Endocytobiosis Cell Res. **15**, 235-245, 2004
"Endosymbiosis and Eukaryotic Organells" eds., Sugiura, M., Oelmüller, R., and Obokata, J.
Finkdruck Apolda, Apolda, pp. 235-245, 2004

Symbiotic aspects of participation of multiple σ factors in functional chloroplasts

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Abstract

The chloroplast, which is responsible for photosynthesis, the conversion of solar energy to chemical energy to be utilized in the cell, is understood to have symbiotically originated from an ancestor of cyanobacteria. Genetic information for building up functional chloroplasts is encoded both in nuclear and chloroplast genomes in eukaryotes. Chloroplast genes for photosynthesis are transcribed mainly by plastid-encoded plastid RNA polymerase (PEP), and σ factors are engaged in determining specificity for the promoters of genes to be transcribed. The function and necessity of multiple σ factors in higher plants are discussed, particularly in the symbiotic aspects.

Introduction

The chloroplast present in plant cells is one form of "plastid" that is plastic and derived from the Greek word "*plastis"* meaning a molder. Morphologically undifferentiated plastids are called proplastids. Differentiated plastids include chloroplasts possessing chlorophylls for photosynthesis; etioplasts in angiosperms grown without light; chromoplasts, which accumulate carotenoids, especially lycopene, in flowers and fruits; and leucoplasts such as amyloplasts, which are colorless and enriched with starch, present in storage tissues including endosperm, cotyledons, root caps, and tubers as well as in cultured plant cells.

Gene expression in the plastid is regulated at multiple steps: DNA replication, transcription, post-transcriptional events, translation, and post-translational mechanisms (Baumgartner et al., 1993; Deng and Gruissem, 1997; Kobayashi, 1991; Kobayashi et al., 1990; Mullet, 1988). Regulation of expression of photosynthesis genes in the chloroplast is compared to that in nongreen plastids, and multiplicity of the nongreen

plastids has made understanding the common mechanisms underlying regulation difficult. We have employed *Arabidopsis thaliana*, which is suited for molecular genetic dissection of higher plants, in focusing on the differences between regulation in chloroplasts in green leaves and regulation in plastids in nongreen roots and calli. Gene expression was most critically regulated at the level of transcription in these particular organs of *Arabidopsis*, in spite of minor regulation at the levels of DNA replication and RNA stability (Isono et al., 1997a). Since any difference in DNA methylation status and activities of *in vitro* transcription with plastid DNA from different sources was not detectable in green leaves, roots, and calli, we concluded that regulation could be ascribed to the difference in activity of transcriptional machinery (Isono et al., 1997a). In 1997, we and other research groups succeeded in cloning cDNAs for σ factors of plastid-encoded plastid RNA polymerase (PEP, multimeric *Escherichia coli*-type RNA polymerase) (Isono et al., 1997b; Tanaka et al., 1997) and nuclear-encoded plastid RNA polymerase (NEP, monomeric T7 or T3 bacteriophage-type RNA polymerase) (Hedtke et al., 1997). Afterward, it has become possible to study the transcriptional machinery in plastids at the molecular level.

1. Plastid RNA Polymerases

There is evidence that photosynthetic genes in the plastid are transcribed by PEP, and housekeeping genes are transcribed by NEP (Allison et al., 1996). The -10 and -35 sequences, 5'-TATAAT-3' and 5'-TTGACA-3', respectively, in promoters of many plastid genes (Kung and Lin, 1985) are recognized by PEP (Igloi and Kössel, 1992). The *E. coli* RNA polymerase is composed of a core complex of α, β, β', and ω subunits and one of a variety of σ factors, the principal one being σ^{70}, which is capable of binding to the -10 and -35 sequences (Burgess et al., 1969; Busby and Ebright, 1992; Helmann and Chamberlin, 1988). Determination of the complete nucleotide sequences of plastid genomes from liverwort (Ohyama et al, 1986), tobacco (Shinozaki et al., 1986), rice (Hiratsuka et al., 1989), and other plants has resulted in finding the genes, *rpoA*, *rpoB*, and *rpoC,* likely encoding the α, β, and β' subunits, respectively, of PEP. In higher plants, *rpoC* is duplicated: *rpoC1* for the β' subunit and *rpoC2* for the β" subunit. Amino acid sequences deduced from maize plastid genes, *rpoC2*, *rpoB*, *rpoC1*, and *rpoA*, have proved to correspond to those of 180-, 120-, 78-, and 38-kDa polypeptides, respectively, of highly purified maize PEP (Hu and Bogorad, 1990; Hu et al., 1991).

2. Cyanobacterial σ Factors

Multiple σ factors have been reported in each cyanobacterial species whose ancestor is thought to be common to that of chloroplasts in higher plants. Each σ factor is responsible through regulation of gene expression for adaptation of cells to changes of environment such as temperature and light, including drastic changes such as stresses. The primary σ factors, the group 1 σ factors (Figure 1), resembling the principal σ^{70}, are involved in propagation and division of cells (Kaneko et al., 1996; Wösten, 1998). The group 2 σ factors, which are homologous to group 1 σ factors except for region 1.1, are known to be present in cyanobacteria. Group 2 σ factors are recognized to be nonessential for stationary growth of cells, since their disruptants seemed to grow as wild-type cells under standard conditions, whereas group 2 σ factors may play roles in adaptation to environmental changes and stresses (Gruber and Bryant, 1998; Khudyakov and Golden, 2001). The group 2 σ factors have been reported in *Synechocystis* PCC 6803, *Anabaena* PCC 7120, and *Synechococcus* PCC 7942 (Figure 1). According to phylogenetic analysis of primary structures of the regions 2.1 to 4.2 of σ factors, cyanobacterial σ factors are classified into 5 subgroups, SigA to SigE (Figure 1). The subgroup SigA is assigned to the group 1, and the subgroups SigB to Sig E constitute the group 2 σ factors. The group 3 σ factor, SigF, which is different from σ factors of groups 1 and 2, has been reported to be important for pilus formation in cyanobacteria (Bhaya et al., 1999). It remains to be revealed whether SigF, which similar to *E. coli* σ^{32}, might respond to a specific stress.

3. Evolution of σ Factors

Nuclear genes for σ factors have been cloned for the first time from eukaryotes, the primitive red algae *Cyanidium caldarium* (Tanaka et al., 1996) and *Galdieria sulphuraria* (Liu and Troxler; 1996), whose conserved domains have homology to those of bacterial primary σ factors. We and others tried to clone genes for counterparts of bacterial σ factors from higher plants based on their possible homology, but have not succeeded in getting clones. Using expressed sequence tags (ESTs) of *Arabidopsis*, cDNAs for σ factors, SIG1, SIG2, and SIG3*[1], were cloned from *Arabidopsis* (Isono et al., 1997b; Tanaka et al., 1997). Afterward, the entire nucleotide sequence of the *Arabidopsis* nuclear genome has been determined, and 6 genes for σ factors, SIG1 to SIG6, have been revealed to exist. The cDNAs and genomic sequences for σ factors have further been cloned from a variety of species in the range of primitive eukaryotic algae to higher plants, among which all genes for σ factors are encoded in nuclear

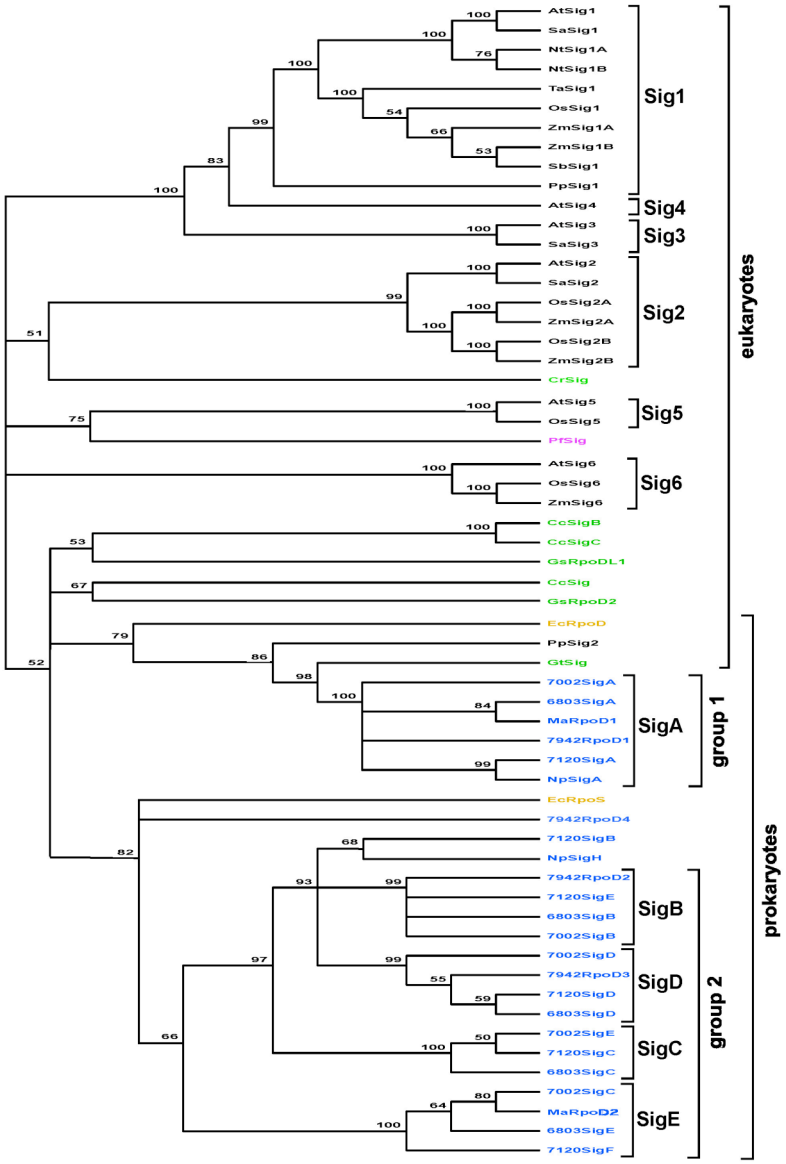

Figure 1. The phylogenetic tree of σ factors (σ^{70} homologues) of cyanobacteria to higher plants.
The phylogenetic tree of σ factors as a component of PEP from organisms with ability of plant-type photosynthesis was built on the basis of amino acid sequences registered in the GenBank (http://www.ncbi.nlm.nih.gov/) and CyanoBase (http://www.kazusa.or.jp/cyano/) databases. Only one independently registered sequences is employed when the sequences are for the same molecular species from the same organism. The calculation was done only for the conserved sequences in the 2.1 to 4.2 regions by the ClustalW alignment and neighbor joining [bootstrap ()ollapse)nodes: <50%); midpoint rootin]] using MacVector 7.2 (Accelrys, Inc.). The numbers in the tree are the percentage occurrence of nodes that occur very frequently in the data resampled 1,000 times.

The species, nomenclatures of the products, and accession numbers are as follows: At, *Arabidopsis thaliana* [Sig1, AB004821; Sig2, AB004820; Sig3, AB004822; Sig4, AB021119; Sig5, AB021120; Sig6, AB029916]; Nt, *Nicotiana tabacum* [Sig1A, AB023571; Sig1B, AB023572]; Os, *Oryza sativa* [Sig1, AB005290; Sig2A, AB095094; Sig2B, AB095095; Sig5, AB096011; Sig6, AB096012]; Sa, *Sinapsis alba* [Sig1, Y15899; Sig2, AJ276656; Sig3, AJ276657]; Sb, *Sorghum bicolor* [Sig1, Y14276];Ta, *Triticum aestivum* [Sig1, AJ132658]; Zm, *Zea mays* [Sig1A, AF058708; Sig1B, AF058709; Sig2A, AF099110; Sig2B, AF099111; Sig6, AF099112]; moss (colored black): Pp, *Physcomitrella patens* [Sig1, AB059354; Sig2, AB046872]; eukaryotic algae (colord green): Cc, *Cyanidium caldarium* RK-1 [Sig, D83179; SigB, AB006798; SigC, AB006799]; Cr, *Chlamydomonas reinhardtii* [Sig, AB049220]; Gs, *Galdieria sulphuraria* (*Cyanidium caldarium*, Allen strain) [RpoDL1, L42639; RpoD2, AF050634]; Gt, *Guillardia theta* [Sig, AAK39807]; protozoa (colored purple): *Plasmodium falciparum* 3D7 [Pf, CAD50481]; cayanobacteria (colored blue): 6803, *Synechocystis* sp. PCC 6803 [SigA, slr0653; SigB, sll0306; SigC, sll0184; SigD, sll2012; SigE, sll1689]; 7002, *Synechococcus* sp. PCC 7002 [SigA, U15574; SigB, U82435; SigC, U82436; SigD, U82484; SigE, U82485]; 7120, *Anabaena* sp. PCC 7120 [SigA, M60046; SigB, M95760; SigC, M95759; SigD, AF262216; SigE, AF262217; SigF, AF262218]; 7942, *Synechococcus* sp. PCC 7942 [RpoD1, D10973; RpoD2, D78583; RpoD3, AB024709; RpoD4, AB024710]; Ma, *Microcystis aeruginosa* K-81 [RpoD1, D85684; RpoD2, D86575]; Np, *Nostoc punctiforme* PCC 73102 [SigA. AF265349; SigH, AF022822]; *Escherichia coli* (colored yellow): Ec, *Escherichia coli* [RpoD, J01687; RpoS, X16400].

genomes. The primary structure of σ factors in eukaryotes also resembles that of σ^{70}. All σ factors reported so far from higher plants belong to any of 6 groups of *Arabidopsis* σ factors (Figure 1). The higher homology is recognized in each group of σ factors among either monocots or dicots (Figure 1).

Higher plants have been more diversified on the structure of σ factors. The homology among σ factors from higher plants, all of which are σ^{70} homologues, is lower than that among σ^{70}-type factors from eubacteria, including cyanobacteria. What about the situation of σ factors from eukaryotic algae, which would evolutionally be on the way from cyanobacteria to higher plants? The σ factors of primitive red algae such as *Cyanidium caldarium* more resemble bacterial ones, whereas σ factor from the green alga *Chlamydomonas reinhardtii* is homologous to Sig2 of higher plants even with a lower percentage of being in the same branch (Figure 1). Two σ factors derived from the moss *Physcomitrella patens* are assigned to Sig1, which is homologous to σ factors from higher plants, and another, Sig2, being similar to those from bacteria (Figure 1). *Physcomitrella* Sig1 belongs to neither the monocot Sig1 group nor its dicot counterpart (Figure 1). A nucleotide sequence for σ^{70} homologue from the protozoa *Plasmodium falciparum* has recently been complied in the database. This homologue is interestingly related to Sig5, although the node in the branch would easily be collapsed depending on parameters for calculation (Figure 1). The overall multiplicity of σ factors supports the speculations: (1) σ factors of cyanobacteria and higher plants first branched from their common ancestor, (2) σ factors from primitive red algae and Sig2 from moss evolved from the cyanobacterial-type σ factors, and (3) σ factors of green algae would have evolved from the plant-type σ factors which have finally more diversified.

4. Participation of PEP in Higher Plants

In vivo and *in vitro* experiments using tobacco plants in which chloroplast-encoded *rpoB* or *rpoA* was knocked out, indicated the possibility that PEP was responsible for the transcription of photosynthesis genes such as *psbA* [for the D1 protein of photosystem (PS) II reaction center] and *rbcL* [for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)] (Allison et al., 1996), whereas NEP was involved in transcription of housekeeping genes including *rpoB* (Liere and Maliga, 1999). Transcriptional activities of *psbA* and *psbD* (encoding the D2 protein of PS II reaction center), *psbC* (for 44-kDa protein in PSII), and *psbE* (for 8-kDa subunit of cytochrome b_{559}) in wheat leaves were determined by run-on assay (Satoh et al., 1999).

Although RNA polymerase in the basal position of dark-adapted leaves was engaged in transcription of *psbA*, *psbD*, *psbC*, and *psbE*, the polymerase present in tips of illuminated leaves transcribed *psbA* and *psbD* (Satoh et al., 1999). The transcription with the basal RNA polymerase required –10 and –35 promoter sequences in the *in vitro* assay, whereas the tip polymerase did not require the –35 sequence (Satoh et al., 1999). Wheat *psbD* is known to have multiple promoters which differentially function depending on different wavelengths of light. Transcription from one of the *psbD* promoters, the light-responsive promoter (LRP), was found to be under circadian regulation (Nakahira et al. 1998). Coincidentally, levels of transcripts for *SigA* (*Sig1*) changed as regulated by a circadian clock (Morikawa et al., 1999). The circadian regulation of chloroplast gene expression would be complicated, since we have observed that the circadian response of the expression was enhanced in transgenic *Arabidopsis* in which *SIG* genes are ectopically expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Shimizu et al., unpublished). The overall findings strongly support the possibility that there are multiple σ factors responsible for specific and refined regulation of chloroplast gene expression.

Involvement of *SIG2* was revealed with an *Arabidopsis* line in which *SIG2* was knocked out (Shirano et al., 2000). The knockout line was pale-green with impaired chloroplast development. The observation by electron microscope suggested that *SIG2* gene was essential for normal photomorphogenesis of chloroplasts but not for skotomorphogenesis of etioplasts. The knockout line has also revealed that at least 4 tRNA species are transcribed with PEP associated with SIG2, among which tRNA-Glu is specially indispensable for both protein and tetrapyrrole (chlorophyll) syntheses (Kanamaru et al., 2001).

We have employed the heterotrophically-cultured plant cell line, tobacco BY-2, which was transformed with each of all 6 *SIG*s from *Arabidopsis* under the control of the CaMV 35S promoter (Yoshimoto et al., unpublished). SIG1 and SIG3 dramatically enhanced expression of photosystem genes, such as *psbA* and *psbD*, whereas SIG2 and SIG4 enhanced expression of all examined photosynthetic genes to a lower degree. In transient expression assays with mutagenized promoter sequences, similar functions were assigned to SIG1 and SIG3 regarding their recognition of common nucleotides in the consensus -35 sequence. On the other hand, the third and fifth nucleotides in the -10 region (5'-TATAAT-3') conferred distinct specificities on SIG1 and SIG3, respectively (Yoshimoto et al., unpublished). The occurrence of multiple species of σ factors with

different promoter specificities is thus crucial for the regulation of expression of a set of plastid genes for the assembly of the photosynthetic machinery.

5. Regulation through Expression of *SIG* Genes

Most plastid photosynthesis genes are expressed in response to light, although pathways of signal transduction starting with light perception for chloroplast gene expression are unknown. Expression of plastid photosynthesis genes is coordinated with that of *SIG* genes either in photomorphogenesis mutants of *Arabidopsis* or in wild-type plants exposed to blue, red, or far-red light (Shimizu et al., unpublished). Expression of *psbA,* among other plastid genes, was strictly repressed by sugar, and that of the gene for SIG2, which specifically promoted transcription of *psbA,* was also suppressed by sugar (Shimizu et al., unpublished). The native and modified *SIG*s were ectopically expressed in *Arabidopsis* under conditions where endogenous *SIG*s were barely expressed. This experiment has proven that levels of SIGs directly drive the expression of plastid photosynthesis genes (Shimizu et al., unpublished).

We have adapted activation tagging to *Arabidopsis* and screened and analyzed mutants in which photosynthesis genes have become expressed in nonphotosynthetic calli, to elucidate the signal transduction underlying buildup of functional chloroplasts. A mutant line, *ces101* (*callus expression of RBCS*), was obtained from a parental line transformed with the hygromycin B phosphotransferase gene under the control of the promoter of *RBCS-3B* (a nuclear gene for the small subunit of Rubisco) (Goto et al., unpublished). The calli of *ces101* were green, and the transcript for *RBCS* was at least 700 times higher and that for *SIG1* was also 6 times higher in comparison with those in the wild-type line. It has been shown by Southern hybridization that a single copy of T-DNA is inserted in the genome. We have amplified and identified the genomic region neighboring the T-DNA by TAIL-PCR (Goto et al., unpublished).

6. Perspective of Regulation by Phosphorylation of σ Factors

Phosphorylation of functional proteins is associated with rapid change of protein activities. There are several examples in which phosphorylation/dephosphorylation regulates expression of genes for photosynthesis. Translation of the *psbA* transcript in *C. reinhardtii* is reported to be suppressed in the dark by inactivation of chloroplast polyadenylate-binding protein (cPABP) following phosphorylation of chloroplast protein disulfide isomerase (cPDI) (Kim and Mayffield, 1997). Phosphorylation would also be involved in transcription from *psbD* LRP in barley (Christopher et al., 1997). It

was observed that treatment of RNA polymerase prepared from etioplasts in dark-grown seedlings of mustard with phosphatase enhanced the ability of binding of its components to promoter of *psbA* (Tiller and Link, 1993). The Ser/Thr protein kinase and its associated form with PEP, the latter designated "plastid transcription kinase, PTK", have been characterized (Baginsky et al., 1997). A cloning strategy to define the PTK gene(s) resulted in the isolation of a full-length cDNA for a protein with overall high homology with the α subunit of cytosolic casein kinase (CK2) that contained an N-terminal extension for a putative plastid transit peptide (Ogrzewalla et al., 2002). The PTK has also been reported to mediate redox control of plastid *in vitro* transcription (Baginsky et al., 1999). The redox state of an electron carrier, plastoquinone, governs the distribution of absorbed light energy between PS I and PS II by controlling the phosphorylation of a mobile, light-harvesting, pigment-protein complex. It has been shown by run-on assay with isolated chloroplasts that the redox state of plastoquinone also controls the rate of transcription of genes, *psbA* and *psaAB*, the latter being for P700 apoproteins A1 and A2 in PS I (Pfannschmidt et al., 1999). As a result of this control, the stoichiometry between the two PSs changes in a way that counteracts the inefficiency produced when either PS limits the rate of the other.

The PTK was found to respond to reversible thiol/disulfide formation of glutathione, GSH/GSSG, in mustard seedlings (Baginsky et al., 1999; Baena-González et al., 2001). Although the activity of PTK was severely inhibited *in vitro* with GSH, it was not influenced by GSSG and DTT (Baginsky et al., 1999). The GSH/GSSG ratio increased under a higher intensity of light, where the absolute amounts of both GSH and GSSG were reduced in comparison with those under a moderate intensity of light. The transcriptional activities of chloroplast genes for photosynthesis were also enhanced under the higher irradiance, whereas activities diminished to near 50% under moderate light (Baena-González et al., 2001). These results lead to the hypothetical scheme that irradiance elevates the GSH/GSSG ratio, which inactivates PTK, followed by maintenance of the dephosphorylated status of PEP, resulting in the enhanced level of chloroplast transcription.

The above experimental results let us suppose the regulation through phosphorylation of σ factors with Ser/Thr protein kinase. The sites of amino acid sequences predicted to be recognized by Ser/Thr protein kinase are mapped on polypeptides of 6 σ factors from *Arabidopsis*, as well as 5 members from *Synechocystis* PCC 6803 (Figure 2). The locations of sites to be phosphorylated are not conserved in all the polypeptides. The conserved site is only ahead of the 1.2 region in SIG1, SIG3,

and SIG5 of *Arabidopsis* (Figure 2). We have made *Arabidopsis* transgenic with SIG1, the major species of σ factors in green leaves of *Arabidopsis*, with or without deletion of putative phosphorylation sites to express them ectopically. The plants were labeled with [^{32}P]orthophosphate *in vivo* and SIG1 was recovered with antibodies. Determination of molecular mass of complexes associated with SIG1, indicated that σ factors competed to bind with core enzyme to generate transcriptionally-active holoenzyme in the light, whereas SIG1 phosphorylated at Thr-170 was more preferentially associated than unphoshorylated one with core enzyme to interfere with transcription in the dark (Kato et al., unpublished).

A Ser/Thr protein kinase partially homologous to *Arabidopsis* MAPKKs and adenylate cyclases is present in *Synechocystis* PCC 6803 (CyanoBase, http://www.kazusa.or.jp/cyano/), although the essential phosphorylation sites are not conserved in *Arabidopsis* and cyanobacterial σ factors. We cannot deny the possibility that the similar regulation through phosphorylation/dephosphorylation of σ factors might participate in transcription in cyanobacteria. In this context, it is worth mentioning that the mutated D1 proteins, which reduce the electron flow, conferred phototolerance on *Synechocystis* PCC 6803 via continuous expression of genes for building up the photosynthetic machinery (Narusaka et al., 1999).

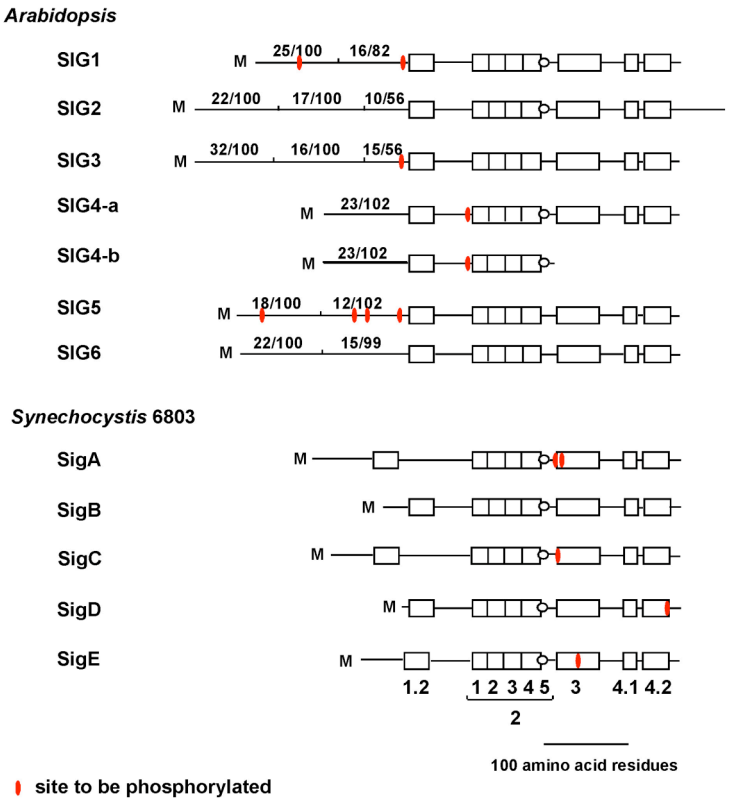

Figure 2. The sites of amino acid sequences predicted to be phosphorylated by Ser/Thr protein kinase in σ factors from *Arabidopsis* and *Synechocystis* PCC 6803. The sites were predicted on the basis of amino acid sequences under the accession numbers shown in the legend for Figure 1 by MacVector 7.2 (Accelrys, Inc.) and Prosite (http://kr.expasy.org/cgi-bin/scanprosite/). The numbers such as "1.2" at the bottom indicate the conserved regions. The other numbers along with each polypeptide are the numbers of Ser plus Thr per 100 residues: the higher scores are characteristic of transit peptides for chloroplast targeting. Two mature transcripts are generated from *Arabidopsis SIG4* gene by alternative splicing.

Concluding Remarks

The photosynthetic machinery in cyanobacteria is basically common to that in chloroplasts of higher plants except for the composition of light-harvesting pigments, and is thought to have evolved from a common ancestor. However, the most striking differences between cyanobacteria and higher plants are: being prokaryotes vs. eukaryotes, in which genes for photosynthesis are split into the plastid and nuclear genomes, and being movable vs. being settled down during their life cycle. The expression of photosynthetic genes in the plastid genome is placed under the control of the nuclear genes in higher plants. Higher plants have evolved to acquire the ability to adapt and respond to environmental changes due to their nature of being settled down. All organisms on the earth's surface are exposed to daily change of day and night, and the light forces the photosynthetic machinery to excite electrons to flow in the PSs, resulting in drastic change in redox in day and night. It is concluded that genes for σ factors migrated into the nuclear genome during evolution with acquiring the nature of response through phosphorylation to day/night change.

Acknowledgements

We are indebted to members at our laboratory, Kohki Yoshimoto, Shingo Goto, and Yasuo Niwa for citation of unpublished data.

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Footnote

*[1] The symbols for genes are italicized, and those for the products are not. The Commission on Plant Gene Nomenclature (CPGN) recommends first capitalized letter followed by lowercase letters for plant nuclear genes, to which plant σ factors belong, and letters starting with lowercase such as *psbA* and *rbcL* for plastid genes. In case of σ factors, therefore, *Sig1*, *Sig2*, *Sig3*, *etc*. satisfy this criterion. On the other hand, "the Steering Committees for the *Arabidopsis* Genome Project" has independently made the common rule that genetic loci are given with designations consisting of three uppercase letters for wild-type genes and lowercase ones for mutated genes, and different loci are with the same three-letter symbols and distinguished by subsequent numbers. Therefore, we here adopt *SIG1*, *SIG2*, *SIG3*, *etc*. for *Arabidopsis* σ factors. Cloning of cDNAs for σ factors from *Arabidopsis* was independently carried out at several laboratories and published coincidentally without refereeing each other, resulting in assigning the different nomenclatures to the same genes. On accepting the CPGN's proposal, primary researchers of plant σ factors discussed and reached the agreement of gene nomenclatures shown in the Figure 1. The details of discussion are shown on "http://sfns.u-shizuoka-ken.ac.jp/pctech/sigma/proposal/" and "http://mbclserver.rutgers.edu/CPGN/". However, in case of cyanobacterial σ factors, the nomenclatures have not yet been approved.