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Cloning and Expression Characteristics of *Pharbitis nil COP1* (*PnCOP1*) During the Floral Induction

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The ubiquitin E3 ligase COP1 (Constitutive Photomorphogenesis 1) is a protein repressor of photomorphogenesis in *Arabidopsis* plants, and it found in various organisms, including animals. The COP1 protein regulates the stability of many of the light-signaling components that are involved in photomorphogenesis and in the developmental processes. To study the effect of COP1 on flowering in a short day plant, we have cloned a full-length of *PnCOP1* (*Pharbitis nil COP1*) cDNA from *Pharbitis nil* Choisy cv. Violet, and we examined its transcript levels under various conditions. A full-length *PnCOP1* cDNA consists of 2,280 bp nucleotides that contain 47 bp of 5'-UTR, 232 bp of 3'-UTR including the poly (A) tail, and 1,998 bp of the coding sequence. The deduced amino acid sequence contains 666 amino acids, giving it a theoretical molecular weight of 75 kD and a isolectric point of 6.2. The PnCOP1 contains three distinct domains, an N-terminal Zn^{2+}-binding RING-finger domain, a coiled-coil structure, and WD40 repeats at the C-terminal, implying that the protein plays a role in protein-protein interactions. The *PnCOP1* transcript was detected in the cotyledon, hypocotyls and leaves, but not in root. The levels of the *PnCOP1* transcript were reduced in leaves that were a farther distance away from the cotyledons. The expression level of the *PnCOP1* gene was inhibited by light, while the expression was increased in the dark. During the floral inductive 16 hour-dark period for *Pharbitis nil*, the expression was increased and it reached its maximum at the 12th hour of the dark period. The levels of *PnCOP1* mRNA were dramatically reduced upon light illumination. These results suggest that PnCOP1 may play an important function in the floral induction of *Pharbitis nil*.

Key words: *Pharbitis nil*, COP1, Flowering, Light-inhibition

INTRODUCTION

Light affects almost all aspects of plant growth and development, with the responses of plants to light playing an especially prominent role in seedling development [1]. In plants, the regulation of protein degradation by the ubiquitin/26S proteasome significantly contributes to plant development by affecting a wide range of cellular processes. Light-regulated protein stability has also been considered to be a major control point of photomorphogenesis. The ubiquitin E3 ligase COP1 regulates the stability of several light-signaling components, and thereby controlling photomorphogenesis [2].

COP1 is one of the single subunit RING/U-box E3s in the family of E3 ubiquitin ligases, and the about 70 amino acid RING (Really Interesting New Gene) finger binds to the E2 in the ubiquitin-dependent pathway [2-5]. The plant *COP1* gene was first isolated from *Arabidopsis* by T-DNA tagging in 1992 [6]. The *Arabidopsis COP1* encodes a protein that contains three recognizable domains: a RING-finger, a coiled-coil domain and multiple WD-40 repeats that are characteristic of the â-subunit of the trimeric G-proteins, which implicates the presence of complex protein-protein interactions. From the study on transgenic plants overexpressing the *COP1* gene, it was demonstrated that COP1 protein acts as a cell-autonomous repressor of photomorphogenic development whose repressive activity is overcome by light [7]. COP1 protein has been localized in the nucleus during darkness and in the cytoplasm upon illumination of light [8-10].

In plants, it is well known that the COP1 protein interacted with a variety of transcription factors and signaling molecules through its three functional domains; a gene encoding a flower-specific transcription factor AtMYB21 [11], the bZIP transcription factor HY5 [12-15], a novel bZIP protein HYH [16], a basic helix-loop-helix protein HFR1 [17], cryptochromes [18], a myb transcription factor LAF1 [19], a repressor of phytochrome A-mediated responses to far red light SPAs [15, 20-22] and phyA [15, 23]. It has been recently demonstrated that the RING-finger protein COP1 can act as ubiquitin-protein ligases (E3) to target proteins for degradation by recruiting the ubiquitin-conjugating enzyme (E2) and transferring the polyubiquitin from E3 to the target proteins; transcription factors HYH [24], HY5 [15], LAF1 [19], phyA [23] and a putative bHLH class

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transcription factor HFR1 that is involved in light signaling [17, 25]. The COP1-mediated ubiquitination of proteins and the subsequent proteasome-mediated degradation of these proteins have also been observed in mammalian systems, and examples of this are the leucine zipper Jun transcription factors [26] and tumor-suppressor protein p53 [27].

Flowering is the transition from the vegetative phase to the reproductive phase, and this process is regulated by various endogenous factors as well as by environmental stimuli. This process might include changes in the expression of many genes. Flowering in *Pharbitis nil* is mainly controlled by the photoperiod, and its cotyledon stages are the most sensitive to induction. *P. nil* can be induced to flower prematurely by expose to a single dark period of at least 14 h just after the cotyledons have fully expanded [28]. These physiological characteristics make it a model plant for studying the short-day photoperiodic induction of flowering [29].

The pleiotropic phenotype of *Arabidopsis cop1* mutants and the multiple interactions of COP1 protein indicate that COP1 protein plays a key role in the transcriptional pathways by regulating not only photomorphogenesis in seedlings, but also by regulating development at later stages such as the floral transition. In addition, the presence of COP1 protein in the nucleus during darkness, but its gradual relocation to the cytoplasm upon illumination may imply that the photomorphogenic repressor can function as a positive regulator during the floral inductive dark period in *Pharbitis*, a short-day plant. To provide fundamental information for the assumption, we have cloned the *COP1* homolog (*PnCOP1*) from *P. nil* and examined its expression in relation to floral induction.

MATERIALS AND METHODS

Plant materials and culture condition

Seeds of *Pharbitis nil* Choisy cv. Violet were stirred with concentrated sulfuric acid for 30 min, rinsed and imbibed overnight in running water. The seeds were sown in prewashed vermiculite in 12 cm diameter pots, and they were grown in a culture room with Hoagland nutrient solution at 26±1°C under continuous illumination for 6-7 days. The light from a mixture of cool white fluorescent lamps and incandescent bulbs was adjusted to an intensity of 15 $w \cdot m^{-2}$. Six or seven-day old cotyledons were exposed to a 16h-dark period for one, three or five days to induce flowering. Control cotyledons were kept under continuous light for the same period. *Arabidopsis thaliana* (L.) Heynh ecotype 'Columbia' seeds were sown in prewashed vermiculite in 9 cm diameter pots, and then they were grown in a non-inductive light cycle (8h L/16h D).

Messenger RNA extraction and construction of a cDNA library

Cotyledons from either non-induced or induced seedlings were ground in liquid nitrogen with a mortar and pestle. The total RNAs were extracted from 5 g of the powder by using the single-step method by Chomczynski and Sacchi [30]. Poly(A^+)-mRNA was isolated from the total RNAs using a PolyAtract mRNA isolation II kit (Promega). A *Pharbitis* cDNA library was constructed by using the Zap-cDNA synthesis kit and Gigapack II gold cloning kit (Stratagene) according to the manufacturer's instruction manual.

PCR-cloning of a partial PnCOP cDNA

To prepare a probe DNA for screening the cDNA library, we designed degenerated primers that were based on the conserved region between the *Arabidopsis thalianaa* (GenBank accension No, L24437) and *Pisum sativum COP1* (GenBank accension No, Y09579) genes: the forward primer was 5'p-GGATCCGATTTGTATGCAGAT-3'OH and the reverse primer was 5'p-GGATCCGCTTTTGGAGGTAAC ATTCTTG-3'OH. RT-PCR was carried out as follows: denaturation at 94 for 3 min, 30 cycles of 94 (1 min), 30 cycles at 52 (2 min), 30 cycles at 72 for 2 min, and an extension step at 72 for 10 min. The PCR product was ligated into the pGEM-T-Easy vector (Promega) and transformed into *E. coli* DH5α competent cells.

Screening of a cDNA library

The library was screened with a random-primed, [α-^{32}P]-dCTP-labeled *PnCOP1* cDNA fragment (780 bp), as a probe by using the standard plaque lift methods [31]. After prehybridization for 4-6 h at 42°C in 50% formamide, 5X SSPE, 10X Denhardt's solution, 1% SDS and 100 μg/mL denatured salmon sperm DNA, the filters were incubated with the ^{32}P-labeled PCR products for 24h under the same conditions. The filters were then washed three times for 10 min each time with low-stringency wash buffer (2X SSPE, 0.1% SDS) at room temperature, and then they were washed twice for ten minutes each time with high-stringency wash buffer (0.2X SSPE, 0.1% SDS) at 65°C. The membrane was exposed to X-ray film. *In vivo* excision using the ExAssist helper phage with SOLR strains was carried out as described in the manufacturer's protocol.

Nucleotide sequencing

Nucleotide sequencing using the dideoxy chain termination method [32] was performed by using a T7 Sequenase version 2.0 sequencing kit (Amersham) according to the protocols provided by the manufacturer. The DNA sequences were analyzed with the Blast DNA search program through the EMBL database and the InterPro from Expasy. Amino acid sequence alignment was carried out using the CLUSTALW multiple alignment program in NPS@ [Network Protein Sequence @nalysis].

Reverse transcription - PCR analysis

The total RNA was isolated from the 3-5 g samples using the single-step method of Chomczynski and Sacchi [30]. RT-PCR experiments were performed by using the Access RT-PCR system (Promega), oligo-dT_{12-18}, and two primers (sense: 5'-GCGACTTGCCCTTGCTGCTCC-3' and antisense: 5'-

GCAGAACGGGAGGAGGGGAA-3'), that amplified the 780 bp fragment. RNA for the 5.8S rRNA gene was used as a control so that an equal amount RNA was applied in the RT-PCR experiment. To design the primers, several known sequences for the 5.8S rRNA genes from GenBank were compared: *Ipomoea nil* (AF110948), *Oryza sativa* (M35384). *Sinapis alba* (AF128106), *Brassica nigra* (AF128094), *Brassica juncea* (AF128094) and *Arabidopsis thaliana* (AJ232900). The sense and antisense primer sequences were 5'-acgactctcg gcaacggata-3' and 5'-gggcgcaacttgcgttcaaa-3', respectively. The PCR conditions were 2 min at 94, then 30 cycles of 30 sec at 94, 1 min at 50 and 2 min at 68, and 1 cycle of 10 min at 68. The PCR products were analyzed on 1.5% agarose gel.

In vitro translation

To determine the *COP1* gene product, *in vitro* translation was carried out with the TNT-coupled reticulocyte lysate system (Promega) by following the manufacturer's protocol. One microgram of plasmid DNA containing the *PnCOP1* cDNA was subjected to the *in vitro* system in the presence of ^{35}S-methionine (Amersham). The product was fractioned on 12% SDS-polyacrylamide gel.

Genomic DNA blot analysis

Genomic DNA was isolated from *Pharbitis* leaves by the method of Dellaporta [33]. The genomic DNA was digested with *Eco*RI, *Hin*dIII, *Xho*I and *Bam*HI, and then separated on 1% agarose gel. The products were then blotted onto a Hybond-N (Amersham). Hybridization and washing of the filters were done as described for the northern blot analysis.

RESULTS

Isolation and molecular characterization of PnCOP1

A partial *PnCOP1* cDNA fragment, 780 bp in size, was obtained from RT-PCR with two degenerate primers, 5'p-GGATCCGATTTGTATGCAGAT-3'OH and 5'p-GGATCCGCT-TTTGGAGGTAACATTCTTG-3'OH. This fragment included a ring-finger motif found in *Arabidopsis* COP1 and its deduced amino acid sequence shows a 75% and 78% similarity with *A. thaliana* and *P. sativum*, respectively (data not shown). The partial clone was used for screening the cDNA library to obtain a full-length cDNA. We obtained several positive clones and sequenced the largest one. A full-length *Pharbitis nil COP1* (*PnCOP1*) cDNA consisted of 2,280 bp nucleotides that contained 47 bp of 5'-UTR, 232 bp of 3'-UTR including the poly (A) tail, and 1,998 bp of the coding sequence. The sequence was deposited in GenBank (accession number, AF315714). The deduced amino acid sequence contained 666 amino acids, giving a theoretical molecular weight of 75 kD and a isolectric point of 6.2. The deduced polypeptide included the IG-MHC motif between amino acid 74 and 80, a zinc (RING)-finger motif, a coiled-coil region, two WD-40 repeats, and two targeting signals, the nuclear and cytoplasmic localization signals (NLS and CLS) (Fig. 1).

At the amino acid sequence level, PnCOP1 showed an identity of 82.4%, 73.8%, 72.6%, 68.1% and 36.9% with tomato (GenBank, AF029984), *Abraidopsis* [6], pea [34], rice [35] and human [26], respectively. The highest identity was observed with tomato COP1 while lowest one was with human COP1. However, the RING-finger domain and WD-40 repeats were highly conserved (data not shown).

To confirm the identity of our cDNA clone, *in vitro* transcription and translation were carried out by using the TNT-coupled reticulocyte lysate system. One microgram of plasmid DNA containing the *PnCOP1* cDNA was subjected to the *in vitro* system in the presence of ^{35}S-methionine, and the product was then fractioned on 12% SDS-polyacrylamide gel. As is shown in Figure 2A, several bands were detected, including ca. 71 kD (based on the MW markers) of the expected band, implying that there is premature termination process at either the transcription or translation levels. As a result of Southern blot analysis, the *PnCOP1* gene appeared to be a single gene (Fig. 2B). PnCOP1 was distantly grouped from *Arabidopsis* and pea COP1 (Fig. 2C). It was very interesting that PnCOP1 was in a group with human COP1 (HuCOP1).

Tissue specific expression and light/dark effect

To investigate the tissue specific expression of the *PnCOP1* gene, the total RNAs isolated from the cotyledons, hypocotyls and roots of seedlings treated with 16 h darkness were hybridized with ^{32}P-labeled full length *PnCOP1* cDNA. As shown in Figure 3, the *PnCOP1* mRNA was detected in the cotyledons and hypocotyls, but not in roots. The transcript level was highest in the cotyledon, which was the most sensitive tissue to photoperiodic floral induction in the *Pharbitis* plants. During germination, the basal level of the *PnCOP1* transcript was detected in the cotyledons that were exposed to the continuous light, whereas the levels increased 3 days after germination in the dark (Fig. 4). It was very interesting that the expression of *PnCOP1* was suppressed by illumination in the *Pharbitis* cotyledons.

PnCOP1 expression in relation to flowering

The mechanism of action for COP1 at the molecular level has recently been proposed [36]. In the dark, COP1 interacts with multiple transcription factors, including HY5, and thereby it inactivates their transcriptional activity. In the light, however, the COP1 translocates to the cytosol, and thus, it activates transcription factor and triggers gene expression, resulting in photomorphogenesis to occur. This hypothesis naturally develops a new hypothesis that COP1 will function as a positive regulator during the floral induction of *Pharbitis nil*, a short day plant for which the dark period is very important. To indirectly test the new hypothesis, we first of all examined the *PnCOP1* expression at the transcription level with respect to the flowering conditions. As shown in Figure

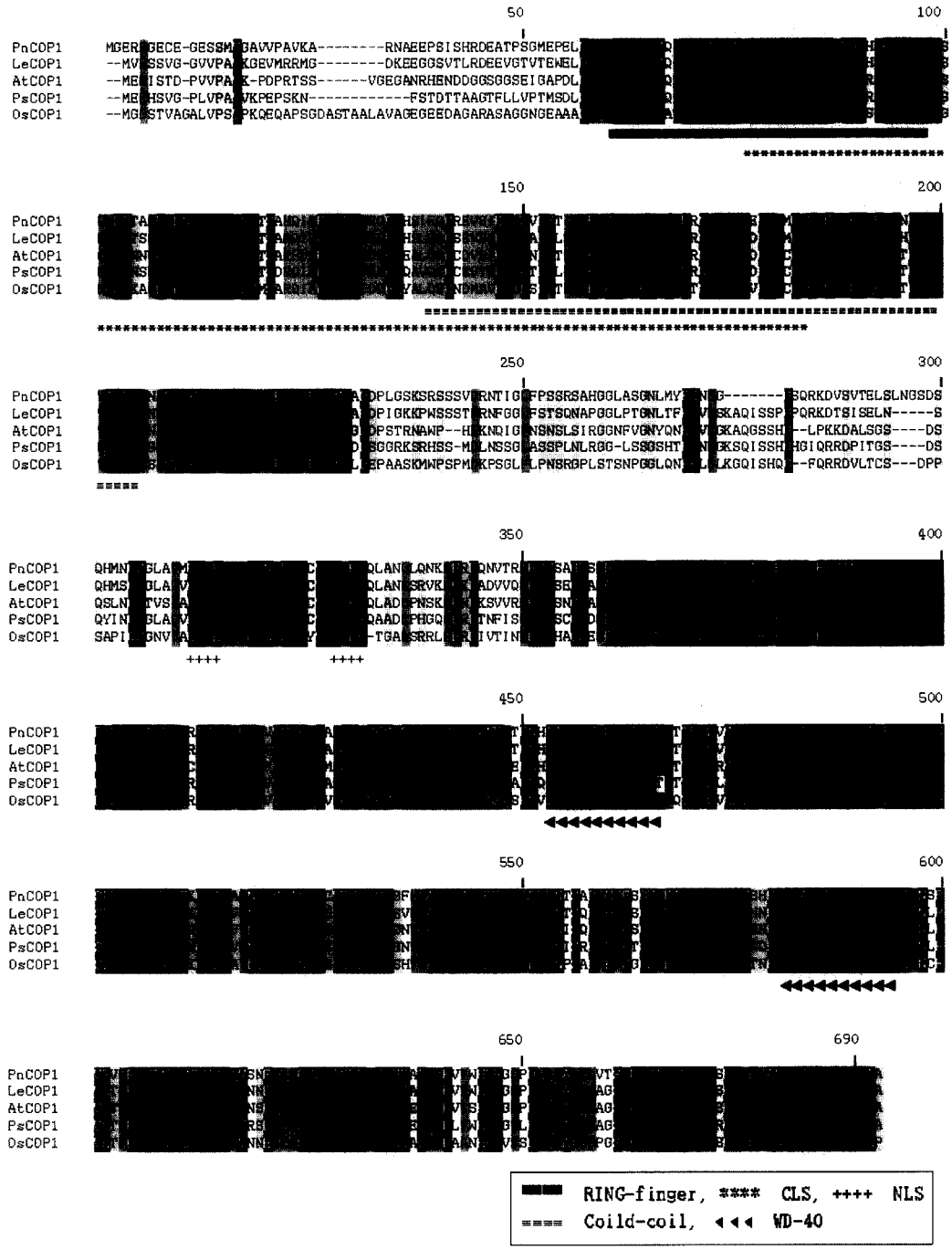

Figure 1. Alignment of the deduced amino acid sequence of *PnCOP1* with tomato (LeCOP1; GenBank, AF029984), *Arabidopsis* (AtCOP1; [6]), pea (PsCOP1; [34]) and rice (OsCOP1; [35]) protein. The amino acid sequences are given as a one letter code and have been aligned by the introduction of gaps (---) to maximize homology. The dark-shadowed boxes indicate identical amino acids, and the shadowed boxes indicate conserved amino acids.

5, during the floral inductive 16 hour-dark period for *Pharbitis nil*, the *PnCOP1* expression was increased and reached its maximum at the 12th hour of the dark period. The levels of *PnCOP1* mRNA were dramatically reduced upon the light illumination. However, the levels of the *COP1* transcripts for *Arabidopsis*, a long day plant, were almost similar for light illumination and dark treatment, which is consistent with the previous report by Deng et al. [6] who indicated that there was no light regulation of the *COP1* gene expression at the mRNA level in *Arabidopsis*. In contrast to the case of photomorphogenesis in *Arabidopsis*, these results suggest that PnCOP1 may have an important function in the floral induction of *Pharbitis nil*.

To investigate the changes in the level of the *PnCOP1* transcript

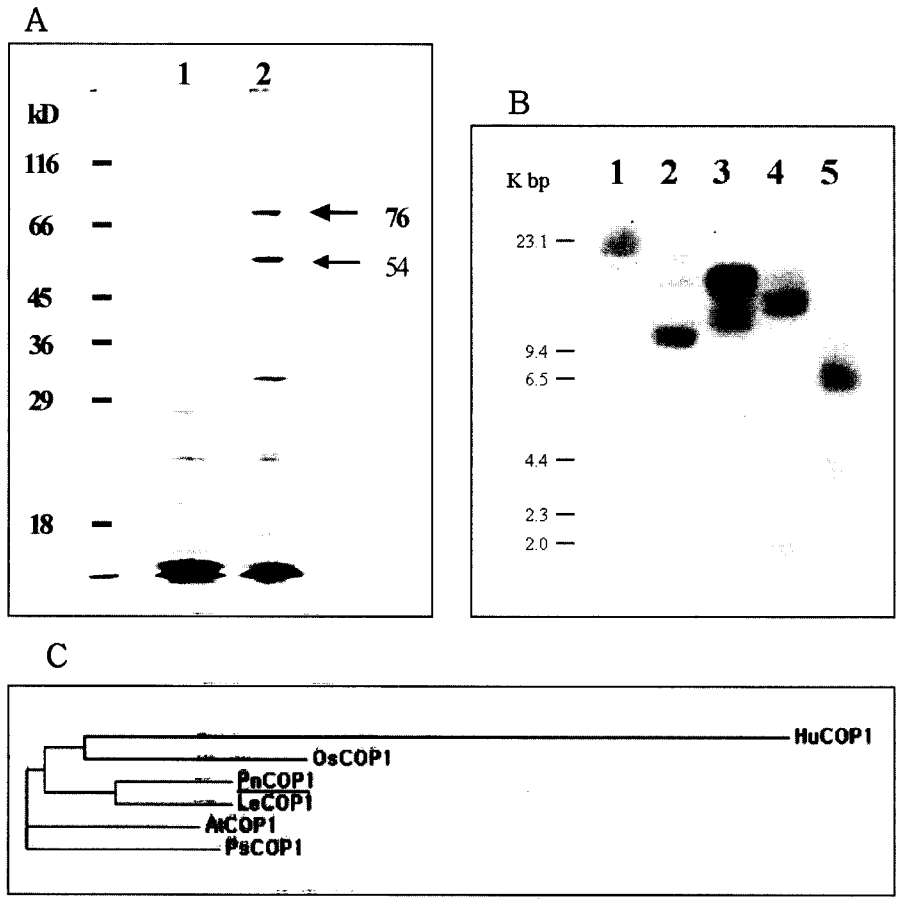

Figure 2. Analysis of PnCOP1. **A.** Autoradiography of the *in vitro* translation product. Ten microliters of *in vitro* translation product were fractionated on 12% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue, dried and then exposed to X-ray film. Lane 1: the control minus *PnCOP1* cDNA. Lane 2: experimental condition plus *PnCOP1* cDNA. **B.** Southern blot analysis of *Pharbitis nil* genomic DNA probed with *PnCOP1* cDNA. Fifteen micrograms of DNA were fractionated on 0.8% agarose gel after 16 h digestion with the given enzymes. 1. Undigested genomic DNA; 2. *Bgl*II; 3. *Eco*RV; 4. *Bam*HI; 5. *Hin*dIII. Size markers are indicated to the left in kbp. **C.** Neighbor-joining tree representing the phylogenetic relationship among the COP1s known to date. The alignment of amino acid sequences was performed using the CLUSTAL W (1.82) multiple sequence alignment program. HuCOP1 (human COP1, [26]); LeCOP1 (tomato COP1, AF029984); AtCOP1, (*Arabidopsis* COP1, [6]); PsCOP1 (pea COP1, [34]); OsCOP1 (rice COP1, [35]); PnCOP1 (*Pharbitis nil* COP1).

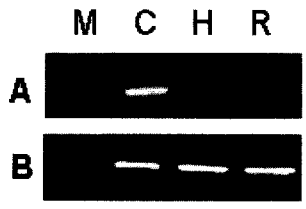

Figure 3. Expression of *PnCOP1* in different tissues. The total RNAs were extracted from cotyledons, hypocotyls and roots, and they subjected to RT-PCR experiment. **A** represents the *PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

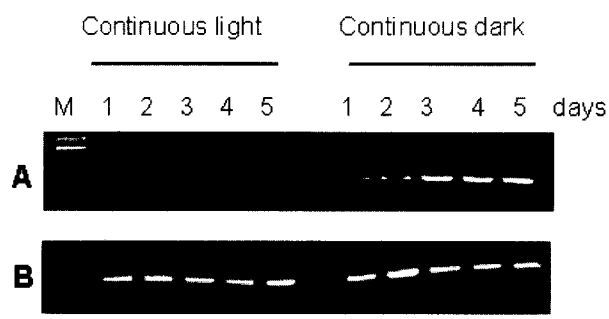

Figure 4. The steady state level of *PnCOP1* transcripts during germination either in continuous light or dark. *Pharbitis* seeds were sown in pots and the cotyledons were harvested every day after it emerged. The total RNAs were extracted from the cotyledons and subjected to RT-PCR experiment. **A** represents *the PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

during consecutive floral induction cycles, a northern blot analysis was carried out with the total RNAs extracted at the end of indicated cycle (Fig. 6). The *PnCOP1* transcript levels were generally lower in the cotyledons exposed to continuous light than in the SD-treated ones. The transcript level did not have a correlation to the number of inductive cycles, and the levels fell at 16 hours illumination and the levels slightly recovered after that. This result suggests that basal level of

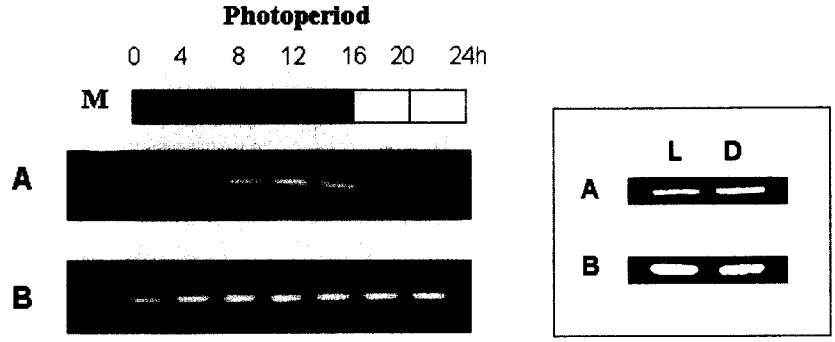

Figure 5. Expression of *PnCOP1* during a floral inductive period (16h-dark/8 h-light). *Pharbitis* plants grown under continuous light for 5 days were exposed to one cycle of the inductive cycle. The total RNAs were extracted from the cotyledons at the given times, and then subjected to RT-PCR experiment and analyzed by 1.5% agarose gel electrophoresis.. **A** represents *the PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

Box: RT-PCR analysis of *Arabidopsis COP1* gene expression. The total RNAs were extracted from leaves exposed to either 8 h light (L) or at the end of the 16-h dark period. **A** represents the *AtCOP1* transcript. **B** indicates the 5.8S rRNA used as a control.

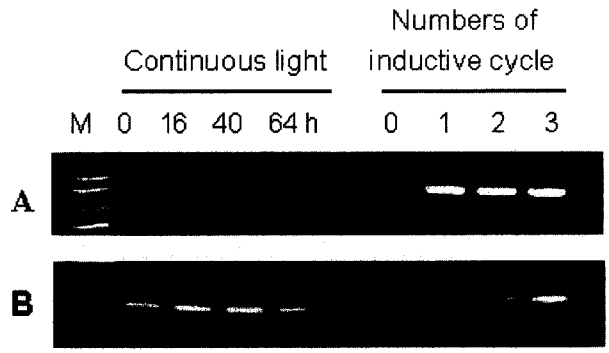

Figure 6. Comparison of the expression of *PnCOP1* between the floral inductive and non-inductive cycles. *Pharbitis* plants grown under continuous light for 5 days were exposed to either 3 cycles of a 16 h-dark/8 h-light period or to continuous light. The total RNAs were extracted from the cotyledons at the given times or at the end of the 16 h-dark period and they were subjected to RT-PCR experiment. **A** represents *the PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

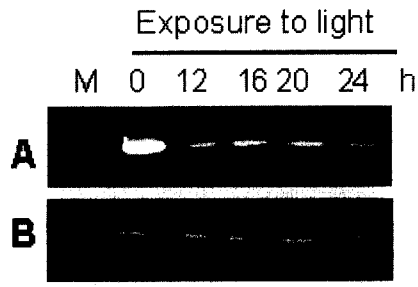

Figure 7. RT-PCR analysis of *PnCOP1* gene expression during the extended photoperiod. *Pharbitis* plants grown under continuous light for 5 days were exposed to 16 h dark and then to the continuous light for 24 h. The total RNAs were extracted from the cotyledons at the given times and then subjected to RT-PCR experiment. **A** represents *the PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

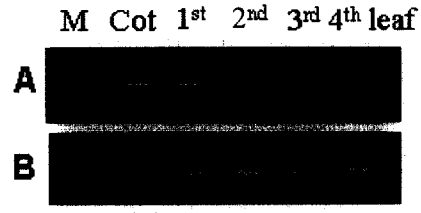

Figure 8. Expression of *PnCOP1* during the developmental stages. *Pharbitis* plants grown under continuous light were exposed to 16 h dark, and then they were kept under continuous light until they had their 4th leaf. Indicated tissues were harvested from 5 individual plants. The total RNAs were extracted from the cotyledons (Cot), the 1st, 2nd, 3rd and 4th leaves, and the RNAs were subjected to RT-PCR experiment. **A** represents *the PnCOP1* transcript. **B** indicates the 5.8S rRNA used as a control. M represents the 1 kbp DNA ladder.

PnCOP1 transcripts in *Pharbitis* cotyledons was maintained, but the levels increased upon dark treatment.

To investigate the change of the *PnCOP1* transcript level by illumination of light after the dark induction, RT-PCR analysis was carried out during the extended photoperiod. *Pharbitis* plants grown under continuous light for 5 days were next exposed to 16 hours dark and then to continuous light for 24 h. As shown in Figure 7, the lower level of the transcript was observed in continuous light than in the dark condition. It was also proved that the level of the *PnCOP1* transcript in cotyledons was higher in the dark than in light.

The cotyledons and leaves of the *Pharbitis* plants have been considered as being the location of the tissues that produce the flower-inducing substance, even though the cotyledons are most sensitive to photoperiodic induction. To examine the relationship between the *PnCOP1* transcript level and the leaf age, a RT-PCR experiment was carried out with plant tissues that were treated with a one-time inductive dark period. As shown in Figure 8, the transcript levels were gradually decreased in the leaves apart from the cotyledons. This result implied that either the level of the *PnCOP1* transcript was higher in the older tissue than the younger tissue or the

transcript level was higher in tissue that was sensitive to the photoperiodic induction of flowering.

DISCUSSION

COP1 was found to have a function as the ubiquitin E3 ligase that regulates the stability of many of the proteins controlling development and differentiation in plants and animals. Recent studies have suggested that in *Arabidopsis*, more than 1400 genes (~5% of the proteome) encode components of the ubiquitin/26S proteasome (UB/26S) pathway [5]. Approximately 90% of these genes encode subunits of the E3 ubiquitin ligases that confer substrate specificity to the pathway. The pleiotropic phenotype of the *Arabidopsis cop1* mutants [9, 37-40] and the multiple protein-protein interactions [2,5] indicate that COP1 plays a key role in transcriptional pathways by regulating not only photomorphogenesis in the seedlings, but also by regulating development at later stages such as the floral transition. All this information has particularly led to an idea that this photomorphogenic repressor, COP1, may function as a positive regulator during the floral inductive dark period in *Pharbitis*, which a short-day plant (SDP). To obtain primary information for COP1's function for the flowering of SDPs, molecular cloning and characterization was performed in *Pharabitis nil*, a model plant for the SDPs.

Isolation and molecular characterization of PnCOP1

The cloned *PnCOP1* includes the typical domains found in *Arabidopsis* COP1; a zinc (RING)-finger motif, a coiled-coil region, two WD-40 repeats, and two targeting signals, the nuclear and cytoplasmic localization signals (NLS and CLS) (Fig. 1). In addition, the protein showed a very high identity to the known plant COP1s and it also shared some important domains, the RING-finger domain and the WD-40 repeats, even with human COP1 which is functionally grouped with PnCOP1 by phylogenetic alignment. All of PnCOP1's characteristics imply that this protein can interact with many proteins and function in the unbiquitin-dependent protein degradation pathway.

There are plenty of reports showing that plant COP1 interacted with transcription factors and signaling molecules, thereby regulating cellular and developmental processes ([2, 4, 5]. In a recent study, mammalian COP1 showed highly conserved functional domains with its plant counterparts, implying that the proteins have similar functions. Human COP1 (huCOP1) interacted with Jun transcription factors, leucine zipper factors and it regulates AP-1 dependent transcription [26]. The COP1 protein was also shown to be a critical negative regulator of p53, the tumor-suppressor protein, and this represents a new pathway for maintaining p53 at low levels in unstressed cells [27]. Despite its low identity with mammalian COP1, PnCOP1 shows strong conservation in the functional domains, implying the multiple functions that may be present.

Tissue specific expression and light/dark effect

In *Arabidopsis*, there was no light regulation of *COP1* gene expression at the mRNA level since the steady-state levels for *COP1* were almost same in the dark-grown and light-grown seedlings [6]. On the other hand, the expression level of *COP1* was positively controlled by light in the etiolated leaves and the green leaves of rice [35]. Our results indicated a tissue specific pattern of expression for *PnCOP1* since the transcripts were detected in cotyledons, hypocotyls and leaves, but not in the root (Fig. 3 and 8). In addition, the level of the transcripts was high in dark-treated cotyledons and this was reduced by light illumination (Fig. 4-7). All these data suggest that expression of *COP1* may slightly differ from species to species at the transcript level even though COP1's function is quite similar. Particularly, the unique expression pattern of *PnCOP1* may imply its critical function in *Pharbitis* plants.

PnCOP1 expression in relation to flowering

Our results may infer either that the floral induction requires for the expression of the *PnCOP1* gene or that the expression of the *PnCOP1* gene is stimulated or induced by darkness, at least in the *Pharbitis* cotyledons (Fig. 5-8). The levels of *PnCOP1* transcript were generally high in the dark treated cotyledons, while the levels became lower upon light illumination. However, we can not explain if this is due to whether the light affects the stability of *PnCOP1* transcripts or if the light affects the rate of transcription itself. In a DNA chip experiment, about 20% of the genome was regulated by COP1 in the dark, which covers over 28 cellular pathways [41]. This microarray data implies that there are more functions for COP1 than we knew existed, and one of these functions includes flowering.

In *Arabidopsis*, a long-day plant, supplying sucrose to the plant induced flowering in dark [42-44]. This dark flowering implies that light provides the energy source for flowering in *Arabidopsis*. The researchers of that study suggested the possible interaction between COP1's function and signal molecules induced by sugars. However, the dark period is critical for flowering in short-day plants (SDPs) like *Pharbitis nil*. It has been suggested that during the dark period, the flowering-inhibitory substance(s) is degraded or flowering-inducing substance(s) is synthesized and accumulated in SDPs [29]. Therefore, PnCOP1 may play an important role in the dark period for floral induction by degradation of proteins that are essential for the synthesis of flowering-inhibitory substance(s).

There are a few reports on the relationship between flowering and COP1 in *Arabidopsis* [11, 44, 45]. The less severe *cop1* mutations were able to induce early flowering and dark flowering. The *cop1* mutations lead to a reduction for the number of leaves that is essential for flowering, suggesting that COP1 has a role for the repression of flowering in *Arabidopsis*. *COP1* represses the transition from the vegetative phase to the reproductive phase, at least in darkness. Nakagawa and Komeda [44] examine the expression of flowering-related genes in the *cop1* mutant

Arabidopsis, and they concluded that COP1 may regulate the CO (CONSTANCE) level, which is a positive regulator of flowering, at post-transcription level. They suggested a possible involvement of the ubiquitin-dependent protein degradation pathway. All these data lead to an idea that PnCOP1 may be involved for flowering in *Pharbitis nil*. Cloning and characterization of genes interacting to PnCOP1 during floral induction in *Pharbitis* cotyledons [46] by using yeast-two hybrid system will be a study of choice.

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