclease protection experiments

A 602bp ClaI-HindIII fragment from pW18E866 and a 1190 bp EcoRI-HindIII fragment from pW18ES1145, which contain 51bp and 41bp vector sequences, respectively, following each plastid DNA sequence, were 5'-end labeled at HindIII site within the vector sequence using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. A 834bp StyI-EcoRI fragment from pW18E866 was also 5'-end labeled at the EcoRI site within the plastid DNA sequence. The resulting radiolabeled ClaI-HindIII and EcoRI-HindIII DNA fragments were used as probes for S1 nuclease protection assays of the *in vitro* synthesized RNAs. The radiolabeled StyI-EcoRI DNA fragment was used for the assay of the mRNAs accumulated *in vivo* in the isolated plastids. Each $^{32}$P probe (20,000cpm

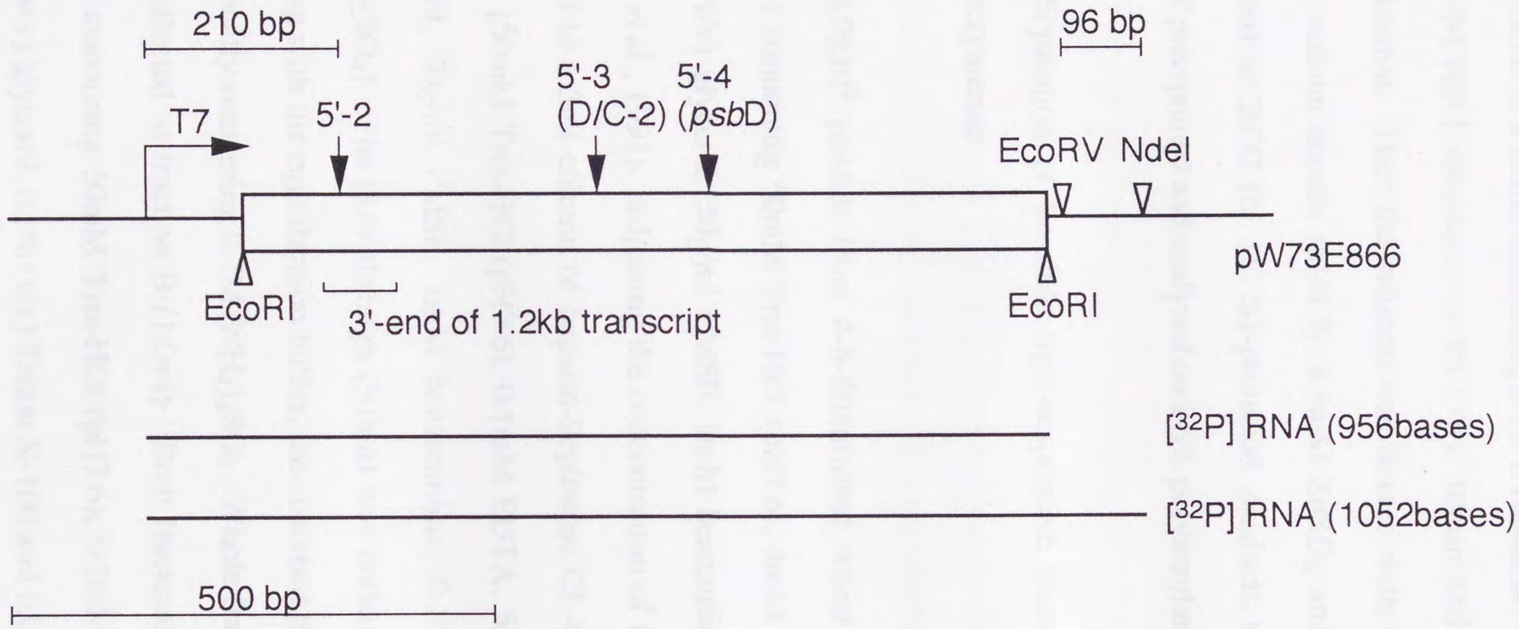



Fig.2-3 Shematic representations of the synthesized RNAs used for in vitro prodessing experiments in the plastid extract.

The 866-bp EcoRI fragment of wheat chloroplast DNA containig 5'-2, 5'-3 (D/C-2) and 5'-4 (*psb*D site) was inserted into pSP73 to give pW73E866. The boxed region indicates the vector sequence. The vertical arrows show the sites of 5'-2, D/C-2 and *psb*D. The possible range of the 3'-end of the 1.2-kb transcript is shown by the horizontal bracket. The 956- and 1052-base RNAs used for in vitro proseccing were synthesized using the T7 RNA polymerase promoter on the templates pW73E866 linearized at the EcoRV and NdeI sites, respectively.

per assay) was mixed with the *in vitro* synthesized RNAs or the RNAs isolated from the plastids and co-precipitated by the addition of ethanol. The resulting pellet was resuspended in a buffer containing 80% formamide, 40mM Pipes (pH6.4), 1mM EDTA and 0.4M NaCl, denatured at 85°C for 10min and incubated at 37°C over night for hybridization. Then the solution was diluted with 100µl of S1 buffer [0.28M NaCl, 0.05M sodium acetate (pH4.5), 4.5mM $ZnSO_4$ and 200units/ml of S1 nuclease] and incubated at 20°C for 1h. S1-protected products were phenol-chloroform extracted, ethanol precipitated and analyzed on a 6% polyacrylamide-7M urea sequencing gel.

2.2.7 Separation of possible light-responsive transcription-regulatory factor(s) from RNA polymerase

$8.9x10^9$ plastids from 4-h-illuminated wheat seedlings were lysed in a buffer (2.7ml) containing 50mM Tris-HCl (pH7.6), 4mM EDTA, 40mM 2-mercaptoethanol, 25% (v/v) glycerol, 50g/ml PMSF, 1mM benzamidine and 1.0% (v/v) Triton X-100 (Tiller *et al*., 1991). Adjusting the concentration of $(NH_4)_2SO_4$ to 0.1M, the lysate was applied to a 3ml column of heparin-Sepharose CL-6B (Pharmacia) equilibrated with a buffer [50mM Tris-HCl (pH7.6), 0.1mM EDTA, 5mM 2-mercaptoethanol, 10% (v/v) glycerol, 50g/ml PMSF, 1mM benzamidine, 0.1% (v/v) Triton X-100 and 0.1M $(NH_4)_2SO_4$]. The flow through (3.0ml) was collected as fraction A. After extensive washing with the equilibration buffer, the absorbed materials were eluted with the buffer additionally containing 0.7M $(NH_4)_2SO_4$. Whole fraction eluted with 0.7M $(NH_4)_2SO_4$ was collected as fraction B (1.0ml). Both fractions A and B were dialyzed against a buffer containing 50mM Tris-HCl (pH7.6), 0.1mM EDTA, 5mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and 0.01M $(NH_4)_2SO_4$ and stored at -80°C for further analysis. *In vitro* transcription assays of each and/or a combination of these fractions were carried out as described above.

## 2.3 Results

In wheat plastids, the *psb*D and *psb*C genes overlap by 50bp (Gray, 1987). Based on the homologous chloroplast genome maps of barley (Sexton *et al.*, 1990b) and rice (Hiratsuka *et al.*, 1989), two photosystem II genes (*psb*I and *psb*K) are predicted to be located upstream of the wheat *psb*D gene (Fig.2-1). From this gene cluster at least nine distinct transcripts ranging from 1.2 to 5.9kb are produced that accumulate in etioplasts of dark-grown seedlings (Fig.2-1) (Kawaguchi *et al.*, 1992). Upon illumination, two novel transcripts of 4.4 and 3.4kb accumulate (Kawaguchi *et al.*, 1992). All of these transcripts share six different 5'-ends, four of which have been suggested to be transcription initiation sites by *in vitro* capping analysis (Kawaguchi *et al.*, 1994). These sites are designated as D/C-1, D/C-2, *psb*D and *psb*C in Fig.2-1. The two light-induced transcripts share a common 5'-end, *psb*D site located 610bp upstream of the *psb*D translation initiation codon.

### 2.3.1 *In vitro* transcription of RNA from the *psb*D/C gene cluster

To elucidate the molecular mechanisms underlying the light induction of transcription from the *psb*D/C gene cluster, we prepared crude plastid extracts from dark-grown wheat seedlings and 4-h-illuminated seedlings, and examined their *in vitro* transcription activities for three transcriptional initiation sites D/C-2, *psb*D and *psb*C. Fig.2-2 shows the structure of the templates used for these analyses. The plasmid pW18E866 contains an 866bp EcoRI restriction fragment of wheat chloroplast DNA spanning the entire putative promoter regions of *psb*D site (LRP) and D/C-2. The plasmid pW18ES1145 bears an 1145bp EcoRI-SacI fragment of wheat chloroplast DNA, that contains the putative promoter region of *psb*C. The latter promoter is located on the

3' coding region of *psb*D and produces *psb*C gene-specific transcripts. After the *in vitro* transcription reactions, the amounts of each transcript were assessed by S1 nuclease protection experiments using the ClaI-HindIII probe or the EcoRI-HindIII probe, both of which were designed not to give a signal from the endogenous chloroplast RNA or DNA, if they exist (see Fig.2-2). We found previously from run-on transcription experiments that the overall *in vivo* transcription rate of the *psb*D/C gene cluster was transiently enhanced about 5-fold during the first 2 to 6h after transfer to the light, and then declined to a level lower than that before illumination (Kawaguchi *et al.*, 1992). Taking into account these findings and those from barley (Sexton *et al.*, 1990a), the effect of illumination was mainly examined by using the plastid extract prepared from 4-h-illuminated seedlings, rather than the chloroplast extract prepared from light-grown seedlings.

In Fig.2-4, the RNAs synthesized *in vitro* in the plastid extracts prepared from 5-day old dark-grown seedlings and 4-h-illuminated seedlings using template pW18E866 are shown (Fig.2-4A) and compared with the RNAs accumulated *in vivo* in the corresponding plastids (Fig.2-4B). S1 protection analysis of the *in vitro* synthesized RNAs from template pW18E866 in the plastid extract prepared from dark-grown seedlings yielded a major signal (522 bases) indicative of the initiation of transcription at the D/C-2 site (Fig.2-4A: lane 1 and lane 2). The full length (602 bases) S1 protected fragment detected as another major signal in lane 1 through lane 3 may be caused by transcription from the *lac*Z promoter in the template, since the signal intensities of their bands are significantly stronger than that of the corresponding bands in the control experiments without template or without extract shown in lane 4 through 7. A significant difference in the intensities of these two signals detected at 522 and 602 bases was not observed between the two different plastid extracts from dark-grown and 4-h-illuminated seedlings. However, a significant difference was observed in the intensity of the signal corresponding to the transcript of 370 bases. The signal intensity at 370 bases was

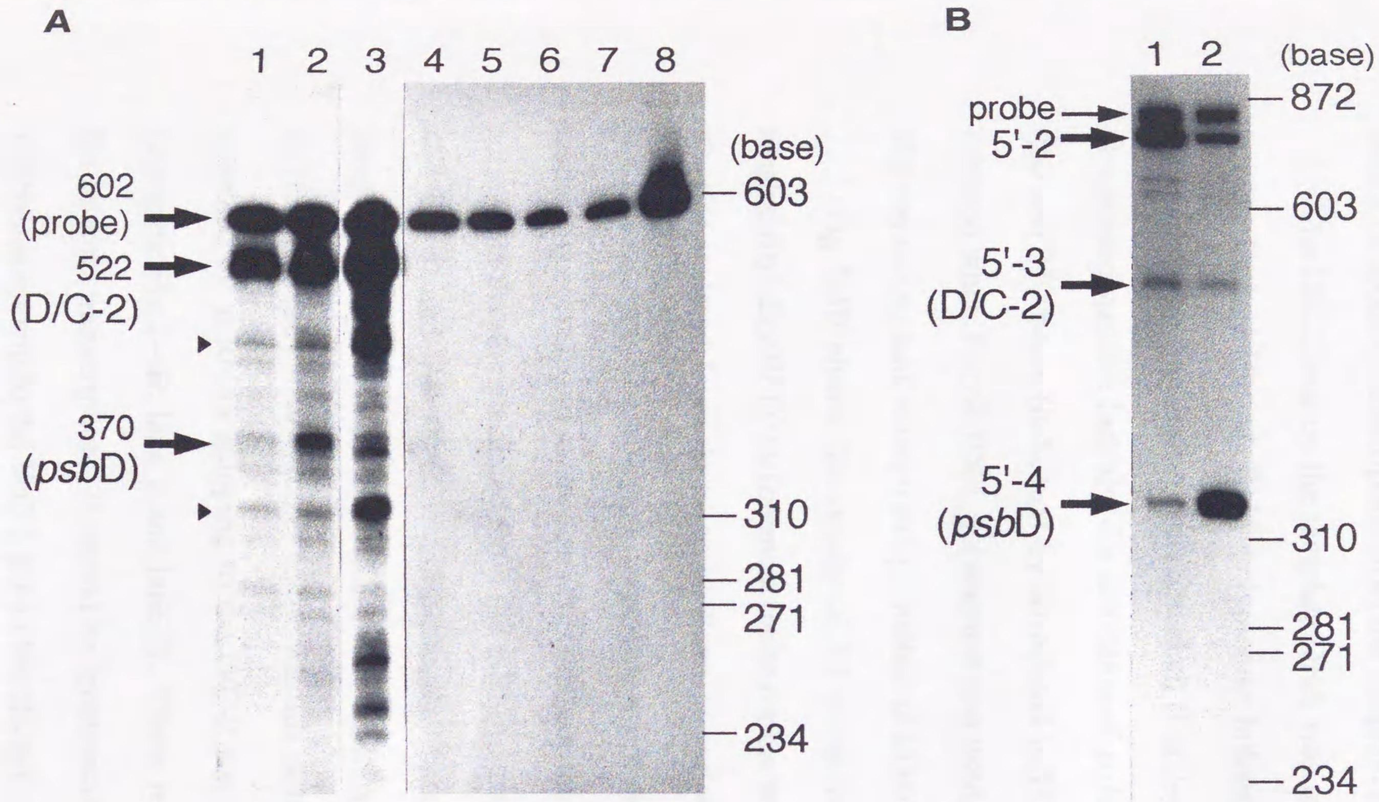



Fig.2-4 Comparison of *in vitro* synthesis of RNAs from D/C-2 and *psb*D sites in the plastid extracts and *in vivo* accumlation of the mRNAs mapping to the corresponding sites.

A. S1 nuclease protection analysis was carried out for the transcripts synthesized in plastid high-salt extract from dark-grown and 4 h-illuminated seedlings, respectively. Lane 3 shows the result with *E. coli* RNApolymerase holoenzyme (0.5 unit/40μl). Lanes 4 and 5 are control reactions where no template DNA was added to the extracts from dark-grown and 4h-illuminated seedlings, respectively. Lane 6 is a control reaction without extract. Probe alone before (approximately 1000cpm) and after treatment with S1 nuclease are also presented, in lanes 8 and 7, respectively. The size of the transcripts synthesized *in vitro* are indicated on the left and the sites of transcription initiation are in parenthesis. The full-length (602 bases) bands correspond to the undigested probe. Numbers to the right indicate the sizes of ϕX174 -HaeIII mol wt makers. B. S1 nuclease protection analysis was carried out for the mRNAs accumlated *in vivo* in the plastids isolated from the dark-grown and 4h-illuminated seedlings. Lanes 1 and 2 show the results with the plastids isolated from the dark-grown and 4h-illuminated seedlings, respectively. 5'-2, 5'-3 (D/C-2) and *psb*D (D/C-3) are indicated by thick arrows and undigested probe by the thin arrow. Numbers to the right indicate the size of ϕX174-HaeIII mol wt makers.

greatly enhanced by using the extract from 4-h-illuminated seedlings (Fig.2-4A: lane 2). The size of the labeled band corresponds to the size of the transcript that is expected to initiate at the *psb*D site. Control reactions without template (Fig.2-4A: lanes 4 and 5) or without extract (Fig.2-4A: lane 6) gave no signal except for the full length signal which may due to probe reannealing, supporting the hypothesis that these signals are indeed the results of specific transcription from the template DNA.

The D/C-2 site on the template DNA was recognized as a transcriptional initiation site by *Escherichia coli* RNA polymerase holoenzyme, but transcripts mapping to the *psb*D site were significantly less abundant (Fig.2-4A: lane 3), suggesting the requirement of a plastid specific factor(s) for activation of *psb*D LRP. Two weak signals detected at 450 and 310 bases (indicated by arrowhead in Fig.2-4A) in both the extracts were also detected when *E. coli* RNA polymerase was used, suggesting that prokaryotic promoter like sequences may act as weak promoters in *in vitro* transcription.

Fig.2-4B shows the results of S1 protection analysis carried out using the $^{32}P$ labeled StyI-EcoRI DNA fragment probe for the mRNAs which accumulate *in vivo* in the plastids isolated from dark-grown (lane 1) and 4-h-illuminated seedlings (lane 2). In both cases, six S1 protected fragments were detected. The sizes of four of these fragments correspond to the sizes expected for the undigested probe and the S1 protected fragments that are produced from the mRNAs mapping to 5'-2 (750 bases), 5'-3 (D/C-2, 471 bases) and 5'-4 (D/C-3: *psb*D site, 319 bases). Two minor fragments may due to insufficient digestion by S1 nuclease. The mRNAs mapping to the *psb*D site (D/C-3 site) increased greatly in abundance in the plastids isolated from 4-h-illuminated seedlings. In contrast, the mRNAs mapping to the D/C-2 site slightly decreased by 4-h-illumination (compare Fig.2-4B: lane 1 and lane 2). These results are consistent with the results of the *in vitro* transcription. It should be mentioned, however, that the *in vivo* level of the mRNAs mapping to the D/C-2 site in the plastids isolated from 4-h-illuminated seedlings appears to be significantly lower than the level of the mRNAs mapping to the *psb*D site.

This behavior can not be explained by the results of the *in vitro* transcription and may be due to the difference in the stability of these two mRNAs *in vivo*. The level of the mRNAs mapping to 5'-2 significantly decreased in the plastids isolated from 4-h-illuminated seedlings compared with that in the plastids from dark-grown seedlings.

Fig.2-5 shows the results of *in vitro* transcription from template pW18ES1145 in three types of plastid extracts from dark-grown, 4-h-illuminated and light-grown seedlings. The level of the transcripts mapping to the *psb*C site (the 397 bases signal) was similar between the plastid extracts from dark-grown and 4-h-illuminated seedlings (Fig.2-5: lanes 1 and 2, respectively). In contrast, the signal obtained using the extract from the mature chloroplasts seems to be considerably weaker than that from the other two extracts (Fig.2-5: lane 3). These results are consistent with the accumulation of the transcripts from the *psb*C site observed *in vivo* (Kawaguchi *et al.*, 1994). *E. coli* RNA polymerase holoenzyme can also initiate transcription at the *psb*C site. Control reactions in the plastid extract from 4-h-illuminated seedlings without template DNA (Fig.2-5: lane 5) and without the plastid extract (Fig.2-5: lane 6) gave no signal.

### 2.3.2 *In vitro* RNA processing in the plastid extract

To confirm that the fragments giving the signals at the positions corresponding to the D/C-2 and *psb*D site in Fig.2-4A are the primary transcripts rather than the products of processing, we examined *in vitro* processing in the plastid extract of the RNAs synthesized using the *psb*D/C templates. In Fig.2-6, the results obtained with the 956 base RNAs synthesized from the template pW73E866 linearized at EcoRV site (see Fig.2-3) are shown. Besides the signal of the synthesized RNAs of 956 bases, two distinct and a few weak signals are detected on the autoradiography. The triangles on the right side of lane 2 in Fig.2-6 show the positions of the expected fragments, if the synthesized RNAs of 956 bases were processed at the 5'-2, 5'-3 (D/C-2) and 5'-4

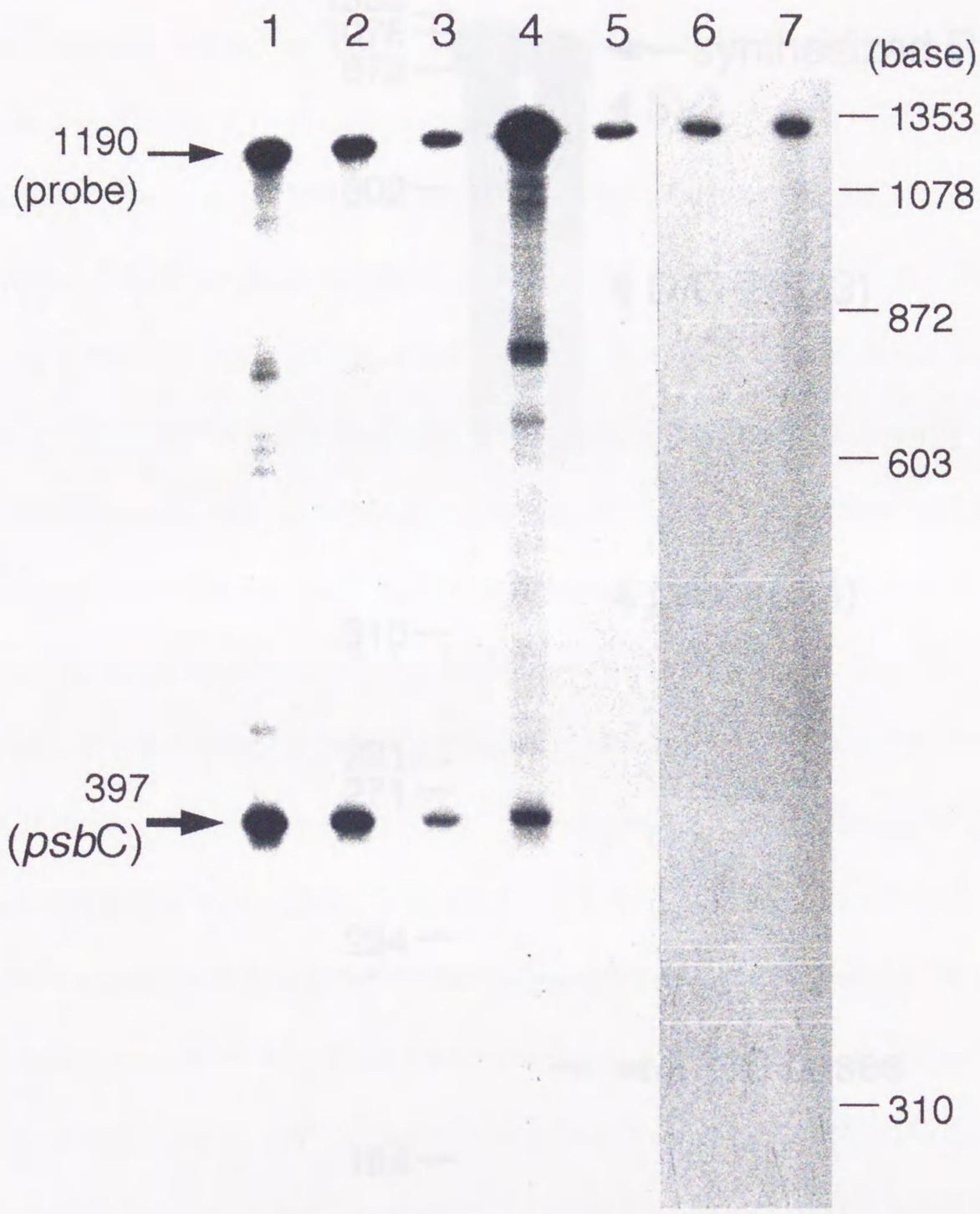



Fig.2-5 S1 nuclease protection analysis of the transcripts synthesized in plastid high-salt extracts from wheat seedlings.

*In vitro* transcription was carried out using template pW18ES1145. Lanes 1 and 2 show the results with the extracts from dark-grown and 4h-illuminated seedlings, respectively. Lane 3 shows the result with the extract from mature chloroplasts isolated from 5-days light-grown seedlings. Lane 4 shows the result with *E. coli* RNA polymerase holoenzyme (0.5 unit/40μl). Lanes 5 and 6 are control reactions without template in the presence of the extract from 4h-illuminated seedlings and without the extract, respectively. Probe alone (approximately 1000 cpm) is also presented , in lane 7.

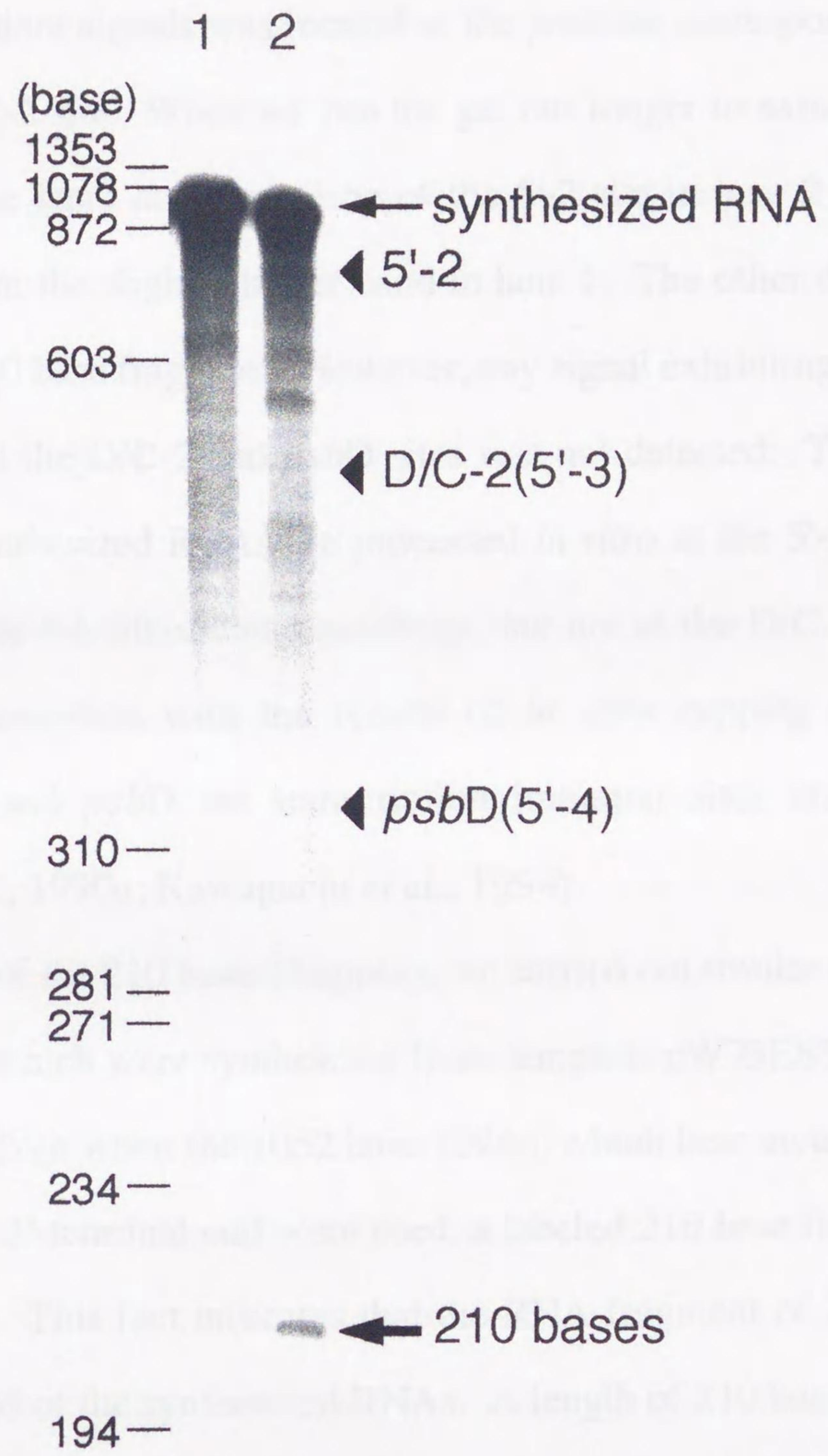



Fig.2-6 *In vitro* RNA processing in the plastid extract.

The 956-base RNAs synthesized from the linearized template (pW73E866) at the EcoRV site were incubated in the mixture used for *in vitro* transcription with the plastid high-salt extract from 4h-illuminated seedlings (lane 2) or without extract (lane 1). The arrowheads on the right side of lane 2 show the positions of the fragments expected if the 956-base RNAs were processed at the 5'-2, D/C-2 and *psb*D sites. Numbers to the right indicate the size of ϕX174-HaeIII mol wt makers.

(*psb*D) sites. One of the distinct signals was located at the position corresponding to the fragment processed at the 5'-2 site. When we run the gel out longer to expand the high molecular weight region, the band at the position of the 5'-2 site in lane 2 was clearly observed to be different from the slightly larger band in lane 1. The other distinct band exhibited production of a 210 base fragment. However, any signal exhibiting production of fragments with 5' ends at the D/C-2 and *psb*D sites was not detected. These results strongly suggest that the synthesized RNAs are processed *in vitro* at the 5'-2 site in the plastid extract prepared from 4-h-illuminated seedlings, but not at the D/C-2 and *psb*D sites. These results are consistent with the results of *in vitro* capping experiments demonstrating that D/C-2 and *psb*D are transcription initiation sites and 5'-2 is a processing site (Sexton *et al.*, 1990a; Kawaguchi *et al.*, 1994).

To identify the origin of the 210 bases fragment, we carried out similar experiments using the 1052 base RNAs which were synthesized from template pW73E866 linearized at NdeI site (see Fig.2-3). Even when the 1052 base RNAs, which bear an additional 96 base vector sequence at the 3'-terminal end were used, a labeled 210 base fragment was produced (data not shown). This fact indicates that the RNA fragment of 210 bases is not produced from the 3'-end of the synthesized RNAs. A length of 210 bases is slightly longer than that expected for the 3'-end fragment of the synthesized RNAs processed at the 5'-2 site (199 bases). As shown in Fig.2-1, a transcript of 1.2kb accumulates in the plastids from both dark-grown and 4-h-illuminated seedlings. If the 210 base fragment arises from the processing 210 bases downstream from the 5'-end of the synthesized RNA, the processing site should be 1030 bases upstream of the initiating ATG of *psb*D gene. As shown in Fig.2-3, this site is coincident with the position of the 3'-end of the 1.2kb transcript (within experimental error). Consequently, it is not unreasonable to suppose that the 210 base fragment arise from a processing event that determines the 3'-end of the 1.2kb transcript.

### 2.3.3 Separation of light-responsive transcription factor from RNA polymerase

As a first step to isolate the light-induced factor(s) which activate *psb*D LRP from the plastid extract of 4-h-illuminated seedlings, we tried to separate the factor(s) from RNA polymerase using a heparin-Sepharose column. Intact plastids from 4-h-illuminated seedlings were disrupted by treatment with Triton X-100. The lysates were subjected to a heparin-Sepharose column and divided into two fractions containing the nonabsorbed components (fraction A) and absorbed components (fraction B). The *in vitro* transcriptional activities of both fractions were then examined. In Fig.2-7, the results obtained with template pW18E866 and with fraction A, fraction B or both are shown. Addition of fraction A into the reaction mixture of *in vitro* transcription did not give any significant signal except for the full length signal (602 bases) which may due to probe reannealing (Fig.2-7: lane 1). In contrast, fraction B produced transcripts from the D/C-2 site (522 bases) and *lac*Z promoter located in the vector sequence (602 bases) (Fig.2-7: lane 2). This indicates that the plastid RNA polymerase is present in fraction B presumably together with a σ-like factor(s), consistent with results previously reported (Eisermann *et al.*, 1990; Tiller and Link, 1993a). Interestingly, however, transcription from the *psb*D site was not observed by the addition of fraction B alone in the reaction mixture. Transcription from the *psb*D site was recovered by the simultaneous addition of fraction A and fraction B (Fig.2-7: lane 3). These results indicate that light-regulated transcription factor(s) were recovered in the fraction containing the nonabsorbed components by heparin-Sepharose (fraction A) as substantially separated from the plastid RNA polymerase. In lane 1 of Fig.2-7, extremely weak signals are detectable at the positions of D/C-2 and *psb*D. These signals may be a result of contamination of very small amounts of the plastid RNA polymerase in fraction A.

To confirm the existence of the factor(s) in fraction A, the ability of fraction A to induce the transcriptional activity from the *psb*D site in the extract from the plastids of

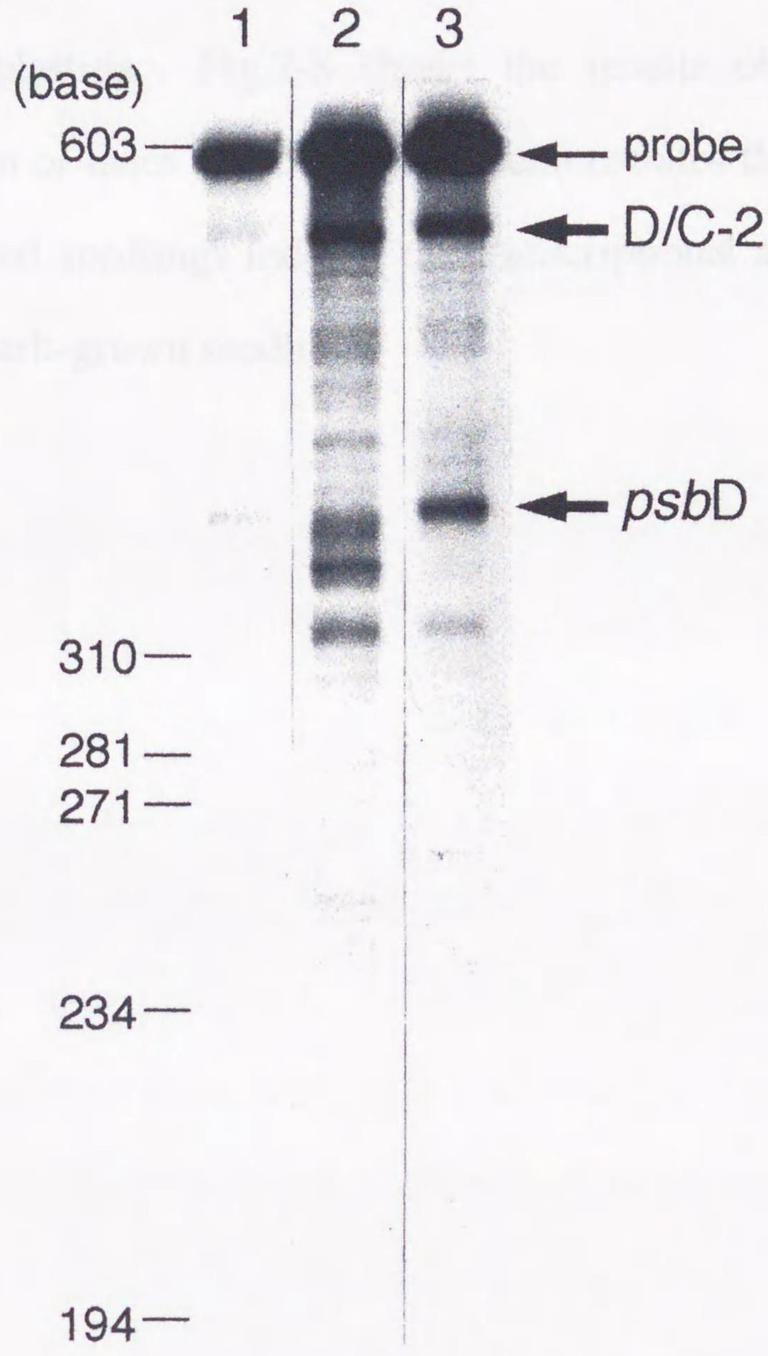



Fig.2-7 *In vitro* transcription activities of fractions A and B from the 4h-illuminated plastid lysate separated by a heparin-Sepharose column.

*In vitro* transcription was carried out using plasmid pW18E866 as a template. The results with 8μl of fraction A (lane 1), 8μl of fraction B (lane 2) and 8μl each of fraction A and B (lane 3) in 40μl of *in vitro* transcription reaction mixture are shown. The signals corresponding to transcription from the D/C-2 and *psb*D sites are designated by thick arrows. Numbers to the left indicate the size of ϕX174-HaeIII mol wt makers.

dark-grown seedlings was examined. In this experiment, the lysate prepared by Triton X-100 treatment of the plastids from dark-grown seedlings was used instead of the high salt extract from the plastids. Fig.2-8 shows the results obtained with template pW18E866. Comparison of lanes 2 and 3 clearly demonstrates that fraction A from the plastids of 4-h-illuminated seedlings induces the transcriptional activity from the *psb*D site in the extract from dark-grown seedlings.

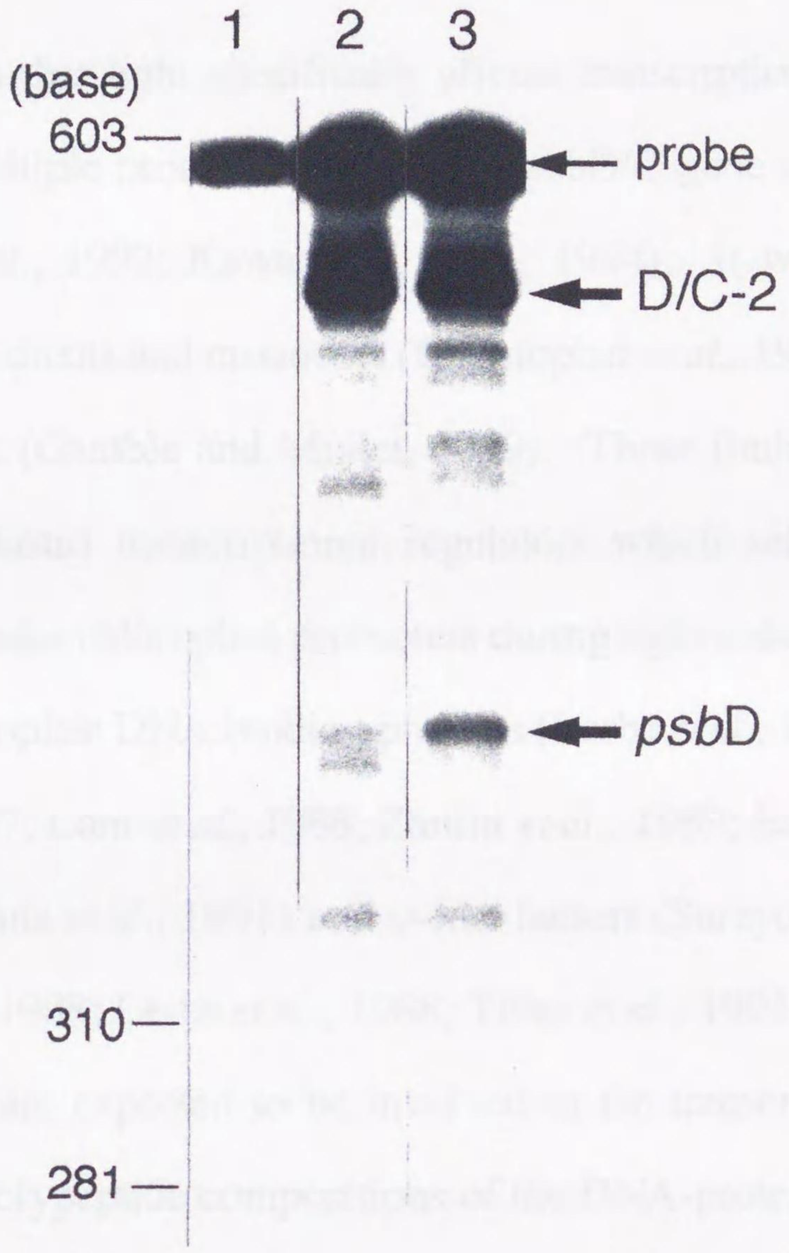



Fig.2-8 Induction of the transcriptional activity from the *psb*D site in the plastid lysate from dark-grown seedlings by the addition of fraction A.

*In vitro* transcription was carried out using plasmid pW18E866 as a template. Lanes 1and 2 show the results with 12μl of fraction A from the plastid lysate of 4h-illuminated seedlings and 4μl of the plastid lysate from dark-grown seedlings, respectively, in 40μl of *in vitro* transcription reaction mixture. Lane 3 shows the results of the simultaneous addition of 4μl of the plastid lysate of dark-grown seedlings and 12μl of fraction A. The signals corresponding to the transcriptions from the D/C-2 and *psb*D sites are designated by the thick arrows. Numbers to the left indicate the size of ϕX174-HaeIII mol wt makers.

## 2.4 Discussion

It has been shown that light specifically affects transcription from one particular promoter among the multiple promoters present in psbD/C gene cluster (Sexton *et al.*, 1990a; Christopher *et al.*, 1992; Kawaguchi *et al.*, 1994). It was reported that this behavior is conserved in dicots and monocots (Christopher *et al*,. 1992) and the promoter is affected by blue light (Gamble and Mullet, 1989). These findings may indicate the existence of specific plastid transcriptional regulators which selectively activate the transcription from particular chloroplast promoters during light-induced development.

A number of chloroplast DNA binding proteins (Lerbs *et al.*, 1983; Reiss and Link, 1985; Bulow *et al.*, 1987; Lam *et al.*, 1988; Zaitlin *et al.*, 1989; Eisermann *et al.*, 1990; Baeza *et al.*, 1991; Khanna *et al.*, 1991) and σ-like factors (Surzycki and Shellenbarger, 1976; Bulow and Link, 1988; Lerbs *et al.*, 1988; Tiller *et al.*, 1991) have been reported. Some of these proteins are expected to be involved in the transcriptional regulation of plastid genes. Indeed, polypeptide compositions of the DNA-protein complexes isolated from the plastids of mustard seedlings were found to vary, depending on the stage of plastid development (Reiss and Link, 1985). The level of the plastid DNA-binding factor 1 in bundle-sheath extracts was reported to be 3-fold higher than in mesophyll cell extracts in Maize (Lam *et al.*, 1988). The proteins binding to the promoter of *psb*A in the plastids of mustard seedlings were suggested to be different between chloroplasts and etioplasts (Eisermann *et al.*, 1990). Thus, the protein components which interact with the promoter region of plastid genes appear to vary depending on the stage of plastid development and the cell type.

In this work, it was observed that among three transcriptional initiation sites (D/C-2, *psb*D and *psb*C sites) in the *psb*D/C gene cluster, *in vitro* transcription from the *psb*D site was selectively enhanced in the plastid extract prepared from 4-h-illuminated wheat seedlings compared with that from dark-grown seedlings. This behavior well reflects the

feature of *in vivo* light-regulated transcription from the *psb*D/C gene cluster (Sexton *et al.*, 1990a; Kawaguchi *et al.*, 1994) indicating that transcriptional regulation is actually involved in light-induced variations of the *psb*D/C gene transcripts.

Mutational analyses using chloroplast *in vitro* transcription systems have suggested that prokaryotic '-35' and '-10' sequences are required for the transcription of many plastid genes (Link, 1984; Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985a; Gruissem and Zurawski, 1985b). Upstream of the transcription initiation sites at D/C-2 and *psb*C in wheat *psb*D/C gene cluster, DNA sequences similar to the prokaryotic promoter motifs are present. These motifs are also present in several other positions of the plastid DNA sequences in the templates, some of which are weakly but significantly recognized as promoters in *in vitro* transcription by the plastid extracts. The activities of *in vitro* transcription from the D/C-2, *psb*C and other prokaryotic promoter-like motifs in the templates are independent of the plastid extracts used, and these promoter-like motifs including the D/C-2 and *psb*C promoters were also recognized by *E. coli* RNA polymerase holoenzyme. *Psb*D LRP is highly conserved within the 60-bp upstream region of the *psb*D site in higher plants (wheat, barley and tobacco), but the putative '-35' element of *psb*D LRP only minimal sequence similarity to the prokaryotic consensus motif and the spacing between them is anomalous (15-bp instead of 17-19 bp). Transcription from the *psb*D site was scarcely observed in the plastid extracts from dark-grown seedlings and was not initiated by *E. coli* RNA polymerase. Judging from the present results, transcription from the *psb*D site requires additional factor(s).

Two possible mechanisms underlying the initiation of transcription from the *psb*D site may be considered. The first possibility is the light induction or activation of transcriptional regulatory factor(s), which specifically activates the promoter at the *psb*D site or releases the specific depression of the *psb*D LRP activity in the plastids of unilluminated seedlings. In either case, such light-induced regulatory factor(s) should be collected in fraction A, because fraction A (no RNA polymerase activity) induces

transcriptional activity from the D/C-3 site in the plastid extract from dark-grown seedlings. Extensive works have been carried out by several groups to identify the transcriptional regulatory *trans* factor(s) and the *cis* element on which the *trans* factor(s) binds.

From a series of mutational analyses, two upstream *cis* elements involved in the *psb*D LRP activity were determined (Nakahira *et al.*, 1996). The first element ( AAAGTAAGT) is located between -54 and -46 and contains short repeated sequences. The presence of a DNA binding factor (designated as AGF) which recognizes the AAGT repeat was demonstrated in barley chloroplast extract (Kim and Mullet, 1995). The second element (GACCTGACT) which also acts as an enhancer for the *psb*D transcription in wheat is positioned between -44 and -36 and contains another repeated sequences (GACC/T). The protein recognizing the GACC repeat was suggested to exist in tobacco chloroplast extract (Allison and Maliga, 1995). At a first sight, presence of a protein(s) which specifically binds to the repeat sequences in the chloroplast extract seems to suggest that regulation of the promoter activity by light is directed *via* interaction between the sequences and the DNA-binding protein(s). Nevertheless, it was finally demonstrated to be unlikely that these sequences themselves are the light switch of *psb*D LRP, because deletion of both of these sequences significantly reduced the transcription in the light extract, but not down to the level in the dark extract (Nakahira *et al.*, 1996). From DNA mobility shift assay with a DNA fragment of -56 through -34 which encompasses the sequences of the two upstream *cis* elements of *psb*D LRP, they demonstrated that the protein(s) which recognizes specifically the upstream *cis* elements and forms DNA/protein complex was detected with the same strength in the light and dark plastid extracts prepared from dark-adapted and re-illuminated seedlings, indicating that the protein(s) equally presents in the chloroplasts of both seedlings, and the affinity of the protein(s) to the upstream *cis* elements was not altered at all by light. Thus, it seems very likely that the two upstream sequences act as general enhancer to maintain the

full activity of the promoter under light rather than the light regulatory element(s) which plays a key role in light- switching of *psb*D LRP.

The second possibility is that there is light-induced modulation of RNA polymerase through the modification of σ-like factors. In prokaryotes, different σ factors are used for transcription of various classes of genes (Helmann and Chamberlin, 1988). In mustard, RNA polymerase preparations from chloroplasts and etioplasts are functionally distinct, with respect to salt requirements for DNA binding, affinity to the *psb*A promoter and the stability of the core enzyme σ-like factor complexes, although they are structurally similar to each other (Tiller and Link, 1993a). Recently, it has been demonstrated that reversible phosphorylation (etioplast) and dephosphorylation (chloroplast) of both the core enzyme and σ-like factors determine the plastid-type specific properties of the RNA polymerase (Tiller and Link, 1993b). In the case of mustard, the σ-like factors dissociated from the plastid RNA polymerase holoenzyme tightly bind to heparin sepharose and are only eluted by very high-salt (1.0-1.3M $(NH_4)_2SO_4$) (Tiller and Link, 1993a). This is in contrast to the case of the light-induced regulatory factor(s) collected in fraction A as nonbinding component(s) on heparin-Sepharose columns. Therefore, if the regulatory factor(s) collected in fraction A is a kind of σ-like factor, they should be very different from the σ-like factors found in mustard in the affinity to heparin-Sepharose.

# 3. Light-Dependent Alternation of Chloroplast RNA Polymerase in Recognition of Core Promoter Structure: A Key Event of Light Responsive Behavior of the *psb*D Promoter

## 3.1 Introduction

Typical plastid promoters contain hexanucleotide sequences resembling bacterial '-10' (TATAAT) and '-35' motifs (TTGACA) (Hanley-Bowdoin and Chua, 1987), indicating that they are structurally and functionally similar to *E.coli* $\sigma^{70}$ type promoters (Reznikoff *et al.*, 1985). Although *E. coli* like promoter elements are required for transcription initiation of plastid genes, some plastid promoters are different in their structure from the general *E. coli* $\sigma^{70}$ type (Gruissem *et al.*, 1986; Neuhaus *et al.*, 1989; Baeza *et al.*, 1991; Klein *et al.*, 1992; Vera and Sugiura, 1995), suggesting the presence of differential promoter usage by multiple types of plastid RNA polymerase. The existence of at least two types of RNA polymerases, *E. coli*-like RNA polymerases (Lerbs *et al.*, 1988; Little and Hallick, 1988) and nuclear-encoded non-*E.coli*-like RNA polymerases (Morden *et al.* 1991; Hess *et al.*, 1993; Lerbs-Mache, 1993; Pfannschmidt and Link, 1994) has been documented in plastids. The former is composed of the subunits dominantly encoded by the plastid genome and is assumed to transcribe preferentially the genes encoding the proteins implicated in the characteristic functions of chloroplast. While, the latter RNA polymerase was recently suggested to function in the transcription of some plastid genes encoding the transcriptional/translational apparatus during early phases of plastid development (Allison *et al.*, 1996; Kapoor *et al.*, 1997).

As mentioned before, the *psb*D LRP, a unique light-responsive promoter in *psb*D/C operon, has the putative '-35' motif which exhibits only minimal sequence similarity to the bacterial consensus motif. It was recently proposed from *in vivo* (Allison and

Maliga, 1995) and *in vitro* (Kim and Mullet, 1995; To *et al.*, 1996; Satoh *et al.*, 1997) analyses that transcription of *psb*D is controlled *via* upstream *cis* elements and specific proteins which recognize these sequences. However, precise mutation analysis of these upstream sequences by means of *in vitro* transcription using the extracts of chloroplasts prepared from dark-adapted and short term illuminated wheat seedlings lead to a conclusion that they act as enhancers, but are not responsible for the light-dependent on/off switch of the *psb*D transcription (Nakahira *et al.*, 1996, Satoh *et al.*, 1997, see Fig.3-1). On the other hand, the *psb*A gene is monocistronically transcribed from a promoter which contains bacterial type '-35' and '-10' motifs in the core promoter region and the apparent transcription activity is independent of light condition at least in mature wheat chloroplasts (Satoh *et al.*, 1997). These results suggest that the sequences in the core promoter region may be responsible for the light-responsive transcription.

In this section, I report evidence indicating light-dependent alternation of chloroplast RNA polymerase in recognition of core promoter structure. Systematic mutational analyses of the *psb*A and *psb*D promoters carried out by means of *in vitro* transcription, using the wheat chloroplast extracts prepared from the dark-adapted and re-illuminated seedlings. It will be shown that the plastid-encoded bacterial type RNA polymerases primarily function in matured chloroplasts and that their promoter recognition pattern differs in the dark-adapted and reilluminated wheat seedlings and this alternation of the RNA polymerase in the core promoter recognition is the main event of the light responsive behavior of the *psb*D LRP.

## 3.2 Materials and Methods

### 3.2.1 Cloned DNAs and plasmid constructions

Plasmids pW18E866, pW18ES472A, and pW18ES1145 containing sequences of *psb*D LRP (-551 to +315) and the *psb*A (-194 to +278) and *psb*C (-793 to +352) promoters, respectively (Fig.3-1) were utilized for preparation of the DNA constructs. A series of 5' deletion fragments which have 5' ends at -234, -119, -78 and -36 and 3' end at +315 of the *psb*D site were amplified by means of PCR from pW18E866 with primers shown in table 3-1 and cloned between BamHI and EcoRI sites of pSP73 to give plasmids pW73BE549, *psb*D/-119, *psb*D/-78 and *psb*D/-36, respectively. A 472-bp EcoRI-SacI fragment of pW18ES472A was subcloned into pSP72 to give a plasmid pW72ES472A. Deletion fragments of the *psb*A promoter which have 5' ends at -118 and -38 and 3' end at +278 were amplified from pW18ES472A, blunted by T4 DNA polymerase, subsequently cut by SacI at 3' end and cloned between EcoRV and SacI sites of pSP72 to give plasmids *psb*A/-118 and *psb*A/-38, respectively. DNA fragments encompassing sequences from -119 through +5 of the *psb*D site and from -118 through +3 of the *psb*A site were also amplified by PCR and cloned between BamHI and EcoRI sites of pSP73 to give a plasmid *psb*D/+5 and EcoRI and SacI sites of pSP72 to give a plasmid *psb*A/+3, respectively.

Mutations in the *psb*A promoter were introduced by PCR using *psb*A/-38 encompassing sequence between -38 and +278 as a template. The PCR primers used for introduction of mutations are listed in table 3-1. The resulting mutated fragments were blunted by T4 DNA polymerase, subsequently cut by SacI at 3' end and cloned between EcoRV and SacI sites of pSP72 to give plasmids *psb*A/MT-1 through *psb*A/MT-7. Double mutations were introduced into the '-35' element and the sequences in spacing region of the *psb*A promoter by PCR to give the plasmids *psb*A/MT-8 and *psb*A/MT-9.

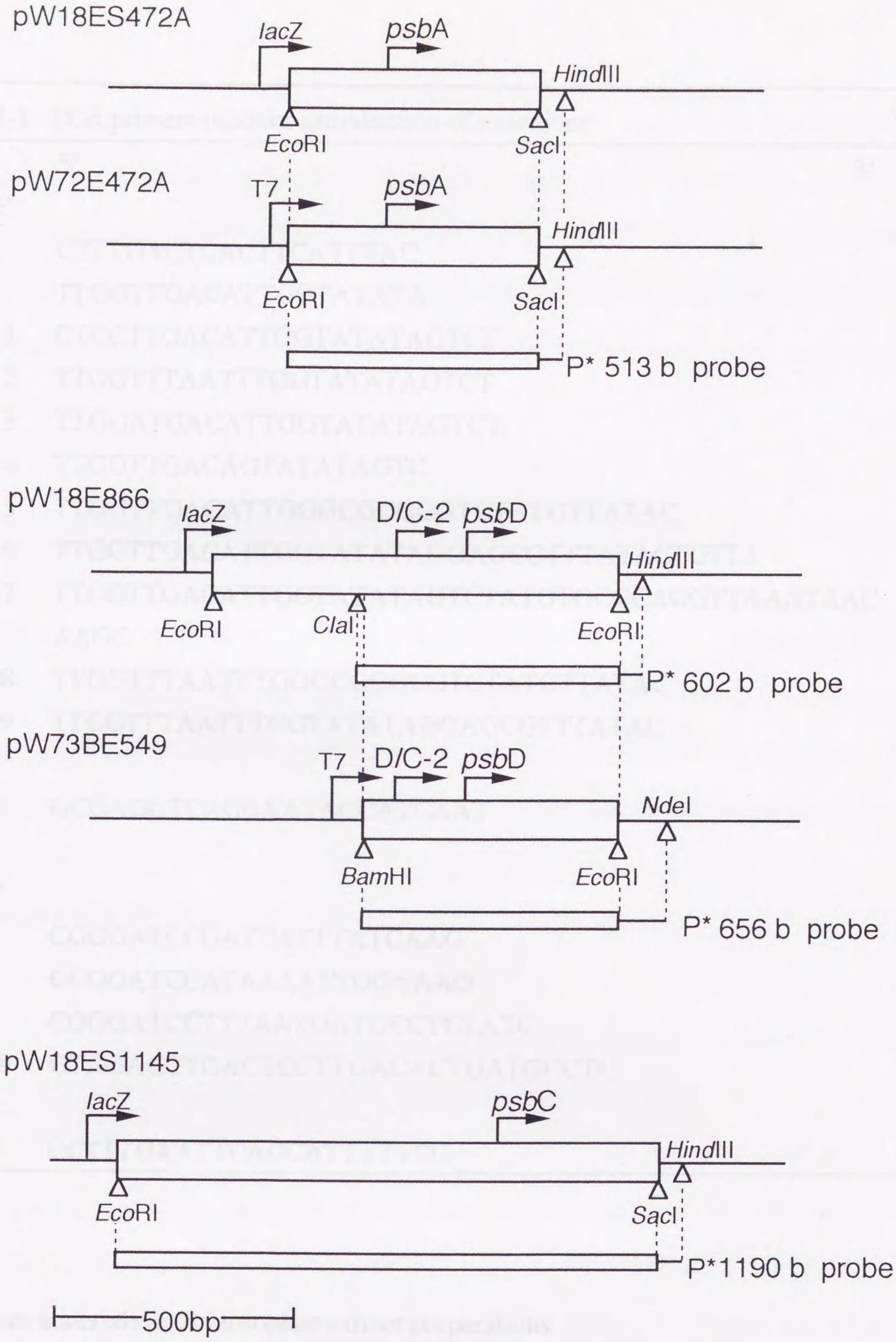



Fig.3-1 Schematic representations of the truncated *psb*A, *psb*D and *psb*C constructs used for analysis of *in vitro* transcription.

The arrows show the direction and sites of transcription initiation. The boxed regions indicate wheat chloroplast DNA sequences and the single lines indicate vecter sequences. Probes used for S1 protection analysis of the *in vitro* synthesized RNAs are shown below the figures of their respective plasmids.

Table 3-1 PCR primers used for introduction of mutations

| | 5' | 3' |
|---|---|---|
| *psb*A 5' | | |
| -118 | CTTTGACTCACTTCATTTAC | |
| -38 | TTGGTTGACATTGGTATATA | |
| MT-1 | CTCCTTGACATTGGTATATAGTCT | |
| MT-2 | TTGGTTTAATTTGGTATATAGTCT | |
| MT-3 | TTGGATGACATTGGTATATAGTCT | |
| MT-4 | TTGGTTGACAGTATATAGTC | |
| MT-5 | TTGGTTGACATTGGGCGCGCGTCTATGTTATAC | |
| MT-6 | TTGGTTGACATTGGTATATAGGAGCGTTTATACTGTTA | |
| MT-7 | TTGGTTGACATTGGTATATAGTCTATGTGCGCAGGTTAAATAAC AAGC | |
| MT-8 | TTGGTTTAATTTGGGCGCGCGTCTATGTTATAC | |
| MT-9 | TTGGTTTAATTTGGTATATAGGAGCGTTTATAC | |
| *psb*A 3' | GCGAGCTCACGAATACCATCAAT | |
| *psb*D 5' | | |
| -119 | CGGGATCCGATTATTTATCAAG | |
| -78 | GCGGATCCATAAAATTGGAAAG | |
| -36 | CGGGATCCTTTAATGATGCCTCTATC | |
| MT-9 | GTAGACCTGACTCCTTGACACTGATGCCTC | |
| *psb*D 3' | CCTTTGAATTCAGCATTATTCC | |

### 3.2.2 Plant materials and chloroplast extract preparations

Wheat (*Triticum aestivum*) were grown under white light (approx 5,000 lx) at 25 °C. The 5-days old light-grown seedlings were shifted to the dark and kept there for 24 h (dark-adapted seedlings) or again transferred to the light for 2 h after 24 h dark-adaptation (re-illuminated seedlings) and harvested. Intact chloroplasts were isolated by

centrifugation of the cell lysates from each of the seedlings on a percoll gradient (10 to 80 %), from which the chloroplast high-salt extracts were prepared as previously described (Satoh *et al.*, 1997; Wada *et al.*, 1994, see section 2). The chloroplast extracts prepared from the dark-adapted seedlings and re-illuminated seedlings are designated as dark extract and light extract, respectively and submitted to the *in vitro* transcription experiments.

### 3.2.3 Cycloheximide treatment of plants

The 24 h dark-adapted leaves cut in the dark were placed in small beakers containing 20 mM Hepes-KOH (pH8.0) solution with or without cycloheximide (20 $\mu$g/mL) and incubated in darkness for 1 h under cool air blowing. After the incubation, the seedlings were illuminated for 1 h and submitted to preparation of the chloroplast extract.

### 3.2.4 *In vitro* transcription

*In vitro* transcription was carried out in the chloroplast extracts with each of the DNA fragments inserted in pSP72 or pSP73 vector as template (super coiled) and RNA synthesized *in vitro* was isolated to be analyzed by S1 nuclease protection assay as described in section 2. In *in vitro* inhibition experiment by tagetitoxin, plasmids pW18E866 containing sequences of *psb*D LRP (-551 to +315) is used as template DNA and 16μl of dark-, light-extracts or 0.1unit of *E.coli* RNA polymerase holoenzyme (Pharmacia) incubated with or without 30μM Tagetin™RNApolymerase inhibitor (Bokusui Brown) in 40μl of *in vitro* transcription reaction mixture. A 512-bp EcoRI-HindIII fragment from pW72ES472A (for *psb*A transcripts) and a 656-bp BamHI-NdeI fragment from pW73BE549 (for *psb*D transcripts) containing 49 and 107-bp vector sequences, respectively, were $^{32}$P labeled at the BamHI and NdeI sites within the vector

sequences and used as S1 probes for *in vitro* transcripts.

### 3.2.5 Partial purification of chloroplast RNA polymerase

Plastids ($1.4x10^{10}$) were dispersed in a 7ml of high-salt buffer (see in section 2.2.3) and homogenated gently with a Teflon pestle 10sec for 5 times and stirred for 1h on ice. All subsequent operations were performed at 0-4 °C. The resulting chloroplast lysate was centrifugated at 30,000rpm (83,000g) for 30 min in a SRP70AT rotor (Hitachi). 6ml of supernatant was adjusted the concentration of $(NH_4)_2SO_4$ to 1M, and applied to a $1.77cm^2$ x 1.4cm of Phenyl-TOYOPEARL 650M (Tosoh TSKgel) hydrophobic column previously equilibrated with DG-25 buffer (50mM Tricine-KOH (pH8.0), 50mM KCl, 0.5mM EDTA, 0.5mM DTT, 0.5mM PMSF, 1mM benzamidine, 5mM ε-amino-n-caproic acid and 25% (v/v) glycerol) containing 1M NaCl and 1M $(NH_4)_2SO_4$. After washing with 20ml of DG-25 buffer containing 1M NaCl and 250mM$(NH_4)_2SO_4$, the absorbed materials were eluted with 15ml linear gradient from 250 to 0mM $(NH_4)_2SO_4$ and fractionated 3ml of each. Proteins in fractions were precipitated with 50% $(NH_4)_2SO_4$, collected by centrifugation at 26,000rpm (64,000g) for 20min in a SRP70AT rotor and resuspended in 0.3ml of DG-25 buffer. The resulting solutions were dialyzed against the DG-25 buffer using Spectra/Por (MWCO:6-8,000) over night at 4°C, frozen in liquid nitrogen and stored at -80°C. Transcriptional activity of each fraction was majored by *in vitro* transcription assay using vectors pW72ES472A (for *psb*A) and pW18E866 (for *psb*D) as templates. SDS-page was performed in 16% SDS-polyacrylamide gel at 250V, 11mA for 16h and stained with Silver Stain Kit (Wako).

## 3.3 Results

### 3.3.1 Comparison of the structures of the psbD LRP and psbA promoter

The third transcription initiation site in the *psb*D/C operon (*psb*D site) is located 610-bp upstream of the *psb*D translational initiation site in wheat. The promoter for this site (*psb*D LRP) reversibly responds to a light/dark regime in mature wheat chloroplasts (Satoh *et al.*, 1997). As shown in Fig.3-2, the 60-bp upstream region of the *psb*D site is highly conserved in higher plants. The conserved region contains putative '-35' and '-10' motifs. But the spacing between them is anomalous (15-bp instead of 17-19 bp) and the putative '-35' element only weakly resembles the prokaryotic consensus motif. Light-dependent transcription with full strength was observed with a template containing the sequences between -78 and +315 of the *psb*D site (Fig.3-2, *psb*D/-78). Deletion of all sequences upstream of the '-35' element (*psb*D/-36), strongly inhibited the transcription from the *psb*D site in the light extract, because *cis* element(s) required for *psb*D LRP to function exists in the upstream region of the core promoter (Nakahira *et al.*, 1996). However, it should be noticed that a weak but significant signal of the transcription from the *psb*D site was still observed in the light extract, even when the sequences in each of the two upstream regions was completely destructed or all sequence upstream of the putative '-35' element was deleted (*psb*D/-36). The signal of the transcription from the *psb*D site was always stronger in the light extract than in the dark one with any templates studied here. These behaviors are not due to the reduction of basic activity of the plastid transcription apparatus by dark adaptation of the seedlings, because the light and dark extracts exhibited equivalent transcription activity for the *psb*A promotor. Furthermore, the transcription from the fourth transcription initiation site (*psb*C site) in the *psb*D/C operon was always stronger in the dark extract than in the light extract (*psb*C/-793).

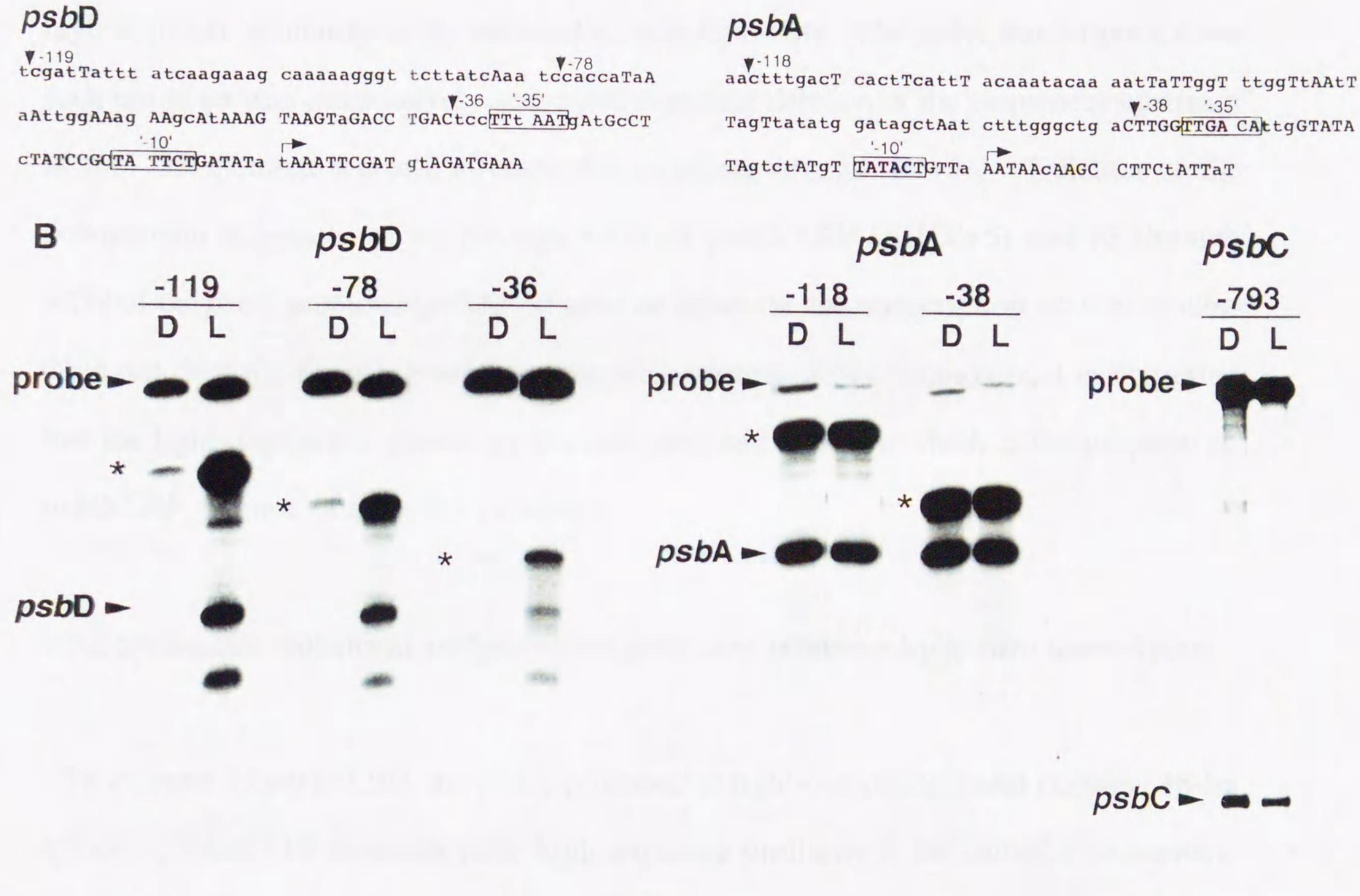


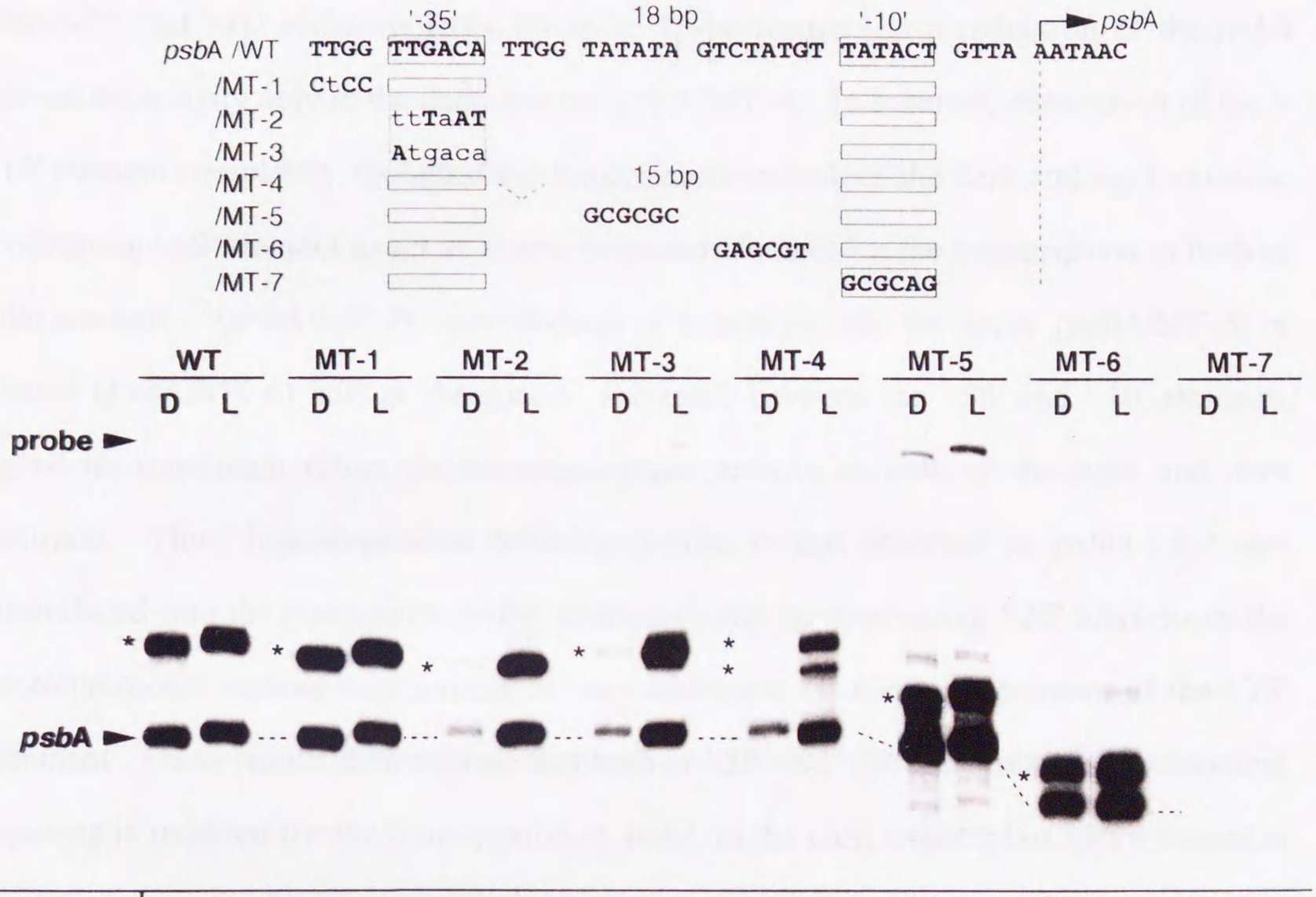

Fig.3-2 ***In vitro*** transcription of *psb*D LRP, the *psb*A and *psb*C promoters in wheat chloroplast extracts.

A, DNA sequences between -120 and +20 of the *psb*D and *psb*A sites. Capital letters depict bases in the sequence which are conserved among wheat, rice and tobacco. Putative '-35' and '-10' elements are boxed. The arrowheads indicate the 5' ends of the deletion constructs used as templates. B, *In vitro* transcription from the *psb*A promoter, psbD LRP and the *psb*C promoter in the dark (D) and light (L) extracts. A 656-bp BamHI-NdeI fragment from pW73CE549, a 512-bp EcoRI-HindIII fragment from pW72ES472A, and a 1190-bp EcoRI-HindIII fragment from pW18ES1145 were used as DNA probes for *in vitro* transcripts from the *psb*D, *psb*A and *psb*C sites, respectively. Experiments for the three promoters were carried out with exactly the same preparations of the plastid extracts. The arrows indicate the primary transcripts synthesized *in vitro*. The full length bands correspond to the undigested probe. Readthrough signals (*) are also seen at the position of the boundary between the vector and chloroplast DNA sequences or mutagenized points.

In contrast, the sequence conservation of the *psb*A promoter is concentrated in the core promoter region which contains the '-35' and '-10' elements spaced by 18-bp with high sequence similarity to the bacterial consensus motifs. The *psb*A transcription from such promoter was constitutively active and complete deletion of the sequences upstream of the '-35' element scarcely affected the promoter strength *in vitro*. Deletion of the downstream sequences of +5 through +315 of *psb*D LRP (*psb*D/+5) and +3 through +278 of the *psb*A promoter (*psb*A/+3) gave no effect on the transcription activity *in vitro* (data not shown). From a comparison between results of two promoters, it is suggested that the light response is caused by the core promote structure which is the property of *psb*D LRP, but not of the *psb*A promoter.

### 3.3.2 Systematic mutational analysis of the psbA core promoter by *in vitro* transcription

In contrast to *psb*D LRP, the *psb*A promoter is light-independent and contains 18-bp spaced '-35' and '-10' elements with high sequence similarity to the bacterial consensus motifs. Complete deletion of the sequence upstream of the '-35' element scarcely affected the *psb*A promoter strength *in vitro* (Fig.3-2). These characteristics of the *psb*A promoter are suitable for mutational analysis to examine the relationship between the core promoter architecture and light dependence of the transcription. The effects of various mutations in the core promoter region on the activity of the *psb*A promoter were investigated in the light and dark extracts (Fig.3-3). Introduction of mutations in the sequence just upstream (from -38 to -35) of the '-35' element never gave effect on the *psb*A transcription activity in both of the light and dark extracts (*psb*A/MT-1). Most interestingly, however,exchange of the *psb*A '-35' element (TTGACA) with the pseudo '-35' element (TTTAAT) of *psb*D LRP severely reduced the *psb*A transcription in the dark extract without affecting the transcription in the light extract (*psb*A/MT-2). The sequence of TTTAAT, however, is not particularly required for introduction of the light




| MT/WT | D | 1 | 1.0 | <0.1 | 0.1 | 0.1 | 1.1 | 0.9 | <0.1 |
|---|---|---|---|---|---|---|---|---|---|
| | L | 1 | 1.0 | 1.2 | 1.1 | 1.0 | 1.1 | 0.9 | <0.1 |

Fig.3-3 Analysis of the *psb*A promoter by *in vitro* transcription with a set of modified promoters (MT-1 through MT-7) in the dark (D) and light (L) extracts.

DNA fragments between -38 to +278 of the *psb*A site having the indicated base pair substitutions at each block in the core promoter region were cloned into pSP72 vector and used as templates. The *psb*A arrow indicates the primary transcripts from the *psb*A site. Details of the figure are same as described in Fig.3-2. The numerical values below the corresponding lanes indicate the transcription activities relative to that of the wild type promoter. The values are given in average of three independent experiments.

response into the *psb*A promoter. A single point mutation within the '-35' element of the *psb*A promoter (substitution of first T to A) reduced the transcription to a virtually undetectable level in the dark extract (*psb*A/MT-3), whereas the transcription activity in the light extract was not affected at all. Furthermore, shortening of the spacing between the '-35' and '-10' elements from 18-bp to 15-bp resulted in a reduction of the *psb*A promoter activity only in the dark extract (*psb*A/MT-4). In contrast, destruction of the '-10' element completely abolished the transcription in both of the dark and light extracts, indicating '-10' element to act as a core promoter element for the transcription in both of the extracts (*psb*A/MT-7). Introduction of mutations into the upper (*psb*A/MT-5) or lower (*psb*A/MT-6) half of the spacer sequence between the '-35' and '-10' elements gave no significant effect on the transcription activity in both of the light and dark extracts. Thus, light-dependent behavior similar to that observed in *psb*D LRP was introduced into the constitutive *psb*A promoter only by destructing '-35' element in the core promoter without requirement of any additional cis element upstream of the '-35' element. These results demonstrate that both of '-35' and '-10' elements with a canonical spacing is required for the transcription of *psb*A in the dark extract, but '-35' element is not necessary for the transcription in the light extract. It is almost a unique explanation for these results that chloroplast RNA polymerase changes from the dark-type which requires bacterial consensus '-35' and '-10' elements into the light-type which does not require '-35' element for transcription initiation from the *psb*A promoter by the shift of the 24 h dark-adapted seedlings to the light.

### 3.3.3 Modification of the core promoter of *psb*D LRP

The above results may indicate that the '-35' element-like sequence in *psb*D LRP is not positively involved in its light responsive behavior, but lacking of the sequence acting as '-35' element is essential for the light response of *psb*D LRP. The evidence shown in

Fig.3-4 supports this proposal. The simultaneous modification of *psb*D LRP by substituting the pseudo '-35' element with the *psb*A '-35' element and changing the space distance to 17-bp converted the character of the promoter to function both in the dark and light extracts with equivalent activity (*psb*D/MT-9).

These results of the mutational analyses led us to a proposal that the light-dependent switching of *psb*D LRP is mediated by the core promoter structure lacking '-35' element which is recognized by the light-type RNA polymerase, but not by the dark-type and the protein(s) which specifically binds to the upstream *cis* elements (Nakahira *et al.*, 1996) acts as enhancers for the light-type RNA polymerase.

### 3.3.4 Effects of tagetitoxin and cycloheximide in *in vitro* inhibition experiment

In order to examine the possibility that either RNA polymerase in the light or dark chloroplasts is different from the bacterial type, inhibitor effects were examined. When we used a plasmid pW18E866 as a template for *in vitro* transcription, two distinct signals from the second transcription initiation site (D/C-2 site) 760-bp upstream of the *psb*D translation initiation codon (Wada *et al.*, 1994, see section 2) and the *psb*D site were observed in the light extract (Fig.3-5A), but only the signal from the D/C-2 site was detected in the dark extract. Tagetitoxin, a potent inhibitor of bacterial type chloroplast RNA polymerases, severely reduced the transcription from the D/C-2 and *psb*D sites in light extract and that from the D/C-2 site in the dark extract, suggesting both of the RNA polymerases in the light and dark to be classified in the bacterial type in tagetitoxin sensitivity.

To gain a insight into the molecular events which induce the change in character of the bacterial type chloroplast RNA polymerase by light, we examined involvement of *de novo* protein synthesis in emergence of the light-type RNA polymerase. As described previously (Satoh *et al.*, 1997), the transcription activity of *psb*D LRP was reduced in the

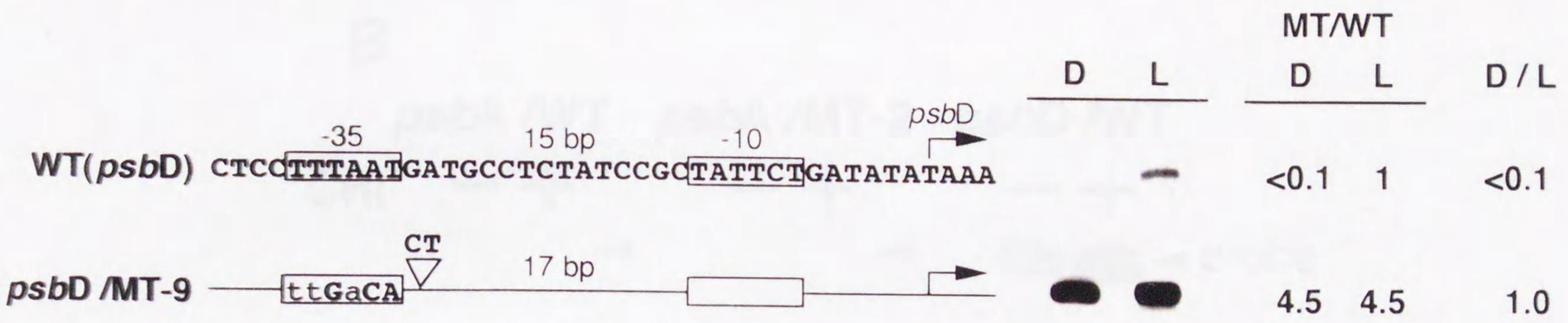



Fig.3-4 Effect of modification of *psb*D LRP to give consensus '-35' element and canonical spacing (17-bp) on *in vitro* transcription in the dark (D) and light (L) extracts.

DNA fragments between -118 and +315 of the *psb*D site having the indicated base pair substitutions in the core promoter region (*psb*D/MT-9) were cloned into pSP73 vector and used as template. The band corresponding to the transcripts from the *psb*D site are shown. The numerical values in the column of MT/WT indicate the transcription activities relative to that observed with the wild type promoter in the light extract. The numerical values in the D/L column indicate the activity of each construct in the dark extract relative to that in the light extract. The values are given in average of three independent experiments.

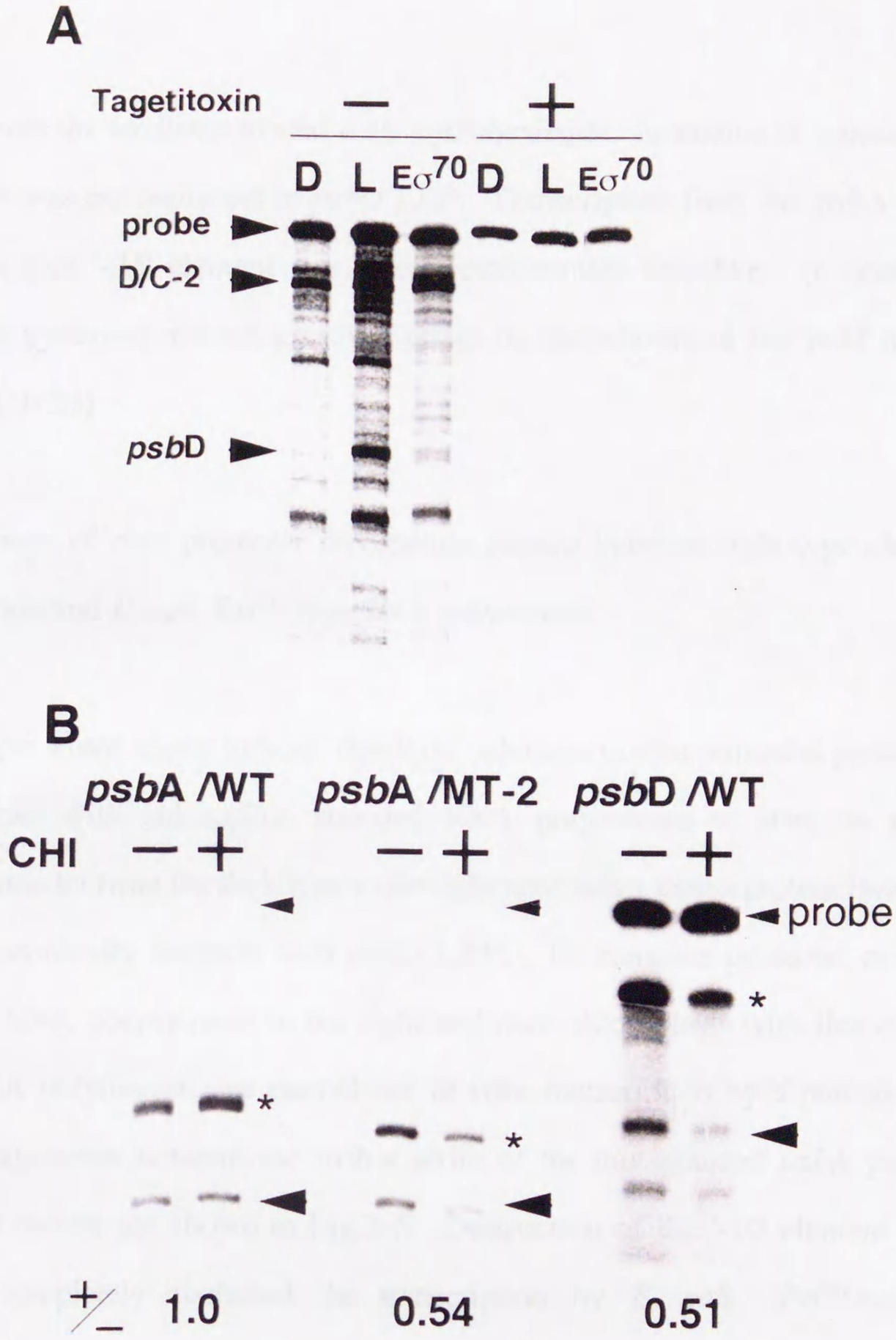



Fig.3-5 Effects of inhibitors on *in vitro* transcription from four types of promoters.

A, Effects of tagetitoxin on the transcription from *psb*D LRP and the D/C-2 promoter in the dark (D) and light (L) extracts. Plasmid pW18E866 encompassing -551 through +315 of the *psb*D site was used as a template. *In vitro* transcription were carried out in the presence (+) or absence (-) of 30 μM tagetitoxin. The D/C-2 and *psb*D arrows indicate the primary transcripts from the D/C-2 and *psb*D site. B, Effects of cycloheximide (CHI) on the *in vitro* transcription from *psb*D /WT (*psb*D/-119), *psb*A/WT (*psb*A/-38) and mutated *psbA psb*A/MT-2) constructs. The arrow indicates the primary transcripts from the *psb*A and *psb*D sites . The numerical values at the bottom indicate the transcription under inhibition (+) relative to that of the control (-). Details of the figure are same as described in Fig.3-2.

light extract from the seedlings treated with cycloheximide. Inhibition of transcription by cycloheximide was not restricted to *psb*D LRP. Transcription from the *psb*A promoter mutated as to lack '-35' element was also cycloheximide sensitive. In contrast, the cycloheximide treatment did not give any effect on the activity of the wild type *psb*A promoter (Fig.3-5B).

### 3.3.5 Comparison of core promoter recognition pattern between light-type chloroplast RNA polymerase and *E. coli* $E\sigma^{70}$-type RNA polymerase

The results presented above indicate that light induces a nuclear-encoded protein factor which associates with chloroplast encoded RNA polymerase to alter its promoter recognition character from the dark type to the light type rather than a protein factor which directly and specifically interacts with *psb*D LRP. To compare promoter recognition pattern of the RNA polymerases in the light and dark chloroplasts with that of *E. coli* $E\sigma^{70}$-type RNA polymerase, we carried out *in vitro* transcription by a purified *E. coli* $E\sigma^{70}$ RNA polymerase holoenzyme with a series of the mutagenized *psb*A promoters. The important results are shown in Fig.3-6. Destruction of the '-10' element in *psb*A (*psb*A/MT7) completely abolished the transcription by *E. coli* $E\sigma^{70}$-type RNA polymerase (data not shown), but introduction of mutations in the '-35' element (*psb*A/MT-2) or spacer sequences (*psb*A/MT5 and *psb*A/MT-6; data not shown) never affected the *psb*A transcription, as observed with the light type RNA polymerase.

It has been known that *E. coli* promoters having TGn motif just upstream of the consensus '-10' element allow transcription initiation by the $E\sigma^{70}$-type holoenzyme, even when they lack the '-35' element (Keilty and Rosenberg, 1987; Kumar *et al.*, 1993). The *psb*A promoter has the TGn motif in addition to well conserved '-35' and '-10' elements. Introduction of double mutations in the TGn motif and the '-35' element reduced the *in vitro* transcription activity by a purified *E. coli* $E\sigma^{70}$-type RNA polymerase as observed

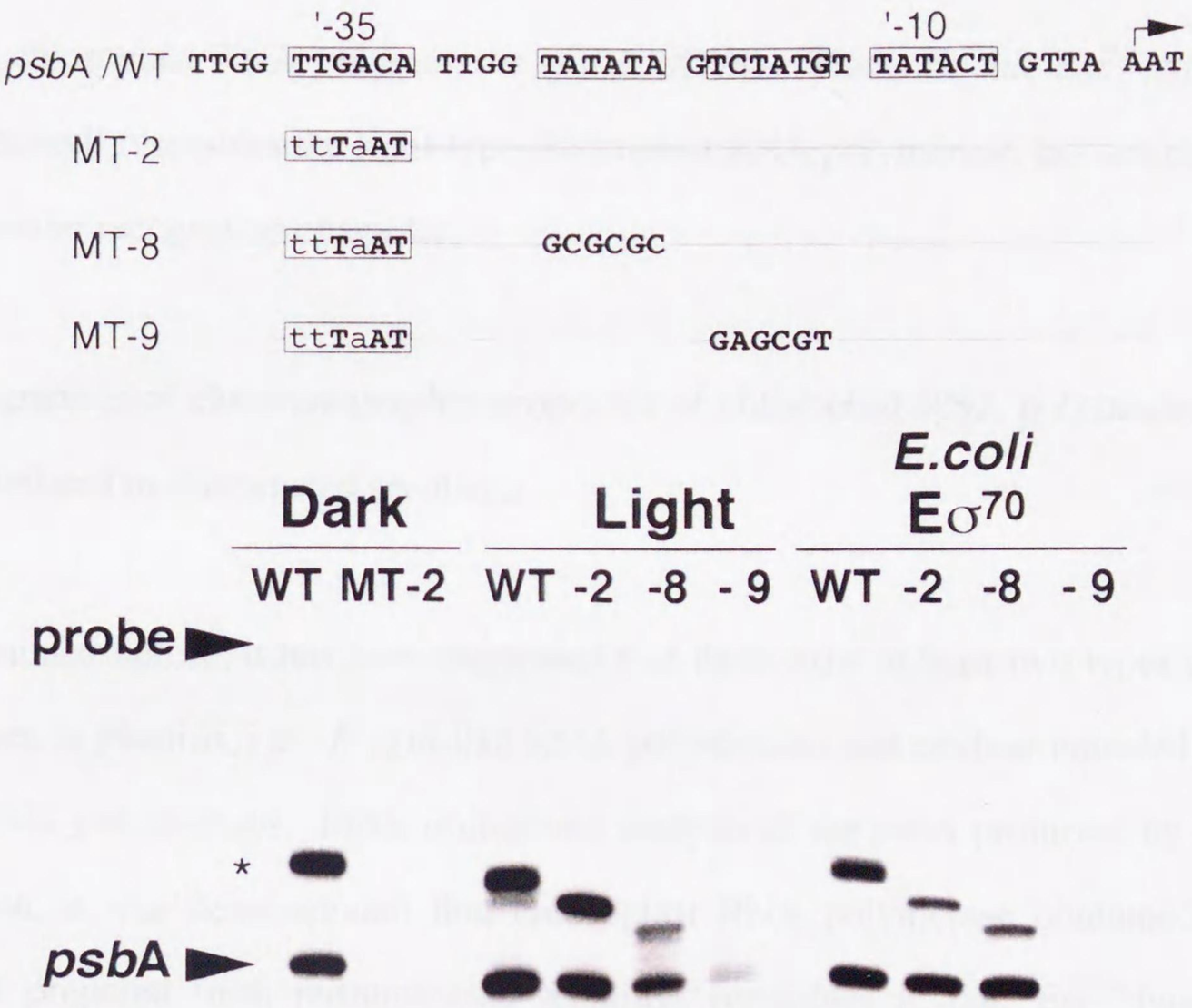



Fig.3-6 *In vitro* transcription from the *psb*A promoters modified by introducing double mutations in both of the '-35' element and spacer sequences in the dark (D) and light (L) extracts and a purified $\sigma^{70}$ type E. *coli* RNA polymerase holoenzyme.

DNA fragments between -38 and +275 of the *psb*A site having the indicated base pair substitutions in the core promoter region were cloned into pSP72 vector and used as templates. The *psb*A arrow indicates the primary transcripts from the *psb*A site. Details of the figure are same as described in Fig.3-2.

with the light-type chloroplast RNA polymerase (*psb*A/MT9) , although double mutation of the '-35' element and the so-called TATA box-like sequence (Link, 1984) in the spacer region caused minor changes in promoter strength for both of the *E. coli* $E\sigma^{70}$-type and light-type chloroplast RNA polymerases (*psb*A/MT/8). Thus, *E. coli* $E\sigma^{70}$-type RNA polymerase well resembles the light-type chloroplast RNA polymerase, but not dark-type in the promoter recognition character.

### 3.3.6 Comparison of chromatographic properties of chloroplast RNA polymerases from dark-adapted and re-illuminated seedlings

As mentioned before, it has been suggested that there exist at least two types of RNA polymerases in plastids, i.e., *E. coli*-like RNA polymerases and nuclear-encoded non-*E. coli*-like RNA polymerases. From mutational analysis of the psbA promoter by *in vitro* transcription, it was demonstrated that chloroplast RNA polymerase contained in the chloroplast prepared from reilluminated seedlings resembles *E. coli* $E\sigma^{70}$-type RNA polymerase on the core promoter recognition. However, there is no sufficient evidence which type of RNA polymerase the dark-type is.

Hydrophobic column chromatographies were carried out for the light and dark extracts and the chromatographic properties of chloroplast RNA polymerases from dark-adapted and reilluminated seedlings were compared.

Chloroplast RNA polymerases were eluted from Phenyl-TOYOPEARL column with linear gradient from 250 to 0mM $(NH_4)_2SO_4$ and fractionated. The components of these fractions were detected by silver staining following SDS-page (Fig.3-7) and transcription activities from the D/C-2, *psb*D and the *psb*A sites were majored by *in vitro* transcription (Fig.3-8). Significant differences in stained pattern between eluents for the dark extract and those for the light extract were not observed (Fig.3-7). Transcripts from the *psb*A and D/C-2 sites were observed in both samples (Fig.3-8). In both eluents prepared from

the dark and light extracts, transcription activities for the D/C-2 and *psb*A promoters were detected in eluents obtained from 100 to 0mM $(NH_4)_2SO_4$ and peaks of the activities were in the vicinity of 30 mM $(NH_4)_2SO_4$. Although absorbed proteins to the column were only less than 17% of proteins in extracts, the transcriptional activities of those absorbed materials preserved above 55% in comparison with those of extracts. Transcripts from the *psb*D site were observed only in eluents prepared from re-illuminated seedlings, but not in those from dark-adapted seedlings. The behaviors of transcription activities of absorbed materials were the same as those of extracts. These results indicate that dark- and light-type chloroplast RNA polymerases quite resemble in the chromatographic properties and factor(s) involved in light-responsive transcription of *psb*D LRP is closely associated with chloroplast RNA polymerase.

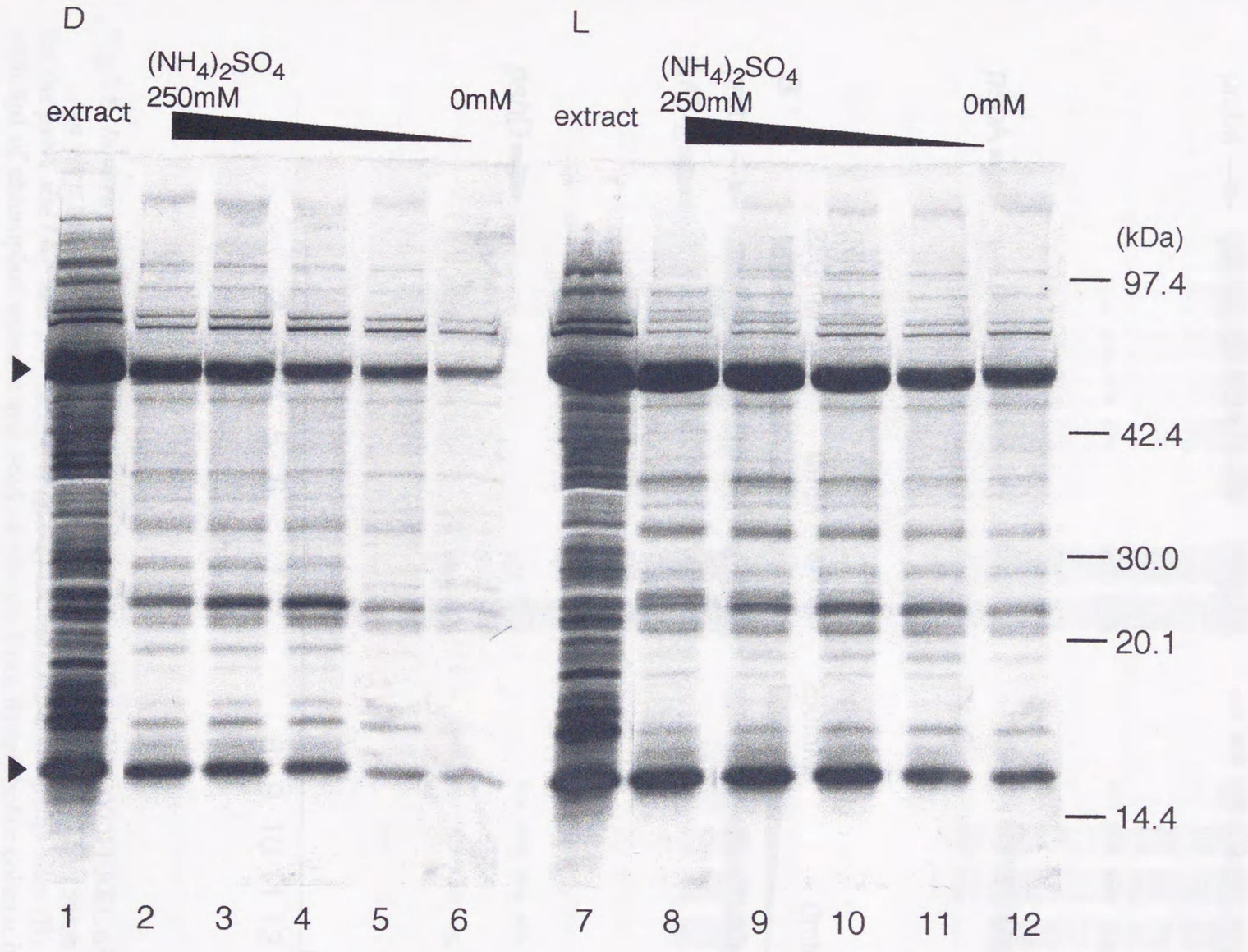



Fig.3-7 Protein contents of extracts and of eluents from hydrophobic column.
5μl of chloroplast extracts (lanes 1and 7) and 20μl of eluents from Phenyl-TOYOPEARL 650 (lanes 2-6 and 8-12) were loaded on 16% polyacrilamide gel electrophoresis and silver stained. Lanes 1-6 and 7-8 shows the results with the extracts and eluents prepared from dark-adapted and 2h reilluminated seedlings, respectively. Arrowheads indicate large and small subunits of Rubisco. Numbers to the right indicate the size of mol wt markers.

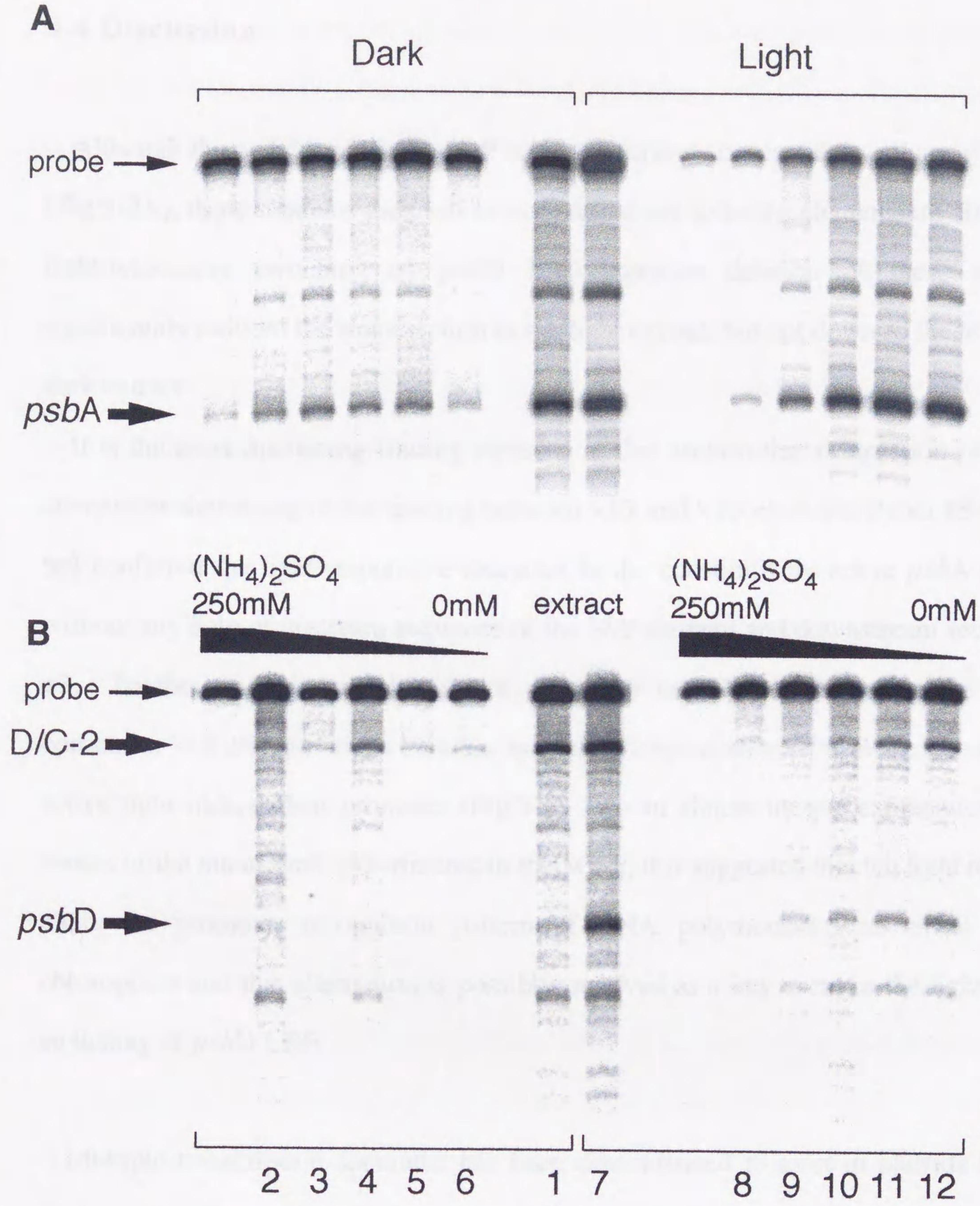



Fig.3-8 *In vitro* transcriotion activities of fractions from Phenyl-TOYOPEARL 650.

*In vitro* transcription was carried out using template pW18ES472A (Satoh *et al.*, 1997) for the *psb*A site (A), and pW18E866 (Fig.2-2) for the D/C-2 and *psb*D sites (B). The results with 8μl of chloroplast extracts and 16μl of eluents from hydrophobic column in 40μl of *in vitro* transcription reaction mixture are shown. Samples prepared from the dark-adapted seedlings and the 2h reilluminated seedlings are designated as Dark and Light, respectively. Signals corresponding to transcripts from the *psb*A, D/C-2 and *psb*D sites are indicated by thick arrows. Numbers below lanes correspond to those of lanes in Fig.3-7.

## 3.4 Discussion

Although the activity of *Psb*D LRP strongly depends on upstream enhancer elements (Fig.3-2A), these elements themselves were found not to be the elements involved in the light-responsive switching of *psb*D LRP, because deletion of these sequences significantly reduced the transcription in the light extract, but not down to the level in the dark extract.

It is the most interesting finding reported in this section that destruction of the '-35' element or shortening of the spacing between '-35' and '-10' elements (from 18-bp to 15-bp) conferred the light responsive character to the constitutively active *psb*A promoter without any help of upstream sequence of the '-35' element and downstream sequence of +3. To the contrary, introduction of mutations in *psb*D LRP to give the bacterial consensus '-35' element and a suitable spacing (17-bp) converted it to the constitutively active light independent promoter (Fig.3-4). As an almost unique explanation for the results of the mutational experiments in this work, it is suggested that the light reversibly alters the promoter recognition pattern of RNA polymerase(s) in wheat matured chloroplasts and that alternation is possibly involved as a key event in the light-induced switching of *psb*D LRP.

Multiple transcription apparatus has been demonstrated to exist in plastids from the works with naturally occurring plants (Morden *et al.*, 1991; Hess *et al.*, 1993) or transplastomic plants which lack plastid-encoded transcription apparatus (Allison *et al.*, 1996) and biochemically characterized (Zailtin *et al.*, 1989; Lerbs-Mache, 1993; Pfannschmidt and Link, 1994). The nuclear-encoded chloroplast RNA polymerases were proposed to transcribe selectively the plastid genes with non-consensus promoters (Allison *et al.*, 1996; Kapoor *et al.*, 1997). However, it is unlikely that *psb*D LRP is recognized by the nuclear-encoded RNA polymerases, because the *psb*D LRP activity

was reported to disappear in tobacco plants by deleting the gene for the β subunit of bacterial type plastid RNA polymerases (*rpo*B) (Allison *et al.*, 1996). Transcription from *psb*D LRP was inhibited by tagetitoxin (Fig.3-5A), a potent and selective inhibitor of bacterial type plastid RNA polymerases (Mathews and Durbin, 1990). Tagetitoxin also completely eliminated transcription from a constitutive promoter in the *psb*D/C operon (D/C-2 promoter) in both of the dark and light extracts, suggesting that plastid-encoded bacterial type RNA polymerases function dominantly in mature chloroplasts in both of the dark-adapted and illuminated wheat plants. This idea was emphasized also by the comparison of the chromatographic properties of chloroplast RNA polymerases from dark-adapted and reilluminated seedlings (Fig.3-7 and 8). Dark-type and light-type chloroplast RNA polymerases were eluted at almost same concentration of $(NH_4)_2SO_4$ from hydrophobic column and behavior of transcription activities of both RNA polymerases was quite similar to that of extracts. Thus, the light seems to alter promoter preference of the plastid-encoded bacterial type RNA polymerase rather than to induce a novel type of nuclear-encoded chloroplast RNA polymerase to activate transcription from *psb*D LRP as well as the mutated *psb*A promoters.

A $E\sigma^{70}$-type *E. coli* RNA polymerase as well as the light-type chloroplast RNA polymerase can recognize the *psb*A promoter, even when the '-35' element is destructed, but not when double mutations are introduced into the '-35' element and the TGn motif (Keilty and Rosenberg, 1987) just in front of the conserved '-10' element. To my knowledge, this is the first report of the so-called extended '-10' promoter (Keilty and Rosenberg, 1987) in chloroplasts. While, the RNA polymerase in the dark-adapted chloroplasts absolutely requires the '-35' element regardless of the presence of TGn motif in the *psb*A promoter. These results indicate that the light-type chloroplast RNA polymerase, but not the dark-type chloroplast RNA polymerase, is similar to the $E\sigma^{70}$-type *E. coli* RNA polymerase in the core promoter recognition. In the case of the

promoter lacking both of the consensus '-35' element and TGn motif, such as *psb*D LRP, it is likely that the light-type RNA polymerase requires additional enhancer sequences and the corresponding trans-factor for the initiation of transcription, in analogy with well characterized *E. coli.* class II catabolite activator protein (CAP)-dependent promoters in which '-35' element is replaced by the CAP binding site (Ushida and Aiba, 1990; Ebright, 1993). The dark-type RNA polymerase absolutely requires the '-35' element and supposedly cannot recognize *psb*D LRP, even in the presence of the upstream enhancer sequences and the binding proteins which recognizes these sequences. In the case of other usual chloroplast promoters having $\sigma^{70}$-type promoter elements such as the *psb*A promoter, it is expected that the change in chloroplast polymerases between the light and dark does not make substantial difference in their transcription activities.

Multiple usage of σ factors having different promoter specificities is known to control the differential gene expression in bacteria (Helmann and Chamberlin, 1988; Errington, 1993). Several plastid-localized σ-like factors have also been identified in plants immunologically (Lerbs *et al.*, 1988; Troxler *et al.*, 1994) and biochemically (Tiller and Link, 1994), and a nuclear-encoded principal type plastid σ factors were cloned recently from unicellular rhodophytes (Liu and Troxler, 1996; Tanaka *et al.*, 1996). Inhibitory effect of cycloheximide on the *in vitro* transcription with *psb*D LRP and the *psb*A promoter mutated as lacking '-35' element in the light extract is suggestive of the light-induced *de novo* synthesis of a nuclear-encoded protein such as σ factors to be involved in the appearance of the light-type chloroplast RNA polymerase.

# 4. Cloning of Light-responsive Chloroplast RNA Polymerase Sigma Subunit

## 4.1 Introduction

Systematic mutational analyses of the *psb*A promoter (see sec.3) and *psb*D LRP (Satoh *et al.*, 1997) provide several evidences demonstrating that the plastid-encoded bacterial type RNA polymerases primarily function in matured chloroplasts.

In bacteria, the RNA polymerase plays a key role for the changes in gene expression. One major mechanism of the promoter selectivity control of RNA polymerase is the replacement of the promoter recognition subunit, σ factor, on the core enzyme for global switching of gene expression in bacteria. The principal σ factor $\sigma^{70}$ in exponentially growing *E. coli* potentiates the transcription of genes controlled by the consensus promoters consisting of two hexanucleotid sequences, '-35' and '-10'motifs. Other minor species of alternative σ factors recognize different sets of promoter sequences, each being associated with a limited number of genes that are mostly expressed in response to various stress conditions.

DNA sequence and phylogenetic analyses indicate that chloroplasts are close relatives of free-living cyanobacteria and are derived from an endosymbiotic event. Chloroplast RNA polymerase is closely related to that of bacteria. From analysis of the complete nucleotide sequences of the plastid genomes, genes for core (α, β, β' and β'') subunits of chloroplast RNA polymerase are encoded by the plastid genome, but not the genes for chloroplast σ factors. This suggests that plastid σ factors are encoded by the nuclear genome. Indeed, nuclear genes encoding chloroplast σ factors were recently cloned from the unicellular rhodophyte *Cyanidium sulphuraria* (Liu and Troxler, 1996) and *Cyanidium caldarium* (Tanaka *et al.*, 1996) and expression of a gene for $\sigma^{70}$-type σ

factor (*rpo*D) from *C.sulphuraria* was shown to be regulated in a light-dependent manner. In mustard (*Sinapis alba L.*), a set of three different σ-like factors was isolated from both chloroplasts (Tiller *et al.*, 1991) and etioplasts (Tiller and Link, 1993), each consisting of polypeptides of 67 (SLF67), 52 (SLF52) and 29kDa (SLF29) and it is proposed that phosphorylation/dephosphorylation control is the initial regulation of transcription in plastids of greening plants (Tiller and Link, 1993). Despite these evidences, no σ factor has been cloned in higher plants.

In this section, the cloning and sequencing of cDNA of a σ factor which is strongly expressed only in the illuminated wheat seedlings are reported.

## 4.2 Materials and Methods

### 4.2.1 Plant materials

Wheat were grown in the dark or under white light (approx 5,000 lx) at 25 °C. After 5 days, a portion of light-grown seedlings was shifted to the dark and kept there for 24 h or again transferred to the light for 15, 30, 60 and 120min after 24 h dark-adaptation. After 6 days, a portion of dark-grown seedlings was transferred to the light for 2h. Seedlings were harvested quickly and freezed immediately.

### 4.2.2 Extraction of total RNA

5g of seedlings frozen in liquid nitrogen were grinded with 50ml of GS buffer containing 4M guanidium thiocyanate, 25mM sodium citrate, 0.5% sodium N-laurylsarcosine and 5mM 2-mercaptoethanol in the mortar at the liquid nitrogen temperature. The homogenized tissues were centrifugated at 15,000rpm (27,000g) for 15min in RPR20-2 (Hitachi) rotor at 20°C. The supernatant was layered onto 6ml of CsCl solution containing 5.7M CsCl and 0.1M EDTA in four 40PA tubes. RNA was collected by centrifugation at 37,000rpm (14,100g) for 24h in RP70T rotor (Hitachi) at 20°C. The pellet was resuspended in 1ml of GTC solution, containing 7.5M guanidium-HCl, 25mM sodium acetate (pH7.0) and 5mM DTT, per tube and transferred to 2ml sample tube. The resulting solution was subjected to ethanol precipitation. The pellet was dissolved in 1ml of sterilized water, 10M LiCl solution was added to a final concentration of 2M and kept at 4°C over night. Total RNA was collected by centrifugation and resuspended in 300μl of sterilized water. Redissolved total RNA was subjected to ethanol precipitation and stored at -20°C until it was needed.

4.2.3 Reverse transcription -PCR

The 1st strand cDNA was synthesized from 10μg of total RNA by cDNA Synthesis Kit (Life sciences, inc.) with AMV reverse transcriptase. Synthesized cDNA was dissolved in 25μl of sterilized water and stored at -20˚C prior to use. Fragments of cDNA were amplified by means of RT-PCR against 4μl of 1st strand cDNA with a couple of 100pM degenerate primers containing deoxyinosine (P1, 5'-GCCTGCAGAYYTIRTICA RGRIGG-3' and P2, 5'-CGCTGCAGSMYTGICKDATCCACCA-3'). These primers were designed based on the conserved amino acid sequences among the principal σ factors of cyanobacteria and red alga (Fig.4-1A). PCR was carried out for 40cycles. Each cycle was at 95˚C for 1min, 60˚C for 1min and 72˚C for 1min. An amplified 122-bp fragment of which length was predicted from the sequence of bacterial σ factors was cloned into pUC18 to yield plasmid pWS122.

4.2.4 cDNA library construction

PolyA-tailed mRNAs were purified from 1mg of total RNA prepared from the 5-days light-grown seedlings by an mRNA Purification Kit (Pharmacia). The double strand cDNA was synthesized from 5μg of poly(A)$^+$ RNA by a TimeSaver cDNA Synthesis Kit (Pharmacia). The resulting cDNA was cloned into the λgt10 vector by cDNA rapid cloning module-λgt10 (Pharmacia) and packaged by λDNA *in vitro* packaging module (Pharmacia).

4.2.5 Hybridization screening from cDNA library

$2x10^5$ plaques of λgt10 phage were infected to $5x10^8$ cells of *E.coli* NM514 by incubation at 37˚C for 20min and plated out $5x10^4$ plaques/plate on 1.5% LBM-plates in

15cm disk dishes containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 10mM $MgSO_4$ and 1.5% (w/v) agar, using 0.8% LBM-top agar. Plates were inverted and incubated at 37°C for 6h and at 4°C for at least 1h. Nitrocellulose membrane Protein BA85 (Schleicher & Schuell) discs were plated on to the surface of the plates and left filters in contact with plates for 1min. Filters were dried up for 10min and laid plaque side up for 5min on a sheet of thick filter paper (Whatman 3MM) soaked in denaturing solution containing 1.5M NaCl and 0.5M NaOH. After denaturing, filters were transferred to a second sheet of thick filter paper soaked in neutralizing solution containing 1.5M NaCl and 500mM Tris-HCl (pH7.0) for 5min, rinsed in 2x SSC (20x SSC containing 333mM NaCl and 333mM Sodium citrate was prepared prior to use) for 5min, dried on the filter paper plaque side up and baked at 80°C for 2h. The 122-bp fragment amplified from plasmid pWS122 by PCR with the sense primer (P3, 5'-GCTGTCCGACCTGATTCAGGGTGG-3') and antisense primer (P4, 5'-TTGAAACA CCCTGACGAATCCACCA-3') containing [$\alpha^{32}$P-dCTP] was used as a probe for hybridization. After preincubation at 42°C for 1h in the hybridization buffer containing 5x SSPE, 0.2% (w/v) SDS, 5x Denhart's solution, 50% formamide and 10mg/ml salmon sperm DNA, hybridization was carried out at 42°C over night with 30,000cpm/ disk heat-denatured probe DNA. 20x SSPE (pH7.4) containing 3.6M NaCl, 0.2M $NaH_2P0_4$ and 20mM EDTA, and 100x Denhart's solution containing 20% (w/v) PVP-360, 20% (w/v) Ficoll-400 and 20% (w/v) BSA were prepared previously. Following hybridization, filters were washed in 2xSSC, 0.1% (w/v) SDS at room temperature for 10 min. This step was repeated with flesh washing solution and then filters were washed twice in 0.2xSSC, 0.1% (w/v) at 42°C for 15min. To identify positive plaques, autoradiography was carried out. Ten independent positive plaques were obtained from about 200 thousands plaques, and one of them was sequenced with a T7 DNA sequencing kit (Pharmacia) and a ALF-red auto sequencer (Pharmacia).

4.2.6 Northern blot hybridization

Total RNA (30 μg) was electrophoresed on a 1.0% agarose/formaldehyde denaturing gel and transferred onto a nitrocellulose membrane with x20 SSC. Blots were hybridized with a 122-bp PCR-amplified fragment with the sense primer (P3, 5'-GCTGTCCGACC TGATTCAGGGTGG-3') and antisense primer (P4, 5'-TTGAAACACCCTGACGAAT CCACCA-3') from wheat *sig*A cDNA in the presence of [$\alpha$-$^{32}$P]dCTP. Hybridization was carried out in a solution containing 5x SSPE, 5x Denhart's solution, 0.3 % SDS, denatured salmon sperm DNA (250 μg/ml) and 50 % formamide at 42 °C over night. Final wash condition was 2x SSC/0.1 % SDS at 37 °C.

## 4.3 Results

### 4.3.1 cDNA of a nuclear encoded chloroplast σ factor

In bacterial RNA polymerases, σ subunit is a crucial factor for transcription initiation and promoter selectivity. In order to examine the possibility that differential usage of σ factors participates in the alternation of the promoter preference of the chloroplast RNA polymerases in the light and dark, we investigated existence of σ factors expressed only under illumination in wheat. Since principal σ factors of a red alga and cyanobacteria are homologous to the $\sigma^{70}$ subunit of *E. coli* RNA polymerase which resembles the chloroplast light-type RNA polymerase in the promoter recognition behavior, firstly we tried to amplify cDNA fragments from the total RNA extracted from the re-illuminated wheat seedlings with a couple of degenerate primers (Fig.4-1A) synthesized based on amino acid sequences conserved among the principal σ factors of cyanobacteria and a red alga by means of RT-PCR (Tanaka *et al.*, 1996). The sequences used for PCR primers are also conserved in the regions 2.2 and 2.4 of *E. coli* $\sigma^{70}$ (Lonetto *et al.*, 1992). A 122-bp cDNA fragment, which was amplified from total RNAs of light-illuminated and reilluminated seedlings rather than that of dark-grown and dark-adapted seedlings, was obtained (Fig.4-1B).

Using the 122-bp cDNA fragment as a screening probe, I have succeeded in cloning a 1875-bp cDNA fragment from the cDNA library constructed against the illuminated wheat seedlings (Fig.4-2). The largest open reading frame in the cDNA clone predicts a protein of 475 amino acids. As compared in Fig.4-3, the deduced amino acid sequence of the cloned cDNA around the region 2, which is the most highly conserved region in the $\sigma^{70}$ family (Lonetto *et al.*, 1992), is highly identical (57%) with the corresponding region of the chloroplast localized principal σ factor (*sig*A) of a unicellular red alga *Cyanidium caldarium* RK-1 (Tanaka *et al.*, 1996), that of cyanobacterial principal σ

**A**

| | | | |
|---|---|---|---|
| *E.coli* | RpoD | FLDLIQEGNI GLMKAVDKFE YRRGYKFSTY ATWWIRQAIT RSIADQARTI | 450 |
| *Synechococcus sp.* | RpoD1 | FQDLIQEGSL GLIRAAEKFD HEKGYKFSTY ATWWIRQAIT RAIADQSRTI | 223 |
| *Synechococcus sp.* | RpoD2 | FLDLIQEGTL GLERGVEKFD PSKGYKFSTY AYWWIRQAIT RAIAQQSRTI | 160 |
| *C.caldarium* | SigA | LQDLIQEGSI GLIRGVEKFD AERGFRFSTY ATWWIRQSIT RAISDSSRSI | 427 |
| *C.caldarium* | RpoDL1 | MQDLIQEGSI GLIKGAERFD YRTGFRFSTY AMWWIRQAMQ RAIADTSRPI | 408 |

P1 → P2 ←

5' 3'

P1 : GCCTG CAGAY YTIRT ICARG RIGG

P2 : CGCTG CAGSM YTGIC KDATC CACCA

**B**

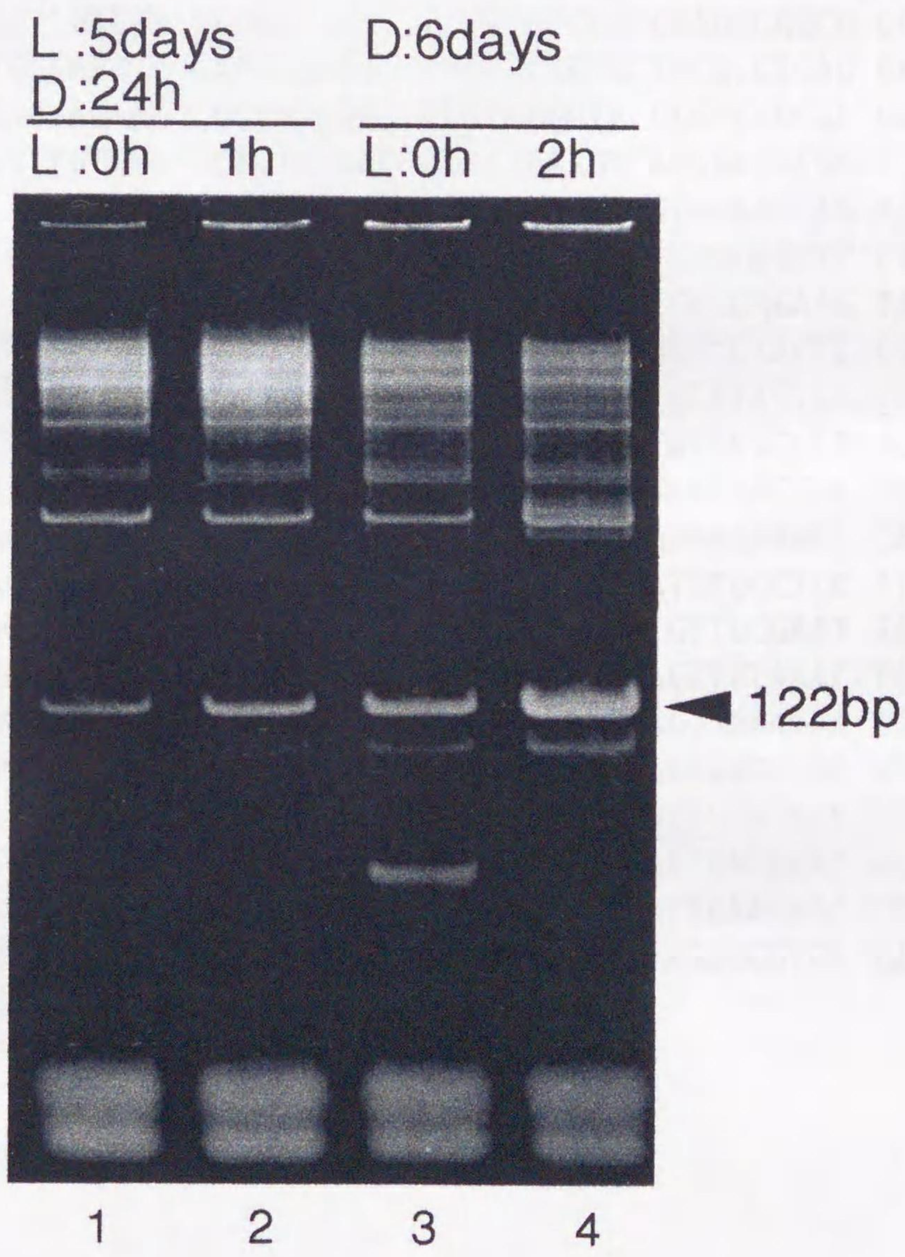



Fig.4-1 Amplification of fragments homologous to the gene encodeing the RNA polymerase σ factor.

A, The amino acid sequence alignment of region 2 among principal σ factors of *E.coli*, cyanobacteria and a red alga. Degenerate primaers containing deoxyinosines(I), P1 and P2, were designed based on the conserved amino acid sequences among principal σ factors. B, RT-PCR was carried out for total RNAs purified from wheat seedlings. PCR products were electrophoresed on 5% acrylamido gel and stained with the ethidium bromide. Lanes 1 and 2 show results with the dark-adapted and the 1h-reilluminated seedlings, respectively. Lanes 3 and 4 show results with the 6days-dark grown and the 2h-illuminated seedlings, respectively. The arrowhead indicate the 122 bp amprified DNA fragment.

```
AACACCAGCA CGCCGCCGTT GGGCCCCAGG AGCACGTCCT GCGCCATCGC CGACCCGCCC     60
AGCAGCATCG CGCTGGCCGC CACGGCGACC GCGGCTTCGC CCCTTTCTTG CCCAGGGAGG    120
CGCGCACCAC CATCCTGCTG CTGCGCATGG GGGTGGCAGC CTTGGCGGCG AAGGAAGGGA    180
CGGACACCGC TGCAGAGGAG AGGGCGGCCA TGGCTGCTAG CTAGCTAAGT AAGTGGTTGG    240
GAAAGAGGGT CAACAGCCAC ATGAGCAGCG CGCGGGAGAC GCCCACGGCT CCTCCTCGCT    300
GCAGTTCGCG CCACCGGCCG CGTCCAAGCT CACCGTCGCC GCGCACAGGC TCAAGCTCTC    360
CCCACATGGC CGCGCGCAGG TCATGCGGGC CCTCAGGCAG AGCGCGCCTG CCTCGCGCCG    420
CAGCCTCCTC CGTTGTTGCC CGCCGACGAG TTCTCCCTCG ATGCCATTAT CCTGCTGCAG    480
AGGTCCATGC TCGAGAAGCA GTGGCAGCTC CCCTTCGAGG AGGAGGACGA CGACGACGGC    540
GGCGGCGATG GCCACCATGT GGAGGCCATT GGTTCGGCCG AGGACGACGA CGGCAAGGGC    600
AGGAGCAGCT CCGTCGTCGT GGCGCCCTCC GGCGTCTCGG CGAGGCAGCG GCGGATGAGC    660
GGCCGGAGGC GTGGGAGGTC CAAGAAAGGC AGCGGCGCCG TGCACCTGAG CATCAGCCCC    720
GAGCTGCTGC AGAGCAGAAA CCGCATCTAC CTTCGCGGTA CCGTCAGCAA GGAGCTCCTC    780
ACGCAAAAGC AGGTTGTCCA CCTCTCCAAG AAGATCAAGG ACGGATATGG CTGCAGCAGC    840
AAAGATCAAA GCTGAAAGAA AAACTGGGGA ATGAGCCGTC GTACAAGCAG ATGGCGCAAT    900
CACTTCGGAT ATCGACGCCG GAGCTACGGT CAAGAATGCG CGAGTCGTTC CTCGCGAGGG    960
AGGTGCTAAC GATGAGCAAC ATCCGTTTGG TCATATCCAT CGCCCAGAAG TATGACAAGC   1020
TGGGGGTTGA GCTGTCCGAC CTGATTCAGG GTGGTCTCAT AGGGCTACTC CGCGGGATCG   1080
AAAAGTTTGA TGCATCTAGG GGCTTCAGAA TTTCCACTTA CGTATACTGG TGGATTCGTC   1140
AGGGTGTTTC AAGAGCACTG GCCGACAACT CGAAAACATT CAGGCTCCCT ACTTACCTGC   1200
ATGAGAGGCT GATTGCGATT CGTGGCGCAA AGTACGCATT GGAGGACCAA GGGGTTTCTC   1260
CGACAACGCA AAACATCGCA AAGTTGCTCA ATATATCGGA GAAGAAAGTG CACAATGCTA   1320
CAGAGGCTGT CAATAAGGCT CTTTCGTTGG ATCAACAGGC ATTCCCCTCC TTAAATGGCC   1380
TACCCGGAGA TACACTCCAC AGCTATATAG AGGATCAAAA TGTTGCGAAT GACCCATGGC   1440
ATGGATTTGA GGAGTGGTAT CTAAAGGAGG AAGTCAACAA ACTTCTAAAC TCAAATCTCA   1500
CTGAACGTGA GAGAGACATT ATTCAGTTAT ACCATGGTAT AGGGAAACAA TGCCATACAT   1560
GGGAGGATAT TAGCAGACAG TTCGGGTTAT CCAGGGAGAG GGTGAGGCAA GTAGGGCTTA   1620
TCGCAATGGA GAAGCTGAAG CAACGCGCGA GGCGGAAAAA GCTGGATGCG TTGCTCCAGG   1680
ATTACTGAGC TGAGGTGTTT TTCATGTTGT GCTGGACCGG ATCCATACAC ACAGTGGTTG   1740
TATATACTCG AAAAGAGAGA GCGACAACAT AGGTTACAAC ATTGAAAGGC TTGTACTGCC   1800
ACTCACCTGC AAATAATCAT ACCTGTACAT TTTGTGACAA TAGGCCGTGT TAACTTACCA   1860
TAACAACAAC TCAAT                                                    1875
```

Fig.4-2 Nucleotide sequence of *sig*A from *Triticum aestivum*.

Putative translation initiation and termination codons are underlined and double underlined, respectively. The largest open reading frame is boxed. The region used as a probe for northern hybridization is shadowed. These sequence data is under submitting to the DDBJ/EMBL/GenBank databases.

```
W   MSSARETPTA PPRCSSRHRP RPSSPSPRTG SSSPHMAARR SCGPSGRARL PRAAASSVVA    60

W   RRRVLPRCHY PAAEVHAREA VAAPLRGGGR RRRRRRWPPC GGHWFGRGRR RQGQEQLRRR   120
W   GALRRLGEAA ADERPEAWEV QERQRRRAPE HQPRAAAEQK PHLPSRYRQQ GAPHAKAGCP   180

W   PLQEDQGRIW LQQQRSKLKE KLGNEPSYKQ MAQSLRISTP ELRSRMRESF LAREVLTMSN   240
                                    R   337 DVE K..EHI.DGR K.K.RMVTA.   360
                                    C   133 PLD .F.R.LFRGR R.KDKMVQ..   156
                                    E   360 INR RMSIGEAKAR R.KKEMVEA.   383
                                                          <----------

W   IRLVISIAQK YDKLGVELSD LIQGGLIGLL RGIEKFDASR GFRISTYVYW WIRQGVSRAL   300
R   L...V...KR .SNR..A.Q. ...E.S...I ..V.....E. ...F...AT. ....SIT..I   420
C   L...V...K. .MNR.LSFQ. ...E.SL..I .AA....HEK .YKF...AT. ....AIT..I   216
E   L.......K. .TNR.LQFL. ...E.N...M KAVD..EYR. .YKF...AT. ....AIT.SI   443
     2.1 ---------->  <----------  2.2 ---------->  <----------  2.3 ---->  <----------

W   ADNSKTFRLP TYLHERLIAI RGAKYALEDQ -GVSPTTQNI AKLLNISEKK VHNATEAVNK   359
R   S.S.RSI... VHV.DTISL. .KQTK..QVE L.RP.SEEE. CESVG.DRA. YRLVM.CSRN   480
C   ..Q.R.I... VH.Y.TISR. KKTTKL.SQE M.RK..EEE. .TRMEMTIE. LRFIAKSAQL   276
E   ..QAR.I.I. VHMI.TINKL NRISRQMLQE M.RE..PEEL .ERMLMP.D. IRKVLKIAKE   503
     2.4 ---------->                  <----------------------------------

W   ALSLDQQAFP SLNGLPGDTL HSYIEDQNVA NDPWHGFEEW Y---LKEEVN KLLNSNLTER   416
R   IV..E----- ----T.LRSG DDVHFLGESL IA.EERA..N CSRDTLR.SI EKVLHC.ST.   531
C   PI..E----- ----T.IGKE EDSRLGDFIE A.GETPED.V A-KN.LR.DL EGVL.T.SP.   326
E   PI.ME----- ----T.IGDD EDSHLGDFIE DTTLELPLDS ATTESLRAAT HDVLAG..A.   554
     3 ---------------------------------------->  <----------

W   ERDIIQLYHG I-GKQCHTWE DISRQFGLSR ERVRQVGLIA MEKLKQRARR KKLDALLQDY   475
R   ..EV.RMRF. LTDGRPR.L. EVGSR.NVT. ..I..IESK. LK..RTPAEN NF..EY.GEV   591
C   ...VLR.RY. LDDGRMK.L. E.GQL.NVT. ..I..IEAK. LR..RHPN.N SI.KEYIR     384
E   .AKVLRMRF. .DMNTDY.L. EVGK..DVT. ..I..IEAK. LR..RHPSRS EV.RSF.D.   613
     4.1 ---------->  <----------  4.2 ---------->
```

Fig.4-3 Alignment of the deduced amino acid sequence of *sig*A from wheat (W) with *sig*A from *C. caldarium* (R), *rpo*D1 from *Synechococcus sp.* strain PCC 7942 (C) and *rpo*D from *Escherichia. coli* (E),.

Residues marked with an dot (.) are identical with those of wheat *sig*A. Evolutionary conserved regions 1.2 through 4.2 are indicated by arrows under sequences. The boxed residues denote conserved amino acid sequences in region 2 used to design PCR primers.

factor (*rpo*D1) from *Synechococcus* sp. strain 7942 ; 52%) and that of *E. coli* .$\sigma^{70}$ subunit (*rpo*D; 46%). Thus, it is likely that this cDNA encodes a protein which is a close relative of principal type σ factors, and we named this gene *sig*A.

### 4.3.2 The accumulation of RNA of wheat *sig*A

Using the cloned 122-bp cDNA fragment (shadowed in Fig.4-2) as a probe, we carried out northern hybridization analyses for the total RNA from the light-grown seedlings, the dark-adapted seedlings and the 2h reilluminated seedlings. As shown in Fig.4-4, a 2.3 kb transcript was detected as a dominant band with the light-grown and reilluminated seedlings, whereas the transcript of this size was not detected at all in the dark-adapted seedlings.

The induction time of the light-induced expression of the 2.3kb transcripts was also examined (Fig.4-5). The transcripts of *sig*A which disappeared in the dark-adapted seedlings began to be induced by illumination for 1h and recovered by 2h-illumination to the level of those in the light-grown seedlings.

These results indicate that at least one chloroplast σ factor (2.3 kb in mRNA) which is expressed only under illumination exists in wheat seedlings and possibly participates in the induction of chloroplast light type-RNA polymerase.

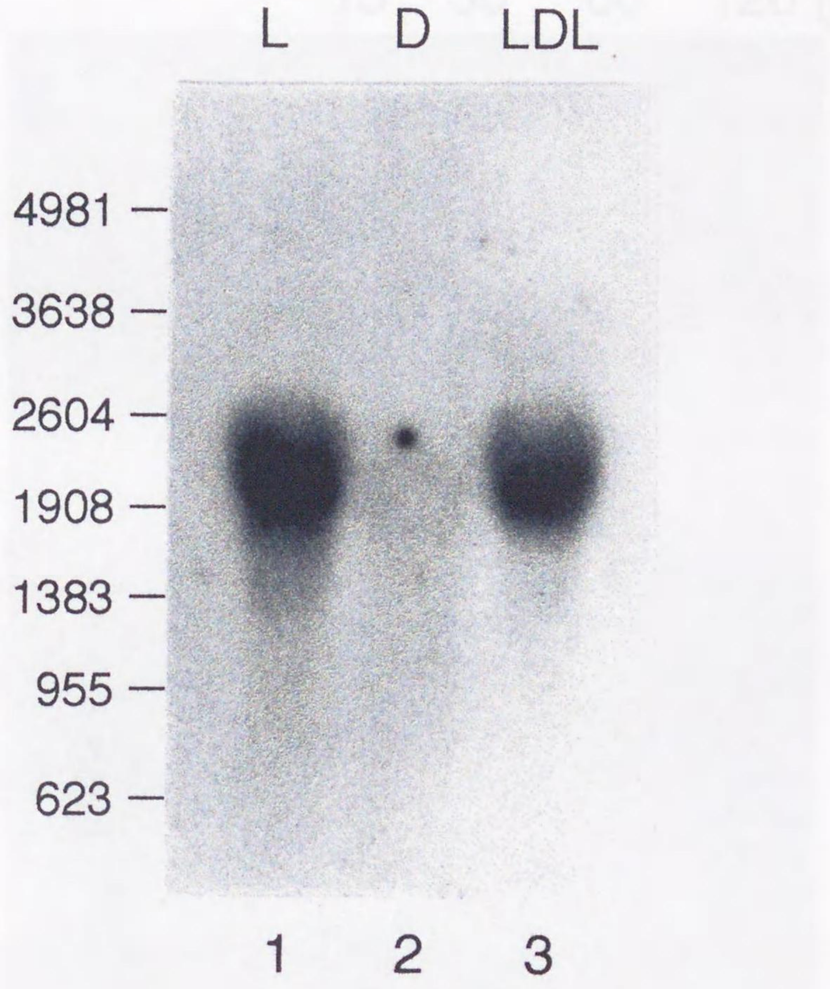



Fig.4-4 Northern blot hybridization analysis of mRNA for *sig*A.

A blot of total RNA (30 μg per lane) from light-grown seedlings (lane 1), dark-adapted seedlings for 24 h (lane 2) and re-illuminated seedlings for 2 h (lane 3) was hybridized to the 122-bp DNA probe amplified with P3 and P4 (see text) as primers from wheat *sig*A cDNA.

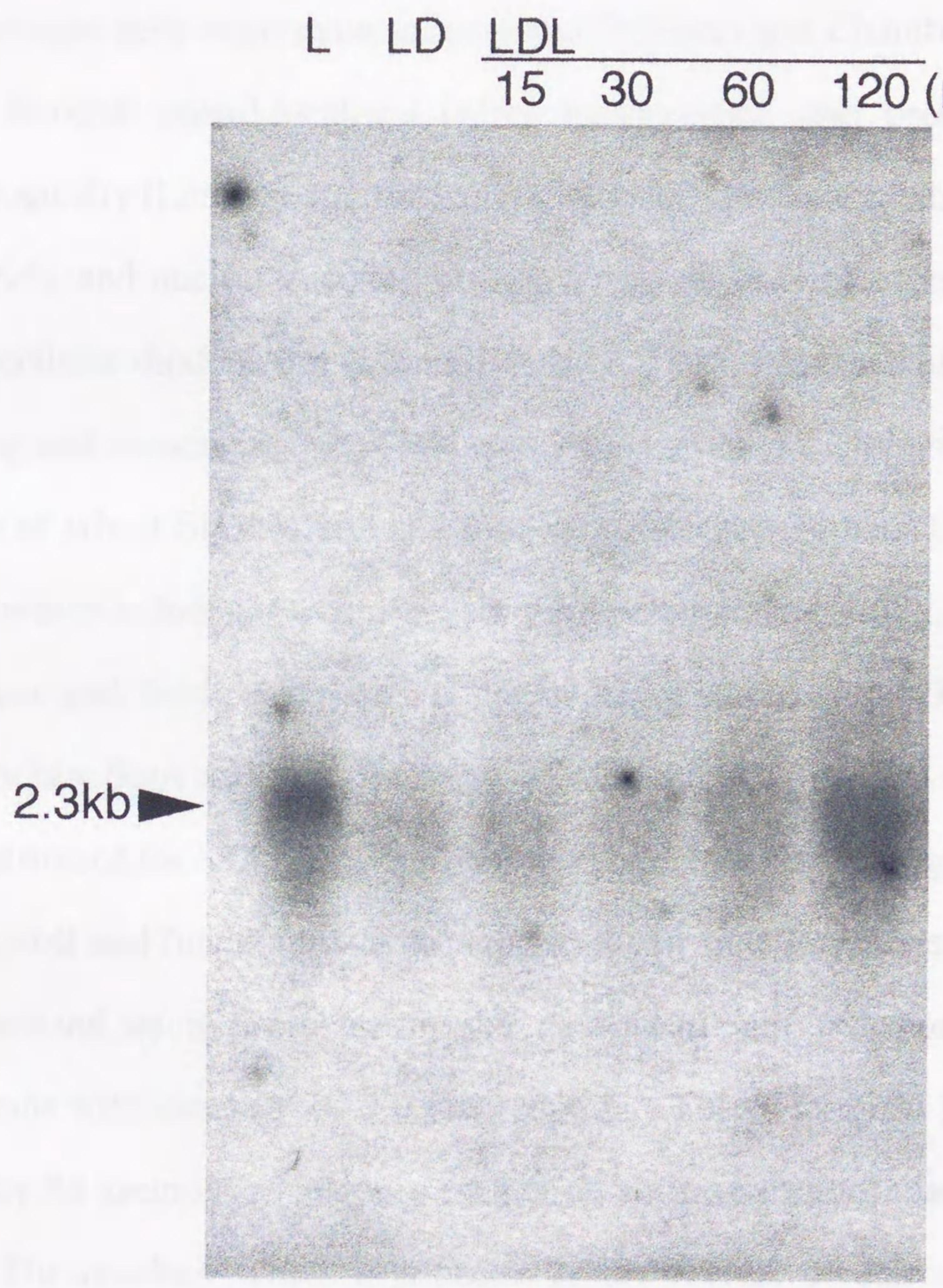



Fig.4-5 Time course of accumulation of *sig*A mRNAs in matured seedlings after the dark to light shift.

A blot of total RNA (30 μg per lane) from light-grown seedlings (lane 1), dark-adapted seedlings for 24 h (lane 2) and re-illuminated seedlings from 15 to 120 min (lane 3-6) was hybridized to the 122-bp DNA probe amplified with P3 and P4 as primers from wheat *sig*A cDNA. The arrowhead indicates the 2.3kb hybridized bands.

## 4.4 Discussion

Multiple usage of σ factors having different promoter specificities is known to control the differential gene expression in bacteria (Helmann and Chamberlin, 1988; Errington, 1993). Several plastid-localized σ-like factors have also been identified in plants immunologically (Lerbs *et al.*, 1988; Troxler *et al.*, 1994) and biochemically (Tiller and Link, 1994), and nuclear-encoded principal type plastid σ factors were cloned recently from unicellular rhodophytes (Liu and Troxler, 1996; Tanaka *et al.*, 1996). I succeeded in cloning and sequencing of cDNA of a higher plant σ factor (*sig*A). The amino acid sequence of wheat SigA is highly homologous to other principal type σ factors in the region 2 which is thought to function in core polymerase binding, promoter melting and recognition and binding of the '-10' promoter element (Fig.4-3). Strong homology between wheat SigA and other principal σ factors is also observed in region 4 implicated in recognition of the '-35' element. Thus, it is likely that wheat SigA is a close relative of $\sigma^{70}$ in *E. coli* and functions as a principal σ factor in chloroplasts. SigA is predicted to be synthesized as a precursor in the cytoplasm and transported into chloroplasts concomitant with cleavage of a transit peptide. The N-terminal region of wheat SigA extends by 84 amino acid residues compared with *Synechococcus rpo*D1 and is rich in Ser and Thr residues, which is a characteristic feature of chloroplast transit peptides (Gavel and Heijne, 1990). A 2.3 kb transcript which hybridizes to the cloned cDNA abundantly accumulated in the light-grown and re-illuminated wheat seedlings, but was not detected in the dark-adapted seedlings (Fig.4-4). These results may suggest that a light-induced σ factor (SigA) encoded by a 2.3 kb mRNA replaces a non identified dark-type σ factor to alter the RNA polymerase(s) from the dark-type to the light type. Satoh *et al.* (1997) demonstrated that the induction time for the *psb*D transcription after shift to the light is 1 to 2 h which is compatible with the induction time of the light-induced expression of the 2.3 kb transcript (Fig.4-5).

Since the 2.3 kb transcripts is expressed only in the illuminated seedlings, there must be other types of σ factors which strictly require the bacterial type '-35' and '-10' elements to function and they should be expressed constitutively or specifically in the dark to recognize the light-independent promoters such as the *psb*A promoter. Indeed, at least three c-DNA clones which resemble cyanobacterial principal σ factors present in *Arabidopsis* EST data base. Very weak bands were also detected on a northern hybridization at the position corresponding other than 2.3 kb with the total RNA extracted from the 24 h dark adapted wheat seedlings, when a probe extended as including 2.1 and 2.4 regions in the cloned cDNA was used (data not shown).

Alternatively, phosphorylation/dephosphorylation of the subunit proteins of the RNA polymerase was demonstrated to be involved in the mechanism of the light dependent modification of the chloroplast RNA polymerase. In mustard, phosphorylation state of plastid RNA polymerase core enzyme as well as σ-like factors was suggested to play an important role in transcriptional regulation of the *psb*A gene during the light-induced de-etiolation of the plastids (Tiller and Link, 1993). In wheat, the activity of the *psb*A promoter is hardly affected by light in mature chloroplasts, but it is enhanced during the light-induced de-etiolation of the dark grown seedlings (Kawaguchi *et al.*, 1992). These results may suggest that phosphorylation/dephosphorylation participates in the regulation of transcription of the *psb*A promoter during the light-induced de-etiolation step of the plastid. In matured chloroplasts, however, the differential expression of several types of chloroplast localized σ factor, depending on the light conditions, would be a key event in light dependent alternation of the chloroplast RNA polymerase in the core promoter recognition, although we have no direct evidence for actual involvement of the cloned σ factor itself in the light-dependent behavior of *psb*D LRP at present stage.

## 5. Summary and Concluding Remarks

*Psb*D promoter is light responsive one among four promoters present in *psb*D/C gene cluster. As described in section 2, it was observed *in vitro* that transcription from the *psb*D site in the *psb*D/C gene cluster is specifically activated in the plastid extracts prepared from the 4h-illuminated wheat seedlings, but not in those from the dark-grown seedlings. This behavior well reflects the feature of *in vivo* light-regulated transcription from the *psb*D/C gene cluster (Sexton *et al*., 1990a; Kawaguchi *et al*., 1994), indicating that transcriptional regulation is actually involved in light-induced variations of the *psb*D/C gene transcripts.

By heparin-Sepharose column chromatography, light-regulated transcription factor(s) was recovered in the fraction containing the nonabsorbed components (fraction A) and that factor(s) substantially separated from the plastid RNA polymerase. Those components in fraction A had the abilities to induce the transcriptional activity from the *psb*D site in the extract from the plastids of the dark-grown seedling. These findings indicated that the specific plastid transcriptional factors selectively activate the transcription from particular chloroplast promoters, such as *psb*D LRP, during the light-induced plastid development. The factor(s) collected in fraction A might be a kind of σ-like factor, but the factor(s) should be very different from the σ-like factors found in mustard in their affinities to heparin-Sepharose.

In the upstream regions of the transcription initiation sites of D/C-2, *psb*C and *psb*A, there are the prokaryotic promoter motifs, and these motifs were also recognized by *E. coli* RNA polymerase holoenzyme. In contrast, *psb*D LRP has unusual '-35' region, and transcription from the *psb*D site was scarcely observed in the plastid extracts from dark-grown seedlings and was not initiated by *E. coli* RNA polymerase.

To give an insight into the light switching mechanism of the *psb*D LRP, mutational analyses were carried out in the core promoter of *psb*A by means of *in vitro* transcription using wheat chloroplast extracts prepared from dark-adapted and re-illuminated seedlings. Mutations in canonical '-35' sequence of the *psb*A promoter, such as the deletion of '-35'motif, the exchange of '-35' element to the pseudo one of psbD LRP and such as the mutation of the length of spacer distance between the '-35' and '-10' elements (Fig.3-3), strictly reduced its transcriptional activity in the chloroplast extract prepared from the dark-adapted plants, but not in the extract from the re-illuminated plants. Introduction of the *psb*A '-35' element and a canonical spacing to the *psb*D LRP resulted in disappearance of light response ability of the *psb*D LRP (Fig.3-4). Destruction of '-10' elements completely abolished transcription of those promoters. These results suggest that the core promoter recognition by chloroplast RNA polymerase is required for light-responsive transcription and that RNA polymerase(s) which requires '-10' element but not '-35' element for transcription initiation is reversibly induced by the light in the mature chloroplast.

Transcription from psbD LRP was inhibited by tagetitoxin (Fig.3-5), a specific inhibitor of bacterial type plastid RNA polymerases. Tagetitoxin completely inhibited transcription from a constitutive promoter in *psb*D/C operon in dark and light extracts. In tobacco, the *psb*D LRP activity was not detected when plants were deleted the gene for the β subunit of bacterial type plastid RNA polymerases (*rpo*B) (Allison *et al.*, 1996). These results suggest that chloroplast RNA polymerases which transcribe genes of PSII components are mainly bacterial type in both chloroplasts from dark-adapted and reilluminated seedlings. Furthermore, the light-type chloroplast RNA polymerase is similar to the E$\sigma^{70}$-type *E.coli* RNA polymerase, since the light-type RNA polymerase recognized the TGn motif just in front of the '-10' element (Fig.3-6).

Highly purified chloroplast RNA polymerases have been obtained from maize (Hu and Bogorad, 1990), spinach (Larbs *et al.*,1985), pea (Rajasekhar *et al*, 1991) and mustard (Pfannschmidt and Link, 1994) containing 7-14 polypeptides (Table 1-3). In maize, some of those polypeptides(180, 120, 78 and 38kDa) were shown to be products of the plastid-encoded *rpo* genes. In pea, some of polypeptides of *rpo* genes (150, 54, and 51kDa) have been confirmed as RNA polymerase subunits by photo-crosslinking. In spinach, all of the major polypeptides were identified. Spinach leaf homogenates contained polypeptides that were immunologically related to $\alpha,\beta,\beta'$ and $\sigma$ subunits of *E.coli* RNA polymerase. 90 and 33kDa proteins cross-reacted with antibodies against $\sigma^{70}$ of *E.coli* in a solid-phase sandwich immunoassay and stimulated correct transcription initiation from rbcL when they were added to fractions of the spinach chloroplast enzyme containing $\beta$- and $\beta'$-like subunits. Troxler et al. (1994) has also shown immunological evidence of the existence of $\sigma$ factors in chloroplast RNA polymerase in maize, rice and red algae by using antibody against the principal $\sigma$ subunit of cyanobacteria RNA polymerase. Chloroplast RNA polymerases from maize and rice contained an immunoreactive 64kDa protein. Although the existence of $\sigma$ factors in chloroplast was suggested in many plant species, $\sigma$ factors have not been isolated from mature plants. Only in mustard on early greening process, 67, 52 and 29kDa $\sigma$-like factors have been found in chloroplasts and etioplasts. Those factors stimulated transcriptions when they were added to *E.coli* core RNA polymerase and enhanced binding activities of the *E.coli* core enzyme to DNA fragments containing the *psb*A, *trn*Q and *rps*16 promoters. However, the immunochemical relationship between $\sigma$-like factors and $\sigma$ factors of bacteria was not known, and there were no primary sequence data concerning $\sigma$-like factors. To confirm the features of $\sigma$ factors of chloroplast RNA polymerase, further refinements of the purification procedures of $\sigma$ factors are required.

Lonetto *et al*. have aligned 31 bacterial $\sigma$ factors and classified them into three groups

(1992): $\sigma^{70}$ superfamily which are essential for exponential growth (group 1), alternative σ factors no-essential for exponential growth (group 2) and σ factors which act for the transcription of genes involved in heat-shock response, flagellar development, and sporulation etc. (group 3). Group1 σ factors, so-called the primary σ factors, are highly similar with each other, having an amino acid sequence identity of at least 51%, and regions of sigma that are involved in promoter recognition are especially highly conserved. Three amino acid segments (region 2-4) are almost completely conserved among group 1 σ factors. Region 2 and region 3 overlap σ recognizing '-10' binding region and core enzyme biding domain. Region 2 contains the promoter recognition region,i.e., the "RpoD box" (Tanaka *et al.*, 1988). Region 4 overlaps the '-35' recognition region. Group 2 σ factors are less similar to the members of group 1. However, they are highly similar to the primary σ factors in their DNA-binding regions suggesting that the two groups of σ factors may recognize similar promoter sequences. Group 1 and 3 σ factors of *E.coli* display a maximum homology of 27% on amino acid sequence level, but functionally equivalent group 3 σ factors from different bacteria display homologies as great as 94%.

Amino acid sequence of SigA in wheat exhibits that SigA protein is the homologue of the primary σ factor. Region 2 and 4 of wheat SigA are highly conserved with those of σ factors of bacteria and red alga (Fig.4-3). This sequence similarity is in agreement with the *psb*A core promoter recognition behavior of light-type RNA polymerase resembling *E.coli* $E\sigma^{70}$-type RNA polymerase holo enzyme.

Although neither dark-induced nor constitutively expressed σ factors have been determined in wheat mature plants at present stage, finally I propose a model for the molecular mechanism of light regulatory transcription of *psb*D LRP (Fig.5-1). In mature chloroplasts, the differential expression of several types of σ factors may act as a key role in the light dependent alternation of the chloroplast RNA polymerase. The light-type chloroplast RNA polymerase recognize *psb*D LRP by associating with the light inducing

σ factor, while the dark-type chloroplast RNA polymerase in the dark-adapted seedlings may contain another σ factors which have different promoter recognition characters. To verify this alternation model, further experiments including the cloning of dark-type alternative σ factor(s) are requested.

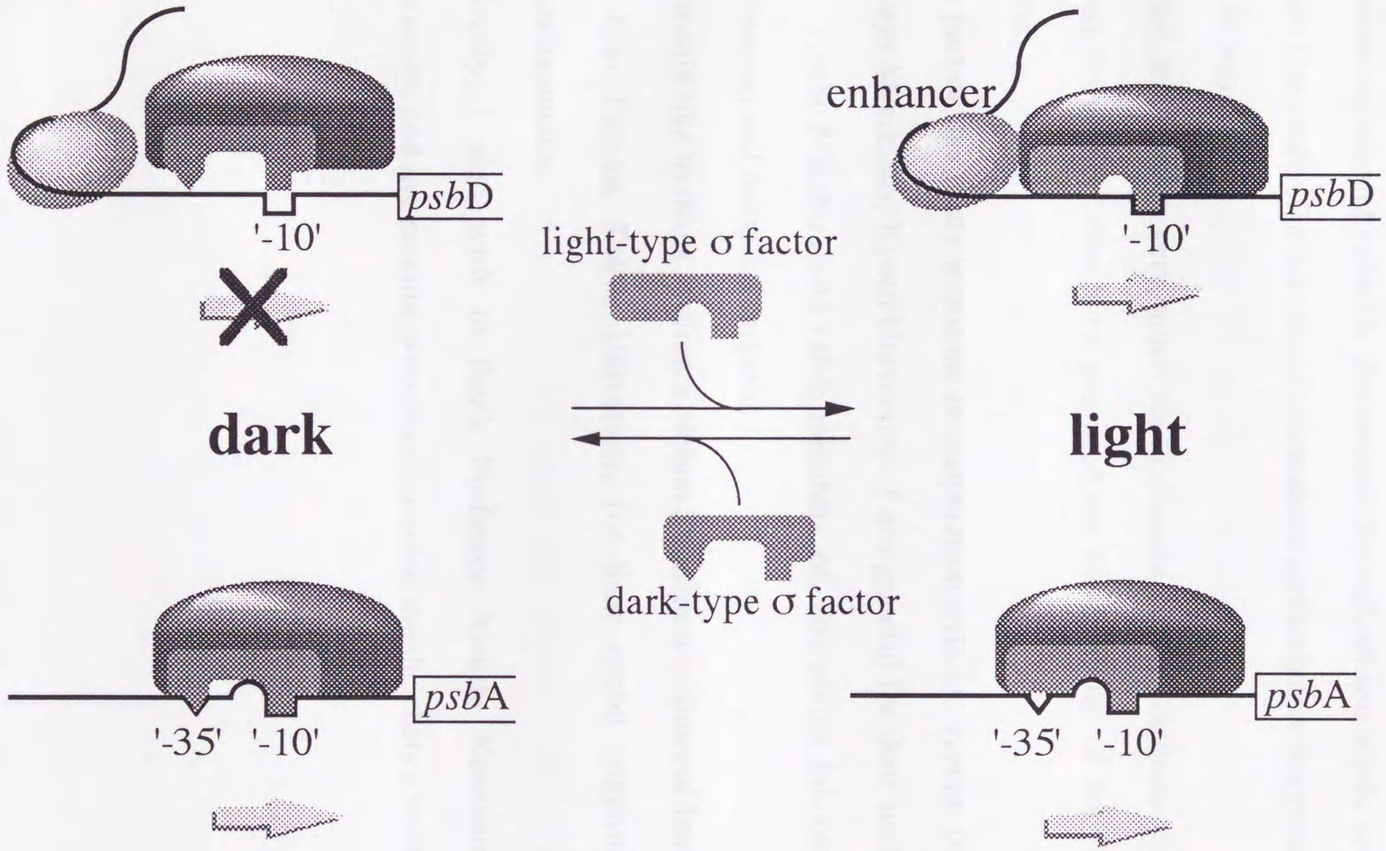



Fig.5-1 Schematic representations of the model for the molecular mechanism of the light regulatory transcription of chloroplast genes.

In chloroplasts, light- and dark-type nuclear-encoded σ factors may act alternatively depending on the light conditions. Both the '-35' and '-10'elements are strictly required for the dark-type σ factor to recognize the core promoter region. The chloroplast RNA polymerase containing the dark-type σ factor can initiate the transcription from the *psb*A site, but not from the *psb*D site which lucks the canonical '-35'motif on the promoter. While, the RNA polymerase containing the light-type σ factor can recognaize the *psb*D LRP. The sequence specific DNA-bindig protein which recognaize the upstream region of the core promoter of the *psb*D LRP acts as an enhancer. The TGn motif just upstream of the '-10'element of the *psb*A promoter allows the RNA polymerase containing the light-type σ factor to initiate the transcription from the mutated *psb*A promoter which lucks the canonical '-35'motif.

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