Characterization of two genes, *Sig1* and *Sig2*, encoding distinct plastid σ factors[1] in the moss *Physcomitrella patens*: phylogenetic relationships to plastid σ factors in higher plants

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Abstract We isolated the cDNA for a σ factor from the moss *Physcomitrella patens*, which possesses unusually large N-terminal extension and the conserved subdomains 1.2–4.2. Phylogenetic analyses indicated that this novel σ factor and PpSIG1*[2], a plastid σ factor previously identified from *Physcomitrella*, were classified into SigA and SigB groups, two major classes of higher plant plastid σ factors, respectively. According to the nomenclature recently proposed, we renamed PpSIG1* into PpSIG2, and named the novel σ factor PpSIG1. A transient expression assay using a green fluorescent protein showed that the N-terminal region of PpSIG1 acts as a chloroplast-targeting signal. Reverse transcription-PCR experiments showed that light induces the expression of the *Sig1* and *Sig2* genes encoding PpSIG1 and PpSIG2, respectively. Thus, PpSIG1 and PpSIG2 are likely plastid σ factors regulating plastid gene expression in response to light signals.

Key words: σ Factor; Transcription; Plastid; *Physcomitrella patens*

1. Introduction

In higher plants, plastid genes are transcribed by at least two types of RNA polymerases (RNAPs), a bacteriophage-type single subunit RNAP and a bacteria-type multisubunit RNAP [1]. The latter consists of a core catalytic complex and a putative σ factor that is supposed to confer promoter selectivity on the core complex based on the functions of bacterial σ factors [2]. The α, β, β′ and β″ subunits of the core complex are encoded by plastid genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, respectively, whereas genes for plastid σ factors were not found on the plastid genome [2]. Recently, nuclear genes for putative plastid σ factors have been identified in red algae [3–5] and higher plants [6–14]. These genes appear to be members of a gene family in each species [5–7,10,12–14], and their expression is tissue- or organ-dependent [7,8,10,12–15], chloroplast development-dependent [15], light-dependent [4–9,11,16] and circadian clock-dependent [11,16]. Moreover, in some cases, each member of the gene family is differentially expressed under the same light conditions [5,7,10,12]. These facts suggest that the plastid σ factors regulate different sets of plastid genes in response to various environmental and endogenous signals. In spite of the supposed functional significance as a critical regulator in plastid gene expression, the in vivo function of plastid σ factors is largely unknown.

Transformation and homologous recombination occur at a high frequency in the moss *Physcomitrella patens* [17–20], which enable efficient analysis of gene functions in vivo by targeted gene disruption [21–24]. Previously we isolated and characterized a cDNA encoding a plastid σ factor PpSIG1* from *Physcomitrella*, and named the corresponding gene *sig1** [25]. Here, we report a newly isolated cDNA species encoding another σ factor in *Physcomitrella*, and show that the moss possesses a gene family for σ factors.

2. Materials and methods

2.1. Plant material and growth conditions

P. patens (Hedw.) was grown under continuous illumination at 40 μmol/m^2/s from white fluorescent lamps as the standard culture conditions described by Nishiyama et al. [26].

2.2. Cloning procedures

We amplified a DNA fragment corresponding to an expressed sequence tag (EST; AW738970) from moss cDNA by PCR using two primers Sig3U1 (5′-TTATGGAGTGGGCCGAGAAGATGGT-3′) and Sig3D1 (5′-TACTGAAAATGTCGTCACATCGCA-3′). The PCR product with the expected size of 352 bp was used as a probe to screen a *Physcomitrella* cDNA library (gift from A. Cuming and S. Bashiardes; 8×10^5 pfu/μl).

The 5′-terminal portion of the *Sig1* cDNA was obtained by the 5′-rapid amplification of cDNA ends (5′-RACE) method using the 5′-RACE System for Rapid Amplification of cDNA Ends Version 2.0 (Gibco BRL, MD, USA) with primers GSP1 (5′-CAGGAGGCGAAGGTTTATCACGGA-3′), GSP2 (5′-TGCGGCTCCAGCAGTGTCTTGTGA-3′) and nested GSP (5′-TGAAATCTGCCCGAGTAACTCTCA-3′).

The *Sig1* and *Sig2* gene-containing regions were amplified from moss genomic DNA using primers based on the corresponding cDNA sequences, respectively. The primers were as follows: Sig2GU2 (5′-GCACGCTGAGCCTTAATTCTCTTG-3′), Sig2GD2 (5′-ATATGTTGGACTTACAAATTGTTCCC-3′) for the *Sig1* region, and Sig1GU2 (5′-CATCGCACCGCTTTTGGCGAGCACCCCAGT-3′)

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[1] EMBL accession no. AB059355 (*Sig1*), AB059356 (*Sig2*) and AB059354 (*Sig1* cDNA).

[2] To avoid confusion, here we refer to the σ factor gene and its protein product previously identified in *Physcomitrella* by our group ([Hara, K. et al. (2001) Biochim. Biophys. Acta 1517, 302–306]; AB046872) as *sig1** and PpSIG1*, respectively; otherwise new names (*Sig2* and PpSIG2, respectively) are used.

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and Sig1GD2 (5′-TCTGTAACAGGATGAACACTCACATAAC-GA-3′) for the *Sig2* region. The PCR products with the lengths of 4590 and 3499 bp containing *Sig1* and *Sig2* genes, respectively, were cloned and sequenced. Exon–intron structures were predicted by comparison of the cDNA and gene sequences with the help of splice site prediction (NetPlantGene, http://www.cbs.dtu.dk/services/NetPGene/).

2.3. Reverse transcription (RT)-PCR experiments

RT was performed with 2.5 μg of total RNA prepared from protonemata using an AMV reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, FL, USA). To detect *Sig* genes-specific amplification, we designed primers based on sequences of both sides of an intron of *sig* genes so that genomic amplification could be distinguished. The primers were as follows: Sig2QUp1 (5′-TGGGTCGC-ATGAGGAAGGCAAAGT-3′) and Sig2QDn1 (5′-ACTGATGCT-CTCTAGTGACA-3′) for *Sig1*, Sig1QUp1 (5′-AAACTACCAGGG-CCGAGGCATGAC-3′) and Sig1QDn1 (5′-AACGCCCTCTATAT-CTTGTC-3′) for *Sig2*. As a control, an *actin* sequence from *Physcomitrella* (AW698983) was amplified using two primers PpAct3U1 (5′-CGGAGAGGAAGTACAGTGTGTGGA-3′) and PpAct3D1 (5′-ACCAGCCGTTAGAATTGAGCCCAG-3′). We used 24 and 27 PCR cycles for *actin* and *Sig* genes, respectively, with which the amplification reactions were in the exponential phase. PCR fragments were electrophoresed on 1.0% agarose gels and stained with ethidium bromide.

2.4. Transient expression assay using the green fluorescent protein (GFP)

The DNA fragment encoding the first 138 amino acid residues of PpSIG1 was amplified from *Physcomitrella* cDNA with primers Sig2TpUp1 (5′-GGATCCCAAGATGGCGCATGCATTAGGTGT-3′; attached restriction site is underlined) and Sig2TpDn1 (5′-CCATGGGGAGAGTAGAGGCGTCAGGCAAAA-3′). The PCR product was cloned in pGEM-T easy vector (Promega, WI, USA). From the resulting plasmid, a ~420 bp *Bam*HI–*Nco*I fragment carrying the PpSIG1 N-terminal region was excised and introduced into *Bam*HI–*Nco*I-cleaved p7133TP-sGFP, a GFP reporter plasmid that was used for the study of PpSIG2 (previously named PpSIG1*) [25]. In the resulting plasmid p7133TP2-sGFP, the expression of the fusion of PpSIG1 N-terminal region and GFP is under the control of a strong promoter E7ΩIn [27]. As a control, plasmid p7133sGFP was used, in which GFP with no fusion is under the control of E7ΩIn [25]. These reporter constructs were introduced into the moss protoplasts by polyethylene glycol-mediated transformation [18,26]. One day after transformation, protoplasts were observed by confocal laser scanning microscopy (LSM 510, Zeiss, Germany).

2.5. DNA techniques and sequence analyses

Escherichia coli (JM109 and DH10B) was handled and DNA was manipulated for molecular cloning as described [28]. DNA sequences were determined with a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, NJ, USA) and a model 373A DNA sequencing system (Applied Biosystems, CA, USA). DNA sequences were analyzed by the program DNASIS (version 3.5; Hitachi Software Engineering, Japan). Multiple sequence alignment and phylogenetic analysis were performed with the program ClustalW [29], and phylogenetic trees were constructed with the program TreeViewPPC (version 1.5.3).

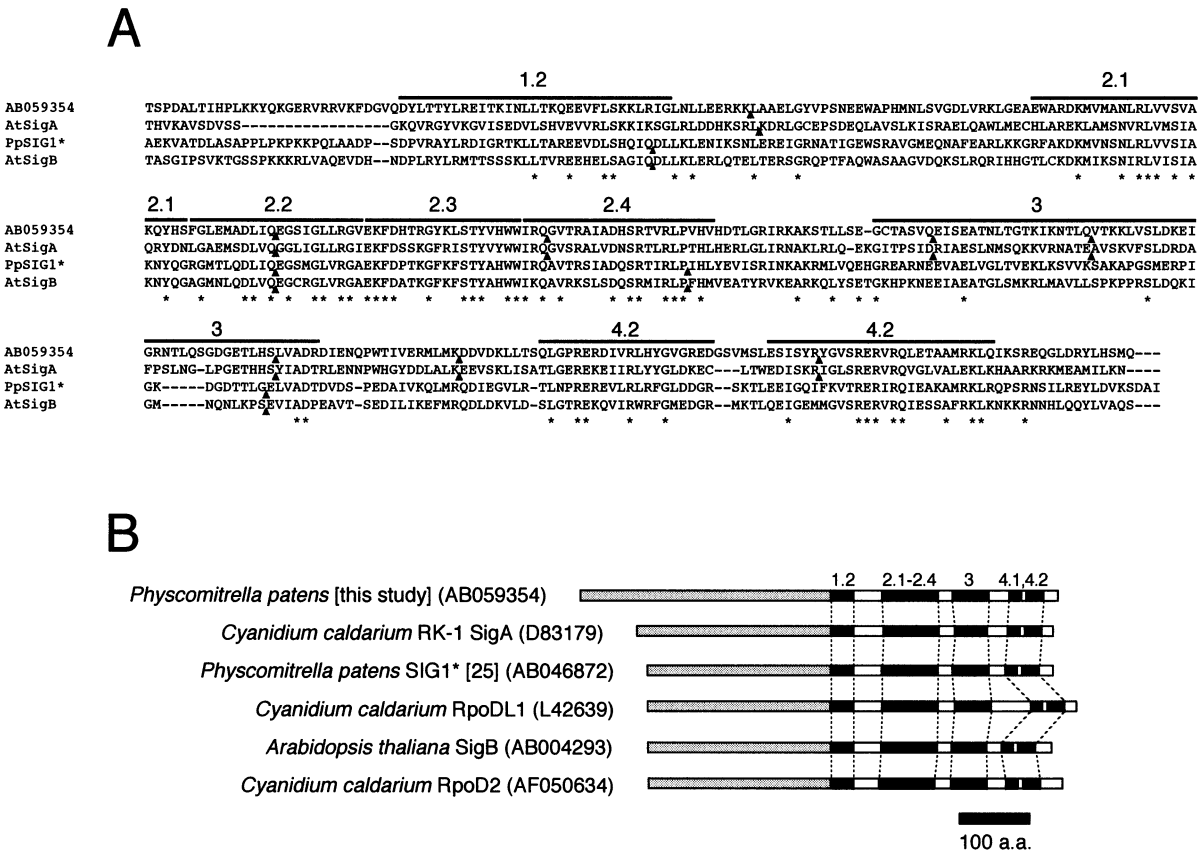


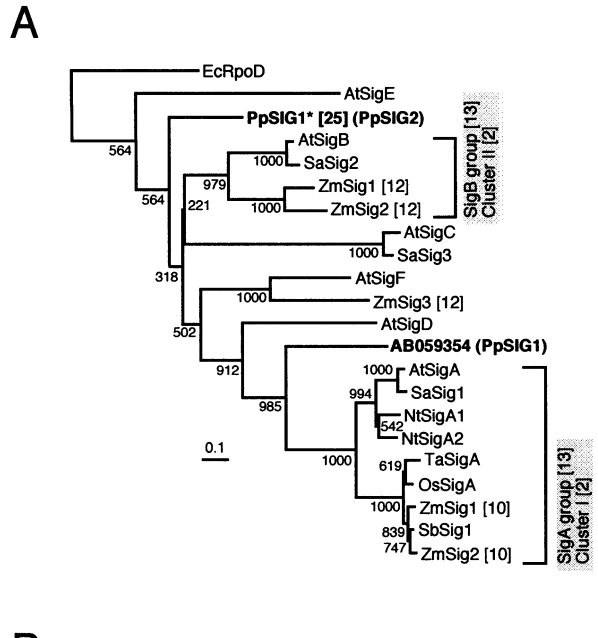
Fig. 1. A: The amino acid sequence alignment of the σ factors from *Physcomitrella* and *Arabidopsis*, and comparison of intron sites. The sequences of conserved C-terminal regions through subdomains 1.2–4.2 of the novel σ factor (shown by its accession no. AB059354) and PpSIG1* from *Physcomitrella* and SigA (AtSigA), SigB (AtSigB) from *Arabidopsis* are aligned. The subdomains are indicated above the sequences. Intron positions are shown by filled triangles. Identical amino acids are indicated by asterisks. B: Structure of N-terminal extensions and conserved domains of putative plastid σ factors. The novel σ factor from the moss and the σ factors with relatively large N-terminal extensions are shown in the order of the sizes of the extensions. N-terminal extensions and conserved domains are shown as gray boxes and closed boxes, respectively. Accession number for each sequence is shown in parentheses.

3. Results and discussion

By a database search, we found an EST sequence from *Physcomitrella* (reported by the *Physcomitrella* EST sequence program (PEP)) similar to the σ factor sequences from various species. The EST sequence was also similar to, but was not identical to, the corresponding sequence of the *sig1** cDNA [25], indicating that it encodes a novel σ factor in *Physcomitrella*. We amplified the EST sequence from *Physcomitrella* cDNA by PCR, and screened a *Physcomitrella* cDNA library with the amplified DNA fragment as a probe. After screening 3.0×10^5 plaques, we isolated six cDNA clones. Since the largest clone still lacked the 5′-portion, it was isolated by the 5′-RACE method. The full-length cDNA sequence (deposited in databases with the accession no. AB059354) was 2484 bp in length and the largest open reading frame (ORF) was predicted to encode a protein of 677 amino acids with an estimated molecular mass of 76.2 kDa. Since conserved amino acid residues were found between the first and the second ATG codons on the ORF (motif I; described below), we suppose that the first one corresponds to the translation start site. On the amino acid sequence alignment with various σ factors, the C-terminal half (amino acids 355–677) obviously exhibited the subdomains supposed to be involved in binding to the core RNAP complex (2.1 and 3), DNA melting (2.3), recognition of −10 and −35 promoters (2.4 and 4.2), respectively (Fig. 1A). Thus, the predicted protein is indicated to be a novel σ factor in *Physcomitrella*. The putative σ factor possesses an unusually large N-terminal extension of 354 amino acids in length upstream from the subdomain 1.2, which is the largest among all the reported plant σ factor sequences (Fig. 1B). This region showed very low similarity to the corresponding N-terminal regions of other plant σ factors as reported to be a general feature of plant σ factors.

Arabidopsis thaliana possesses six genes (*sigA* to *sigF*) for σ factors on the genome, and their intron structures have been studied [13,16]. To study the evolutional relationship among the moss and higher plant σ factors, we cloned the genes encoding PpSIG1* [25] and the novel σ factor isolated in the present study, respectively, and compared the intron sites between the moss and *Arabidopsis* genes (Fig. 1A). Positions of four introns of the *sig1** gene encoding PpSIG1* on the alignment of the conserved regions through 1.2–4.2 were identical to those of *Arabidopsis sigB*, *sigC* and *sigF*, indicating the close relatedness of these four genes (Fig. 1A; data using *sigC* and *sigF* sequences are not shown). The positions of seven out of eight introns of the gene encoding the novel σ factor (AB059354) were identical to those of *Arabidopsis sigA* on the alignment (Fig. 1A). The other intron of this gene was also very close in position to a corresponding intron of *sigA* with only one amino acid gap. These results indicate the close relatedness of the *Physcomitrella* novel σ factor gene and the *Arabidopsis sigA* gene.

To further investigate the phylogenetic relationships, we constructed a phylogenetic tree with 21 land plant σ factors and *E. coli* RpoD as the outgroup (Fig. 2A). In the tree, PpSIG1* [25] and the novel σ factor of this study are distantly located among the higher plant σ factors. PpSIG1* is clustered with all the land plant σ factors except for *Arabidopsis* SigE. However, relationship among PpSIG1* and other σ factors is not conclusively judged due to low bootstrap values. We suppose that PpSIG1* is classified into the SigB group



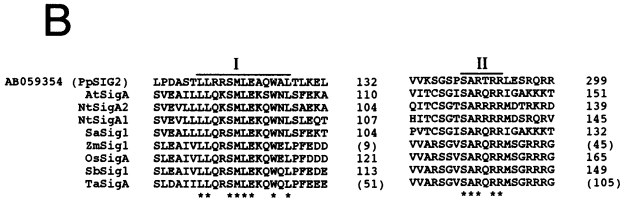


Fig. 2. A: Phylogenetic tree constructed using the σ factor sequences from various species. The tree was constructed by the neighbor-joining method [34] using ∼260 aligned residues corresponding to the conserved regions 1.2 and 2.1–4.2. Numbers at each node represent bootstrap values out of 1000 bootstrap resamplings. The bar indicates the distance corresponding to 10 changes per 100 amino acid positions. The PpSIG1* and the novel σ factor identified in this study (AB059354) from *Physcomitrella* are indicated by bold letters and their names proposed in this study are shown in parentheses. The other σ factors are as follows: EcRpoD, *E. coli* RpoD (J01687); AtSigA, AtSigB, AtSigC, AtSigD, AtSigE and AtSigF, *A. thaliana* SigA (D89993), SigB (AB004293), SigC (D89994), SigD (AB021119), SigE (AB021120) and SigF (AB029916); SbSig1, *Sorghum bicolor* Sig1 (Y14276); SaSig1, SaSig2 and SaSig3, *Sinapis alba* Sig1 (Y15899), Sig2 (AJ276656) and Sig3 (AJ276657); OsSigA, *Oryza sativa* SigA (AB005290); NtSigA1 and NtSigA2, *Nicotiana tabacum* SigA1 (AB023571) and SigA2 (AB023572); TaSigA, *Triticum aestivum* SigA (AJ132658); ZmSig1 and ZmSig2 [10], *Zea mays* Sig1 (AF058708) and Sig2 (AF058709); ZmSig1, ZmSig2 and ZmSig3[12], *Z. mays* Sig1 (AF099110), Sig2 (AF099111) and Sig3 (AF099112). B: Conserved motifs (I and II) in the N-terminal extensions of the σ factors in the SigA group. Amino acid stretches in the N-terminal regions conserved among the SigA group sequences are aligned and presented as in Fig. 1. The number at the end of each line shows the first amino acid of each line. The N-terminal regions of ZmSig1 and TaSigA have not yet been completely isolated [10].

comprising orthologs of the *Arabidopsis sigB* gene product [13] (Fig. 2A), because, (1) the amino acid sequence of PpSIG1* showed higher homology to the SigB group sequences than to other plant σ factor sequences [25], and (2) the intron sites of the gene encoding PpSIG1* and *sigB* were identical on the amino acid sequence alignment (Fig. 1A). In support of this idea, PpSIG1* is clustered with the SigB group σ factors in another phylogenetic tree constructed on the basis of the most highly conserved subdomains 2.1–2.4 (data not shown). On the other hand, the novel σ factor

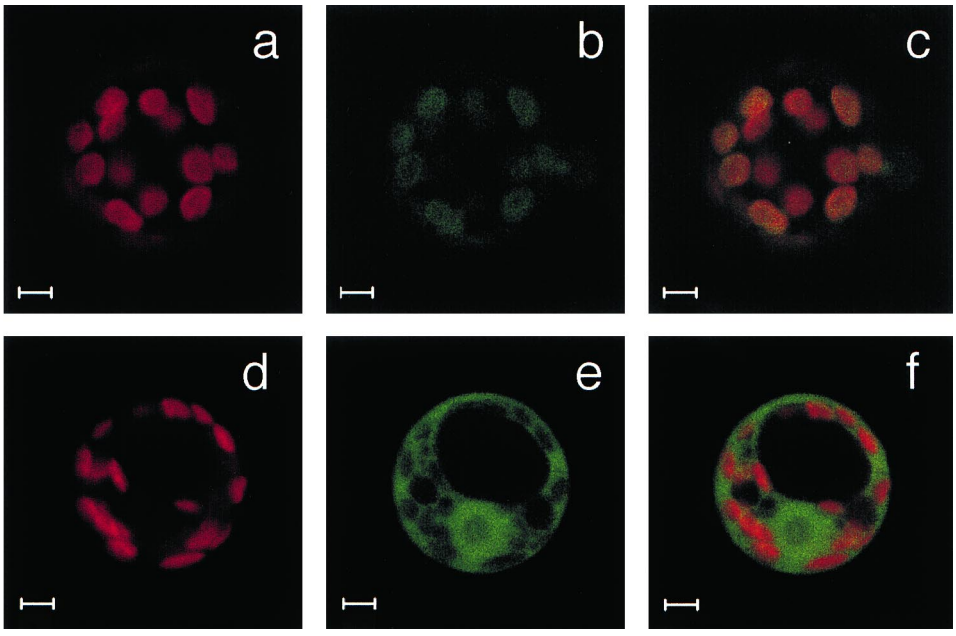


Fig. 3. Intracellular localization of the PpSIG1 transit peptide–GFP fusion. Shown are cells transformed with GFP fusion with the PpSIG1 N-terminal region (a, b and c) or with GFP with no fusion (d, e and f). Cells were illuminated for excitation at 488 and 543 nm to observe fluorescence of GFP and chlorophyll, respectively. Images were taken at 505～530 nm and at over 560 nm to observe GFP (b, e) and chlorophyll (a, d), respectively. Merged images are shown in (c, f). Bars, 5 μm.

(AB059354) is clustered with orthologs of the *Arabidopsis sigA* gene product (SigA group [13]) (Fig. 2A), consistent with the result of the comparison of intron sites. Moreover, a conserved motif, present in the N-terminal regions of higher plant SigA group σ factors [14], was also found in the N-terminal region of the novel σ factor (Fig. 2B, motif I). This motif was not found in any of the higher plant sequences other than SigA orthologs, supporting the idea that the novel σ factor from the moss is a member of the SigA group. We identified another motif immediately upstream of subdomain 1.2, which is conserved among the novel σ factor and other σ factors from the SigA group (Fig. 2B, motif II). This motif was also present in the σ factor sequences from the SigB group with less similarity (data not shown). Thus, PpSIG1* and the novel σ factor of *Physcomitrella* were most likely classified into the SigB and SigA groups, respectively.

Recently, an increasing number of cDNA sequences encoding putative σ factors have been reported in various plant species [2]. They were named independently by each research group usually according to the order of isolation in each species. As a result, a very complicated σ factor nomenclature has been generated [2]. To avoid confusion, a consistent nomenclature for plant σ factors was proposed recently [30], which reflected phylogenetic relationship among individual σ factors from different plant species. According to this nomenclature and the results of phylogenetic analyses (Figs. 1A and 2), here we renamed the *sig1** gene and its product PpSIG1* [25] into *Sig2* and PpSIG2, respectively, and named the novel σ factor (AB059354) and the corresponding gene (AB059355) isolated in this study as PpSIG1 and *Sig1*, respectively.

If PpSIG1 is a plastid σ factor, it must be localized to the plastid compartment to regulate plastid gene expression. The N-terminal region of PpSIG1 protein does not seem to share typical characteristics with plastid targeting signals (transit peptides), e.g. an abundance of hydroxylated amino acid residues [31]. Thus, we examined whether or not the N-terminal region acts as a transit peptide by fusing an N-terminal region of PpSIG1 (1–138) to the GFP reporter (sGFP(S65T), a synthetic GFP [32]), and it was transiently expressed in the moss protoplasts. The fluorescence of the GFP fusion was faint but clearly overlapped with chlorophyll fluorescence (Fig. 3a–c). In the control experiment, GFP with no fusion was expressed and detected in the cytoplasm and nucleus (Fig. 3d–f; [25]). These observations indicated that the N-terminal region of PpSIG1 can function as a transit peptide, and that PpSIG1 is a plastid σ factor.

Expression of many plastid σ factor genes is regulated by light [4–9,11,14]. We examined by RT-PCR whether the expression of the *Sig1* and *Sig2* genes encoding PpSIG1 and PpSIG2, respectively, is also light-regulated (Fig. 4). After incubation in the dark for 24 h, 2 h of light (40 μmol/m^2/s from white fluorescent lamps) or dark period was adminis-

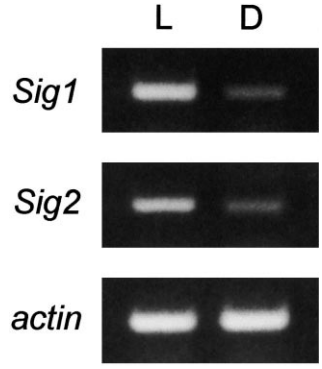


Fig. 4. Light induction of the *Sig1* and *Sig2* gene expression. RT-PCR was performed to detect the expression of *Sig1* (upper), *Sig2* (middle) and *actin* (lower) genes. Total RNA prepared from cells grown in the light (L) or dark (D) was used.

tered to the *Physcomitrella* protonemata cells. RNA was extracted from the illuminated or dark-grown cells, and used for RT-PCR analyses using the *Sig1* or *Sig2* cDNA-specific primers. Amplifications from both *Sig1* and *Sig2* transcripts obviously increased in the illuminated cells (Fig. 4), indicating that the *Sig1* and *Sig2* genes are positively light-regulated. This result, together with the observations that PpSIG1 (this study) and PpSIG2 [25] were targeted into chloroplasts, strongly suggest that the moss σ factors are involved in light-regulated expression of plastid genes.

Phylogenetic analyses demonstrated that higher plant plastid σ factors are classified into structurally conserved classes beyond species [2,13]. Fujiwara et al. classified the σ factors into at least four classes, SigA, SigB, SigC and SigF groups, consisting of orthologs of *Arabidopsis sigA*, *sigB*, *sigC* and *sigF* gene products, respectively [13]. Allison classified the σ factors into at least three classes, clusters I, II and III, corresponding to the SigA, SigB and SigF groups, respectively [2]. The present study showed that the two distinct σ factors PpSIG1 and PpSIG2 from *Physcomitrella* could be classified into the SigA (cluster I) and SigB (cluster II) groups, respectively (Figs. 1 and 2). Therefore, the SigA and SigB groups, the two major classes of σ factors in higher plants [2], were already present in the common ancestor of moss and higher plants. Previously, the analysis of the genomic DNA of *Physcomitrella* by Southern blotting experiments using a *Sig2*-specific probe revealed five to seven bands [25]. The number of *Sig* genes in *Physcomitrella* predicted by this result is in good agreement with the number of *sig* genes in *Arabidopsis*. In addition to *Sig1* and *Sig2* genes, *Physcomitrella* may possess other σ factor genes whose products are classified into the SigC or SigF (cluster III) groups, or those that have not yet been classified into any groups such as SigD or SigE in *Arabidopsis* [13]. It is an interesting question whether or not the classification based on sequence similarity of the plastid σ factors reflects functional differences. Recently, Shirano et al. reported that chloroplast development was impaired in an *Arabidopsis* T-DNA insertion mutant that lacked *sigB* gene function [33]. Efficient transformation and homologous recombination of *Physcomitrella* should be useful for the analyses of in vivo functions for the *Sig1* and *Sig2* genes.

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