In the model seed plant Arabidopsis thaliana, a subfamily of B-box containing transcriptional factors (BBXs), which is classified in the BBX-IV group based on the domain structure, contains two tandem B-box domains and plays crucial roles in early photomorphogenesis under the control of blue light receptors, cry1 and cry2. The results of an examination of light responsiveness of representative Physcomitrella BBX-IV genes and their heterologous expression in Arabidopsis suggested that the light signaling-related characteristics of the BBX-IV subfamily are evolutionarily conserved in a moss, which is a basal lineage of land plants.

Key words: Arabidopsis thaliana; light signaling; Physcomitrella patens; transcriptional factor

Plants possess several classes of photoreceptors that can monitor light from UV-B to near infrared. Genetic and biological studies of the model seed plant Arabidopsis thaliana have shown that these light sensors mediate numerous adaptive responses. From an evolutionary viewpoint, it is of interest to characterize such light-signaling systems in Physcomitrella patens, which is a basal lineage of land plants. Here we attempted to gain insight into blue light signaling-related transcription factors of Physcomitrella patens, taking advantage of the current comprehensive information on A. thaliana (Fig. 1A).

The moss has two cryptochromes (PpCRY1a and PpCRY1b) the amino acid sequences of which are highly similar to those of A. thaliana. The results of genetic studies indicated that Physcomitrella cry1a and cry1b coordinateably regulate many steps in growth and development of mosses, including induction of side branching on protonema and gametophore development. It was also reported that PpCRY1a and PpCRY1b act negatively on the transcript levels of several SBP-box genes, some of which appear to act as negative regulators of side branching. Based on these, we investigated to know whether the cryptochrome-mediated signaling network is conserved between A. thaliana and Physcomitrella patens.

In A. thaliana, several signal-integrators function downstream of the blue photoreceptors. The best studied is COP1 (see Fig. 1A), loss-of-function mutations of which result in the phenotype of constitutive photomorphogenesis even in the dark. COP1 acts as an ubiquitin E3 ligase, and it plays a crucial role in protein degradation through interacting with a number of downstream target proteins. A. thaliana has a single COP1 gene, whereas P. patens has nine COP1 homologues. In A. thaliana, the best-characterized target of COP1 is HY5, a primary light-signaling transcription factor. HY5 belongs to a member of the family of basic leucine zipper transcriptional factors (bZIP). We recently reported that P. patens has two HY5 homologues, and that they play roles in caulonema development. In this study, we focused on another putative COP1-target, a B-box-containing transcriptional factor (BBX).

In A. thaliana, BBXs form a family consisting of 32 members. The founding example is CONSTANS (CO or BBX1) (Fig. 1B), which plays a pivotal role in the photoperiodic control of flowering time. Among 32 Arabidopsis BBX (Ar/BBX) family proteins, eight members contain two tandem B-box domains at the N-terminal end, but unlike CO, they lack the C-terminal CCT motif (Fig. 1B). The members of this sub-family has been classified into the BBX-IV group. The function of the BBX-IV sub-family appears to be involved in blue light signal responses. ArBBX24/STO acts as a negative regulator of photomorphogenesis, whereas ArBBX21/STH2 and ArBBX22/STH3/LZF1 function as positive regulators, acting in concert with HY5. ArBBX18/DBB1 is involved in gibberellin homeostasis and the regulation of hypocotyl elongation in response to blue light. Interestingly, ArBBX21/STH2 and ArBBX22/STH3/LZF1 appear to be targets of COP1. Based on knowledge from the seed plant A. thaliana, here we asked a fundamental question as to whether BBX-IV homologues are conserved in P. patens.

An extensive analysis of the P. patens genome databases was performed, and it was found that there were seven genes, each encoding a member of the Physcomitrella BBX-IV (PpBBX-IV) sub-family (Fig. 1C). The results of phylogenetic analyses showed that they were highly homologous to those of A. thaliana. According to the phylogenetic tree, the BBX-IV sub-families of Arabidopsis and Physcomitrella are classified into two subgroups, subgroup A and subgroup B. Five Physcomitrella BBX-IV proteins belonging to subgroup A, PpBBX-1Va-e, form a cluster, sister to ArBBX24 and
At BBX25. Two Physcomitrella BBX-IV proteins belonging to subgroup B, PpBBX-IVf and PpBBX-IVg, form a cluster, sister to AtBBX18 and AtBBX19. A search by BLASTP on the Physcomitrella protein database (Phytozome ver. 7.0) identified PpBBX-IVa as one of the proteins most significantly similar to AtBBX24 (E-value = 2.8e-36), and PpBBX-IVg to AtBBX18 (E-value = 3.7e-40) (Fig. 1D). Considering these, we selected PpBBX-IVa and PpBBX-IVg as representative proteins for subgroup A and subgroup B respectively to characterize their properties.

First we cloned the corresponding cDNAs to confirm the inferred amino acid sequences. Then their light responsive transcriptional profiles were examined in P. patens. In response to both blue and red light, transcription of PpBBX-IVa was gradually induced (Fig. 2A), but we do not know whether this induction is to be attributed to the primary response to the light signal. Transcription of PpBBX-IVg was more clearly and rapidly induced in response to both blue and red light (Fig. 2B). AtBBX25/DBB1a is primarily responsive to blue light and AtBBX22/STH3/LZF1 to far-red light. It is suggested, apart from regulatory mechanisms, the property of light induction in PpBBX-IVg expression is conserved in P. patens. Here, another critical question is whether expression of them is subject to the diurnal oscillation in light and dark cycles, because it is known that most AtBBX-IV members, including AtBBX18 and AtBBX24, are under the control of the circadian clock. Hence their diurnal expression profiles were examined for P. patens plants grown under 12 h light and 12 h dark cycles (Fig. 2C and D). It was found that the expression of both the PpBBX-IVa and PpBBX-IVg gene oscillated robustly in response to the diurnal light and dark cycles with peaks at noon and dawn respectively. The peak phases of PpBBX-IVa and PpBBX-IVg are

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**Fig. 1.** Schematic Representation of a Pair of Blue Light-Signaling Factors Conserved between A. thaliana and P. patens.

A. Inferred blue light signaling pathway in P. patens. P. patens has two blue light photoreceptors, cry1a/1b. It appears that the downstream light signal integrator, COP1, is conserved in P patens. The transcription factors involved in this study, HYS-homologues and BBX-IV-homologues, are highlighted in the black rectangle. P. patens has two HYS-homologues, HY5a/b, and seven BBX-IV-homologues, as indicated in parentheses. Information on these genes can be retrieved from PHYTOZOME (http://www.phytozome.net/physcomitrella). For A. thaliana BBX-IV, the AGI codes are shown in parentheses. For P. patens BBX-IV, the transcript names in the Phytozome ver. 7.0 database are shown in parentheses.

B. Schematic representation of the domain structure of the BBX-IV subgroup in comparison with a representative BBX-I subgroup, Arabidopsis CONSTANCE (AtCO). B1, B2, and CCT denote B-box 1, B-box 2, and CCT domain respectively.

C. Phylogenetic relationships among eight AtBBX-IVs (AtBBX18-25) and seven PpBBX-IVs (PpBBX-IVa-g). A rooted N-J tree with branch length was constructed with the Clustal W program, based on the amino acid sequences of B1 and B2 domains. Following the phylogenetic tree, we focused on PpBBX-IVa and PpBBX-IVg (indicated by asterisks) in this study. D. Amino acid sequence alignment between PpBBX-IVg and AtBBX18. Two B-box domains are indicated by B1 and B2. Identical and similar amino acids are denoted by asterisks and colons respectively.
**BBX Transcriptional Factors in *P. patens***

Fig. 2. Characterization of Light-Responsive Expression of Representative *Pp*BBX-IV Genes.

A, Examination of the blue and red light responses of the *PpBBX-IVa* gene. Protonema cells grown on a solid BCDAT medium for 5 d under light with an intensity of 45 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) were transferred to darkness for another 3 d, and then the plants were exposed to 15 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) blue (\( \lambda_{\text{max}} = 470 \text{ nm} \)) and 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) red (\( \lambda_{\text{max}} = 660 \text{ nm} \)) light by using the light-emitting diodes (STICK LED, EYELA, Tokyo). B, Examination of the blue and red light responses of the *PpBBX-IVg* gene. The same experiment as in A was performed. C and D, Examination of diurnal expression. Protonema cells were grown on a solid BCDAT medium for 5 d under a 12 h white light with an intensity of 45 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 12 h dark cycle conditions, and mRNA samples were prepared at 3 h intervals. E, Examination of the free-running rhythm of the *PpBBX-IVa* and *PpBBX-IVg* genes under constant dark conditions. After the same diurnal condition as in C and D, the plants were released to darkness and mRNA samples were prepared in intervals. In all of these experiments, the expression levels of the *Pp*BBX-IVa and *Pp*BBX-IVg genes relative to the *PpACT3* transcripts (the highest expression level was set to 1.0) were analyzed by qRT-PCR.

*PpBBX-IVg* expression coincides with those of the homologues, *AtBBX24* and *AtBBX18*, respectively.**

Next we found that expression of the *PpBBX-IVg* gene exhibited a robust free-running rhythm in continuous darkness, suggesting that it is under the control of the circadian clock, as has been reported for *AtBBX18* (Fig. 2E). In sum, *PpBBXg* and *AtBBX18* are closely homologous in the sense that the expression of them is induced by blue light and also has circadian rhythm.

It is of importance to determine whether *PpBBX-IVa* and *PpBBX-IVg* play light-associated roles. To analyze the physiological functions of these *Pp*BBX-IV members, the best way is to isolate loss-of-function mutants in *P. patens*, but it is necessary to construct *PpBBX-IV* multiple mutants in order to observe overt phenotypes, because these *PpBBX-IVs* most likely play redundant roles. Hence we decided to take an alternative approach. It is well known that when some *At*BBX-IV members are overexpressed in *A. thaliana*, the resulting transgenic plants exhibit aberrant phenotypes with regard to the early photomorphogenesis of young seedlings. For instance, transgenic seedlings overexpressing *AtBBX18* exhibit extremely long hypocotyls when they were germinated under a light and dark cycle, as compared with reference wild-type seedlings. Based on this, to gain a preliminary insight into the possible functions of the moss BBX-IV members, several independent *Arabidopsis* transgenic lines overexpressing *PpBBX-IVa* and *PpBBX-IVg* under the CaMV35S promoters (*PpBBX-IVa*-ox and *PpBBX-IVg*-ox) were constructed by using binary vector pSK1 with the hygromycin resistance gene as selection marker.
Characterization of Arabidopsis Transgenic Seedlings Overexpressing PpBBX-IVa and PpBBX-IVg.

A. Phenotype of PpBBX-IVa-ox. Three independent T2 Arabidopsis transgenic lines overexpressing PpBBX-IVa (denoted by PpBBX-IVa-ox L4, L5, and L7) and the Hyg reference strain (denoted by Col) were established. They were germinated and grown on MS agar-medium containing 20 µg/mL Hygromycin B under 16 h light and 8 h dark cycles for 7 d. Expression of the transgene was examined by RT-PCR experiments with primers 5'-CACCACCTTAGGTGAGAAATGCAGGTAATC-3' and 5'-ACCCTGATGATGAGAATGCAGGTAATC-3'. Representative seedlings were photographed. The resulting lengths of the hypocotyls were analyzed quantitatively (n > 20). B. Phenotype of PpBBX-IVg-ox Three independent T2 Arabidopsis transgenic lines overexpressing PpBBX-IVg (denoted by PpBBX-IVg-ox L2, L3, and L5) and the Hyg reference strain (denoted by Col) were established. Expression of the transgene was examined by RT-PCR experiments with primers, 5'-CTCATACTTGTATGAGAACCCTTTGTGATGTG-3' and 5'-GCCCTGATGAGCAGCGCCGCTCAACGACAGGCTTTGG-3'. They were germinated and grown on MS agar-medium containing 20 µg/mL Hygromycin B under 16 h light and 8 h dark cycles for 7 d. Representative seedlings were photographed. The resulting lengths of the hypocotyls were analyzed quantitatively (n > 20). The phenotype was further confirmed with the homozygous T3 lines established (n > 20).

Phenotypes of the Hyg T2 seedlings were examined with reference to elongation of the hypocotyls during early photomorphogenesis. Though the phenotypic alteration of PpBBX-IVa-ox was very subtle, the PpBBX-IVg-ox seedlings clearly showed a phenotype of long hypocotyls as compared with that of the Hyg reference strains. The wild-type Col seedlings transformed with the same binary vector (Fig. 3A and B). The results were further confirmed by employing a set of homozygous T3 PpBBX-IVg-ox lines grown on a Hygromycin-free medium (Fig. 3A and B, bottom panels). This phenotype of PpBBX-IVg-ox is similar to that of AtBBX18-ox, suggesting that the function of PpBBX-IVg is conserved and that it plays a light signaling-related role in Arabidopsis.13,16

Based on the results of this and previous studies, the early blue light-signaling pathway, consisting of photoreceptors (cry1a/1b), integrators (e.g., COP1), and transcription factors (e.g., HY5-homologues and BBX-IV-homologues) appears to be conserved evolutionarily between the seed plant A. thaliana and the moss P. patens.21 Although we do not know whether PpBBX-IV homologues do function downstream of the cryptochromes and PpCOP1, it would be interesting to unravel whether PpBBX-IVa and PpBBX-IVg are regulated by the PpHY5 and PpCOP1 families, because AtBBX21/STH2 and AtBBX22/STH3/LZF1 interact with HY5 in vivo and show COP1-dependent localization to nuclear speckles by B-box domain.14,15 At this stage, however, we know that (i) transcription of the PpBBX-IVg gene is regulated by light; (ii) the PpBBX-IVg gene is under the control of the circadian clock; and (iii) when the PpBBX-IVg gene is introduced into the heterologous seed plant A. thaliana, it affects the photomorphogenic programs of the host. These facts, observed with respect to the PpBBX-IVg, are in good agreement with that of its homologous gene, AtBBX18.

The morphological and developmental programs of P. patens are quite different from those of A. thaliana. In contrast with seed plants, the period of haploid generation is much longer than that of diploid generation. P. patens germinates from a haploid spore, producing a linear array of cells that branch and generate a filamentous, two-dimensional network known as protonema. These predominant life cycles are regulated through the actions of phytohormones (e.g., auxin and cytokinin) and light signals.19 It is of
importance to perform genetic studies on PpBBX-IVs to characterize their physiological functions with regard to every developmental step. To this end, the results of this study will provide a common platform.

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