Expression of Calcium-Dependent Protein Kinase Gene (*PnCDPK1*) is Affected by Various Light Conditions in *Pharbitis nil* Seedlings

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Abstract Calcium-dependent protein kinases (CDPKs) are central calcium signal decoders. Here we report the isolation and characterization of the Japanese Morning Glory's (*Pharbitis nil*) CDPK gene, termed *PnCDPK1*. The full-length cDNA of 1943 bp contains an open reading frame for PnCDPK1 consisting of 514 amino acid residues and a calculated molecular mass of 57.9 kDa. The deduced amino acid sequence suggests that this protein contains the kinase domain at the amino terminus and autoregulatory and calmodulin-like domain at the carboxy terminus. Sequence alignment indicated that PnCDPK1 shared high similarities with other CDPKs. Biochemical analyses showed that bacterially expressed recombinant protein was catalytically active and was able to phosphorylate the histone III-S in a calcium-dependent manner. Besides the identification of PnCDPK1 as a member of the CDPK family, it was shown that a transcript of *PnCDPK1* was modulated during germination and seedling growth. Moreover, *PnCDPK1* mRNA was present in every tested organ, including root, hypocotyl, and cotyledon of the light- and dark-grown plant; however, the higher expression level was found in dark-treated ones. The *PnCDPK1* mRNA level in the cotyledons exhibited a significant increase after moving seedlings into the dark, peaking 2 and 8 h after dark exposure, and then it gradually decreased. Expression was significantly changed by exposure to red light after 8 h in the dark. These data unequivocally identify the product of the *PnCDPK1* gene as a calcium-dependent protein kinase and emphasize the potential that this gene can be an element of light signal transduction involved in growth and development of *P. nil*.

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Introduction

Calcium signaling is one of the best documented pathways in plants. It has been confirmed to be operative in a series of biological processes from cell division to plant responses to a wide range of stimuli, including phytohormones, light, pathogen elicitors, and abiotic stresses (Reddy 2001; Mazars and others 2009). Different calcium sensors recognize specific calcium signals and transduce them into downstream effects, including altered protein phosphorylation and gene expression (Rudd and Franklin-Tong 2001). Among the downstream targets of calcium in plants, calcium-dependent protein kinases (CDPKs) form an interesting class of kinases that are activated by Ca^{2+} binding. Since the first characterization of plant CDPK (Harmon and others 1987), many isoforms have been found and some corresponding genes have been cloned (Hrabak and others 2003; Chehab and others 2004; Kumar and others 2004; Li and others 2008) however, the precise role of each specific CDPK is still largely unknown.

CDPKs are found in various subcellular locations, suggesting that individual isoforms are implicated in a diverse array of responses to endo- and exogenous stimuli. The absence of specific inhibitors for CDPKs, the lack of dominant negative constructs, and the possibility of functional redundancy have made it difficult to assign functions to individual CDPKs in distinct signaling pathways (Cheng and others 2002; Raíces and others 2003). CDPKs are known to be involved in various physiological processes and at different stages of plant growth and development (Cheng and others 2002; Ivashuta and others 2005). For example, CDPKs participate in pollen germination and tube growth (Estruch and others 1994; Yoon and others 2006; Myers and others 2009), hormone signaling (Mori and others 2006; Zhu and others 2007; Ishida and others 2008), self-incompatibility regulation in tobacco (Kuntz and others 1996), the early stages of potato growth (Raíces and others 2001), and embryogenesis, seed development, and germination in sandalwood (Anil and others 2000).

It is known that CDPK formation may be regulated at the transcriptional and post-transcriptional levels (Frattini and others 1999). The mechanisms by which plant CDPKs can be regulated are important questions that touch upon molecular cloning of the CDPK gene(s) as well as analysis of the expression of CDPK coding gene(s), especially because expression of the CDPK was not tested during seedling growth in various light regimes and photoperiodic flower induction. Studies focusing on such problems and identification of the CDPK gene in *Pharbitis nil* are the subject of this report. The biochemical characterization of recombinant protein is described and confirms that the analyzed enzyme belongs to a CDPK family. Moreover, it is shown that expression of this gene is regulated by light conditions during germination and seedling growth.

Photoperiodism is the reaction of a plant to the changing duration of light over 24 h and the periodic succession of light and darkness. Photoperiodic induction of flowering is based on the perception by the plant of the appropriate photoperiod that results in the differentiation of a vegetative shoot meristem into a generative one leading to the creation of a flower (Szmidt-Jaworska and others 2006). Studies on the mechanism of photoperiodic flower induction should be conducted on a plant that shows a clear response to photoperiodicity. The Japanese Morning Glory (*Pharbitis nil*) is such a plant and one of the most intensively studied. It maintains vegetative growth under long-day or continuous light conditions and can be induced to flowering in an early stage by a single 16-h dark treatment just after the cotyledons have fully expanded (Liu and others 2001; Szmidt-Jaworska and others 2004). The control mechanisms of photoinduction of flowering are not clear. There are some suggestions that calcium and calcium-binding protein are involved in the flowering process (Tretyn and others 1994; Jaworski and others 2003; Szmidt-Jaworska and others 2006).

To the authors' knowledge, there is no information about the role of CDPK in photoperiodic flower induction. This gap in the literature provided us with a strong rationale for analyzing the level of expression of *PnCDPK1* during various light/dark conditions in *Pharbitis nil*.

Materials and Methods

Plant Material and Light Treatments

The experiments were conducted on Morning Glory (*Pharbitis nil* Chois. cv. Violet, synonym *Ipomoea nil*) (Marutane Co., Japan), which is a model short-day plant. CDPK was assayed at daily intervals during different developmental stages: dry seeds, imbibed seeds, germination, and seedling growth. Seeds were soaked in concentrated sulfuric acid for 45 min and then washed with running water for 3 h. They were left in ddH_2O for 24 h at $25 \pm 2°C$ in white light or dark. The imbibed seeds were planted on a mixture of vermiculite and sand (2:1 w/w) and grown at $25 \pm 2°C$ in continuous light (green plants) (130 μmol m^{-2} s^{-1}; cool white fluorescent tubes, Polam, Poland) or dark (etiolated plants) for 5 days. Roots, hypocotyls, and cotyledons were harvested from 5-day-old seedlings growing in continuous light or dark.

For expression analysis in various light/dark regimes, plants were grown in continuous light for 5 days. A portion of 5-day-old plants were left in such conditions. The rest of the plants were exposed to a 16-h-long dark period (induction) and then moved to light. For some plants the 16-h-long night was disrupted by a 5-min pulse of red light (R, 1.5 μmol m^{-2} s^{-1}, fluorescent tubes TLD 15R/18 W, Philips) at the eighth hour or by red light followed by a 10-min-long pulse of far-red light (FR, 0.1 μmol m^{-2} s^{-1}; narrow-band filter FR = 730 ± 2; half-band width = 9 nm). Afterward, cotyledons were harvested every hour, immediately frozen in liquid nitrogen, and stored at $-80°C$.

Molecular Cloning of *PnCDPK1*

Total RNA was isolated from etiolated roots of *P. nil* seedlings using Tri Reagent (Sigma). The first-strand cDNA for RT-PCR was synthesized with RevertAid M-MuLV RT (Fermentas) following the manufacturer's instructions. PCR was performed with 50 ng of cDNA and 100 ng of degenerate primers, 5′-GTYGGNAGTGCWT AYTATGTKGC-3′ and 5′-RTTCTTCRTANGTDATNGT NCCRCT-3′, corresponding to the conserved amino acid

sequences VGSAYYV and SGTITYEE, respectively. PCR parameters are 50 s at 95°C for denaturing, 30 s at 55°C for annealing, and 50 s at 74°C for extension for 35 cycles, and a final extension step of 7 min at 74°C. PCR products were separated on a 1% agarose gel, eluted, and sequenced.

A full-length cDNA amplification for *PnCDPK1* was performed using the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech), and mRNA was purified from total RNA using the Oligotex mRNA Mini Kit (Qiagen). 5′-RACE and 3′-RACE primers (5′-CTTT GATGCTGTTCTTCGGGGACATC-3′ and 5′-GGCTTGT CAGATGCATCACCATCTTC-3′, respectively), specific for the *PnCDPK1* cDNA fragment, were used. PCR reactions were performed using the Advantage 2 PCR Enzyme System (Clontech). The PCR products were purified and cloned into a pTZ57R vector (Fermentas) for sequencing. To confirm a full-length *PnCDPK1* cDNA assemblage, PCR amplification was carried out with primers designed according to the 5′- and 3′-RACE product sequences. The total volume of 25 μl PCR reaction solution contained 2.5 μl 10× Pfu buffer, 1.5 μl 50 mmol MgCl$_2$, 1 μl 10 mmol/μl each of dNTPs, 1 μl 10 μmol/μl PnCPK1 (5′-GAACAGATCTTCCTCTCCCAGTT-3′) and PnCPK2 (5′-AGTTTGACTTGGGTCGTCTACCT-3′) primers, 1 μl cDNA, and 2.5 units Pfu DNA polymerase (Invitrogen). The following protocol was used: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 74°C for 2 min, with a final extension of 7 min at 72°C.

The nucleotide sequence of *PnCDPK1* reported here is available in GenBank under accession No. DQ319188. Data analyses were performed in ClustalW (www.ebi.ac.uk/clustalw) and BLAST 2.2 (www.ncbi.nlm.nih.gov/BLAST).

Gene Expression Analysis

Semiquantitative RT-PCR analysis was performed to analyze the expression of the *PnCDPK1* gene. Total RNA was isolated from particular plant material with the GeneMA-TRIX Universal RNA Purification Kit (Eurx), then the RNA sample (1 μg) was reverse transcribed with the MMLV RT enzyme (Epicentre) at 37°C for 1 h. PCR was conducted at the linearity phase of the exponential reaction for each gene. The gene-specific primer pairs homologous to the 3′ UTR (untranslated region) of *PnCDPK1* cDNA were as follows: for *PnCDPK1*, the forward primer was 5′-TTGTGTCGTGTTGCAATGTG-3′ and the reverse primer was 5′-CAGCAGCACAACCTCTCAAA-3′, and for the *actin4* gene, the forward primer was 5′-GAATTCGA-TATCCGAAAAGACTTGTATGG-3′ and the reverse primer was 5′-GAATTCCATACTCTGCCTTGGCAATC-3′. The *Actin4* expression level was used as a quantitative control. The specificity of the *PnCDPK1* RT-PCR product was confirmed by cloning and DNA sequencing.

The computer application used for the analysis was Quantity One (BioRad), and for the calculations and graphs we used SigmaPlot 2001 v7.0 (SPSS Inc.).

Expression and Purification of GST-PnCDPK1 Fusion Protein

The open reading frame (ORF) of *PnCDPK1* cDNA was amplified by PCR with primers introducing *Xho*I restriction sites (5′-AAACTCGAGAAATGGGGGAGTTGTAACAGC ATAC-3′ and 5′-AAA*CTCGAG*TCATCTACGACGCCTA TTAATGACC-3′). The PCR product was introduced into the plasmid pTZ57R/T (Fermentas) and verified by DNA sequencing. For expression of *GST-PnCDPK1* in bacteria, the *PnCDPK1* ORF was cut from pTZ57R/T and inserted into the pGEX-6P2 (GE Healthcare) expression vector at the *Xho*I restriction site and the proper orientation of the cloned cDNA was checked by sequencing. The *E. coli* BL21 strain, transformed with the resulting plasmid, was used to produce the GST-tagged protein. The expression of the fusion protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and incubated at 24°C for 3.5 h. The bacteria cells were collected by centrifugation, suspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) NP-40, 1 mM PMSF, and 0.2 mg ml^{-1} lysozyme], and disrupted by sonication. The soluble fraction was separated by centrifugation at 12,000 *g* for 10 min at 4°C and the GST-tagged proteins were adsorbed onto glutathione–Sepharose 4B beads (GE Healthcare). After washing the column with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, the GST-PnCDPK1 complex was either eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0), or PnCDPK1 was released from the fusion protein by proteolytic cleavage of the protein with Pre-Scission protease (GE Healthcare Life Sciences) following the manufacturer's instructions. For control expression the pGEX-6P2 vector was used or GST alone was purified as described above.

Electrophoresis and Western Blot Analysis

The homogeneity and purity of eluted protein fractions were analyzed by 12% (v/v) SDS–PAGE (Laemmli 1970) and gels were stained with Coomassie Blue. For Western blot analysis, proteins resolved by SDS–PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Hybond-P, GE Healthcare) using the semidry system (BioRad) in 25 mM Tris, 192 mM glycine buffer

(pH 8.3) with 20% (v/v) methanol. After blocking in TBS buffer (20 mM Tris, 100 mM NaCl) containing 3% (w/v) nonfat dry milk, the membrane was incubated with poly-clonal anti-GST antibodies (1:15,000) (GE Healthcare) or anti-CDPK antibodies (1:10,000) (Bachmann and others 1996). The Western blots were visualized with horseradish peroxidase-conjugated secondary anti-goat IgG (1:60,000) for GST and anti-rabbit IgG antibodies (1:30,000) for CDPK (Sigma-Aldrich). The blots were detected using a chemiluminescence kit (ECL plus) following the manu-facturer's instructions (GE Healthcare).

PnCDPK1 Activity

Protein kinase activity was determined as described pre-viously by (Jaworski and others 2003), with minor modi-fications. The standard reaction mix (50 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 0.5 mg ml^{-1} histone III-S as a substrate, and 0.2 mM EGTA with or without 0.25 mM CaCl$_2$. The reaction was initiated by the addition of 25 μM [γ-^{32}P] ATP (270 cpm/pmol) and the mixture was incubated for 5 min at 30°C. Samples were spotted onto a P-81 filter and washed with 5% (w/v) tri-chloroacetic acid with 1 mM sodium pyrophosphate. Radioactivity on the dried filters was determined by scin-tillation counting (Wallac 1409, PerkinElmer). For some experiments the reaction was terminated by addition of sample buffer. The samples were boiled for 5 min and subjected to 12% acrylamide gels. The dried gels were autoradiographed with X-ray film.

To investigate the effect of CDPK inhibitors on the PnCDPK1 activity, calmidozolium, trifluoperazine, chlor-promazine, and W-7 (Sigma-Aldrich) were added into the reaction system at 0.1 mM concentration. The kinetic analysis of PnCDPK1 was conducted as described above, taking 0.2 μg of the purified enzyme. Assays were per-formed in the presence of different concentrations of cal-cium ions (0.2–0.3 mM) and histone III-S (0.025–1 mg ml^{-1}). The $K_{0.5}$ and V_{max} were determined from the slopes and intercepts of the Lineweaver–Burk plots. Kinetic parameters were determined by averaging two independent assay results. Free calcium ion concentrations were calculated by the MaxChelator (MAXCLITE v1.15) computer program.

For assay kinase activity in-gel, 0.2 μg purified PnCDPK1 protein was separated in 12% SDS-PAGE, with histone III-S (0.5 mg ml^{-1}) added to the separation gel just prior to polymerization. Proteins were denatured, rena-tured, labeled in the presence of 50 μCi [$^{32}\gamma$-P] ATP (4500 Ci/mmol) with 1 mM CaCl$_2$ or 1 mM EGTA, and washed as described by Jaworski and others (2003). Gels were dried and autoradiography was performed.

Results

Molecular Cloning of PnCDPK1

Pharbitis nil cDNA was isolated from etiolated roots using a combination of RT-PCR strategies. PCR for the core fragment was carried out using degenerate primers based on conserved regions of CDPK. From the initial RT-PCR reaction, PCR products (490 bp) corresponding to the core fragment were cloned and sequenced. A full-length cDNA, designated as *PnCDPK1*, was isolated using the RACE-PCR. The *PnCDPK1* cDNA consisted of 1943 bp, including a 36-bp 5′ UTR, a 331-bp 3′ UTR, and a poly(A) tail of 30 bp. The ORF of *PnCDPK* cDNA contained a 1545-bp that encoded a 514-amino-acid peptide with a theoretical molecular mass of 57.9 kDa and a pI of 5.8. Using the program PESTFind (http://www.at.embnet.org/embnet/tools/bio/PESTfind/), we identified a conserved potential region called PEST sequence in the variable N-terminal region of the PnCDPK1 protein. This specific amino acid sequence within the polypeptide is rich in proline, glutamic acid, serine, and threonine and enhances the rapid degradation of many proteins (Fig. 1). The PEST region in PnCDPK1 (residues 1-27) scored +6.91 (PEST scores greater than +5 are considered significant). More-over, the potential myristoylation and palmitoylation sites were noted in the N terminal.

The deduced *P. nil* CDPK protein has shown all the characteristics of CDPKs of other plants. It contains a highly variable N-terminal region, a conserved kinase catalytic domain with 11 signature protein kinase subdo-mains typical of the serine/threonine protein kinase family,

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 (1)   MGSCNSIPSSPFAATSAGGESDPPPPKNDITVLPPSQPPPPRPFLSGVGR
 (51)  VLGRPMEDVHSTYIFGGELGRGQFGVTYLVTHRKTRERLACKSIATRKLL
(101)  SKDDVDDVRREVQIMHHLTGHRNIVELKGTYEDRNHVHLVMELCAGGELF
(151)  DRIIAKGHYSERAAAGLCRQMVTVLHYCHSMGVMHRDLKPENFLFLSSDE
(201)  NSPLKATDFGLSVFFKPGDTFKDLVGSAYYVAPEVLRRNYGPEADIWSAG
(251)  VILYILLSGVPPFWGENEQSIFDAVLRGHLDFSSDPWPSISSSAKDLVKK
(301)  MLRSDPKERLSATDVLNHPWMREDGDASDKPIDIAALSRMKQFRAMNKLK
(351)  KVALKVIAENLSEEEIIGLKEMFKSIDTDDSGTITYEELKAGLTKMGTKL
(401)  SESEVRQLMEAADVDGNGTIDYLEFITATMHMNRVEREDHLYKAFEYFDK
(451)  DKSGYITMEELEHSLKKYNITDEKTIKEIIVEVDTDNDGKINYDEFVAMM
(501)  RKGTPDLVINRRRR*
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Fig. 1 The predicted amino acid sequence of PnCDPK1. The kinase domain is boxed and shaded, the autoinhibitory domain is boxed and bolded, EF hands in the calmodulin-like domain are bolded and underlined. The potential myristoylation and palmitoylation sites in the N-terminal variable domain are in *italics*. The PEST sequence is double underlined. Stop is indicated by an *asterisk*

and an autoinhibitory domain joined to the C-terminal CaM-like domain with four conserved Ca^{2+}-binding EF hands. Searching protein databases using BLASTX in GenBank, it was found that the deduced protein sequence of PnCDPK1 exhibited high homology to other CDPKs, including *Cucumis sativus* CsCDPK5 (81% identity), *Arabidopsis thaliana* AtCDPK6 (79%), *Capsella rubella* CrCDPK (80%), and *Triticum vulgare* TaCDPK1 (77%) (Fig. 2).

To assess the properties of PnCDPK1, the ORF of the gene was expressed in *E. coli* BL21 as a glutathione S-transferase (GST) fusion protein. The *PnCDPK1* ORF was cloned into a pGEX-6P2 expression vector and the recombinant *PnCDPK1* was expressed in *E. coli* using the GST fusion system. When protein expression was induced by addition of IPTG, GST-PnCDPK1 emerged as a clear main band with a molecular mass of 83 kDa, which was not observed in control fractions (pGEX-PnCDPK1 without
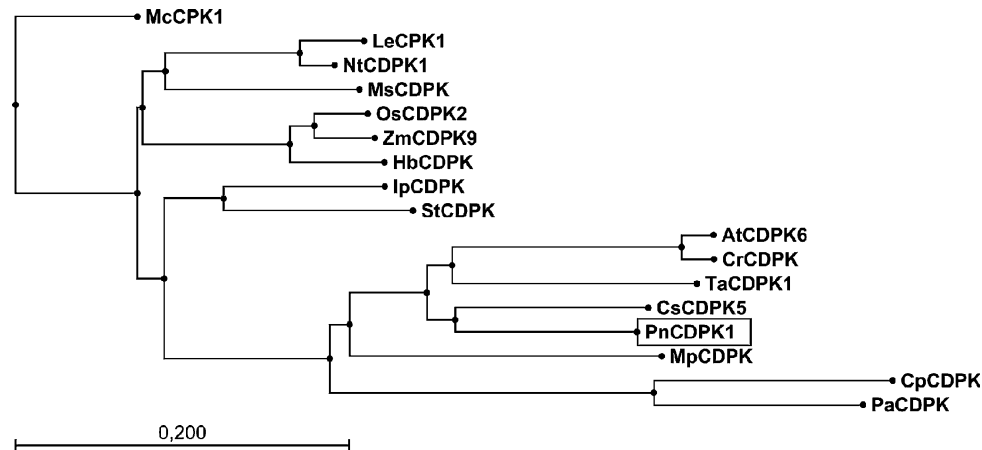

Fig. 2 Rooted phylogenetic tree based on the amino acid sequences illustrating the relationship between PnCDPK1 with selected CDPKs. The length of each pair of branches represents the distance between sequence pairs. The neighbor-joining tree was created in the MEGA4.1 program using complete protein sequences of different CDPKs. The tree was rooted with the MpCDPK sequence. The amino acid sequences and accession numbers are from PnCDPK1 (*Ipomoea nil*, DQ319188), CsCDPK5 (*Cucumis sativus*, AY0227885), At-CDPK6 (*Arabidopsis thaliana*, NM118496), AtCDPK6 (*Arabidopsis* *thaliana*, NM118496), CpCDPK (*Cucurbita pepo*, U90262), LeCPK1 (*Solanum lycopersicum*, AJ308296), MsCDPK (*Medicago sativa*, X96723), StCDPK (*Solanum tuberosum*, AF115406), IpCDPK (*Ipomoea batatas*, D87707), CrCDPK (*Capsella rubella*, EF197847), NtCDPK1 (*Nicotiana tabacum*, AF072908), ZmCDPK9 (*Zea mays*, D85039), McCPK1 (*Mesembryanthemum crystallinum*, AF09835), PaCDPK1 (*Phalaenopsis amabilis*, EF555574), HbCDPK (*Hordeum brevisubulatum*, DQ250026), OsCDPK2 (*Oryza sativa*, X81394), TaCDPK1 (*Triticum aestivum*, AJ621356)
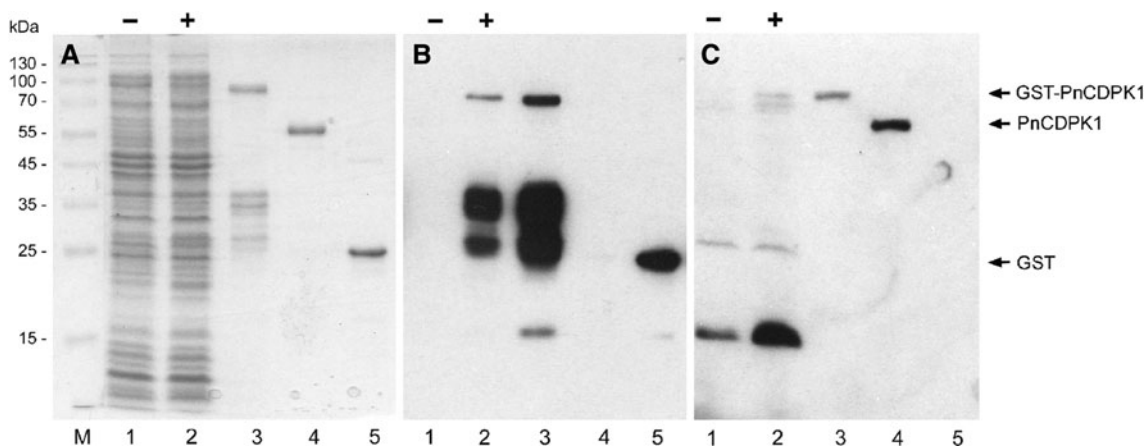

Fig. 3 Purification of GST-PnCDPK1 fusion protein and GST-PnCDPK1 digested with PreScission protease. **a** Coomassie brilliant blue-stained gel after protein separation on 12% SDS-PAGE. *Lines 1 and 2*: supernatant of non-IPTG-induced (−) and IPTG-induced (+) *E. coli* BL21 containing the expression vector pGEX-6P2-PnCDPK1, respectively. *Line 3*: purified GST-PnCDPK1 fusion protein using GST-Sepharose 4B. *Line 4*: PnCDPK1 recombinant protein after digestion of fusion protein with PreScission protease. *Line 5*: GST protein. **b, c** Immunoblots of the same samples as in (**a**) using a GST-specific antibody and anti-CDPK antibodies from soybean, respectively. *M* protein molecular weight markers (kDa)

IPTG application) (Fig. 3a, lane 3). When a sample of the GST-PnCDPK1 fusion protein was digested with PreScission protease, one main 56.5-kDa band appeared, corresponding to PnCDPK1 (Fig. 3a, lane 4). Western blot analysis showed that an anti-GST antibody reacted with the 83-kDa peptide as well as with GST (26 kDa) (Fig. 3b, lanes 2, 3, and 5), demonstrating that the induced protein was GST-PnCDPK1. Moreover, anti-soybean CDPK reacted with both the 83-kDa fusion protein and the 56.5-kDa protein (Fig. 3c, lanes 3 and 4). Based on the ability of these polyclonal antibodies to recognize different CDPK isoforms in many plant species (Anil and others 2000; Pang and others 2007), it was assumed that the detected *Pharbitis nil* protein was also CDPK.

The possibility that the recombinant protein was an active enzyme was examined by in vitro and in-gel analyses in the presence of histone III-S as a substrate. The recombinant kinase was able to phosphorylate exogenous substrate in a calcium-dependent manner, and its activity was inhibited by EGTA (Fig. 4a). V_{max} in the presence of histone III-S was 0.5 μmol min^{-1} mg^{-1} protein (Fig. 4b). Activity of PnCDPK1 was totally dependent upon free calcium ions and 50% of full activity was observed at the concentration of 1.57 μM (Fig. 4c). Moreover, as shown in Fig. 4d, the influence of CDPK inhibitors (0.1 mM) (chlorpromazine, trifluoperazine, calmidozolium, and W-7) on the activity of the recombinant enzyme was tested. All of them significantly decreased the enzyme activity, with

Fig. 4 Biochemical properties of recombinant PnCDPK1. **a** Phosphorylation of histone III-S by PnCDPK1 in vitro (I) and in gel (II). Activity was assayed in the presence of 1 mM Ca^{2+} (+) or 1 mM EGTA (−). *Arrows* indicate the position of histone III-S phosphorylated by PnCDPK1 (I) and the position of kinase activity against histone on the gel (II). **b** Effect of increasing concentration of substrate (histone III-S, range = 0-1.25 mg ml^{-1}) and **c** micromolar Ca^{2+} ions (range = 0-30 μM) on the recombinant PnCDPK1 activity. **d** Effects of various antagonists on PnCDPK1 activity. The enzyme was tested in the presence of 1 mM CaCl$_2$ using 0.5 mg ml^{-1} of histone III-S as substrate. One-hundred percent activity represents a specific activity of 0.365 μmol min^{-1} mg^{-1}. Values are the means of three replicates. *Bars* represent standard error
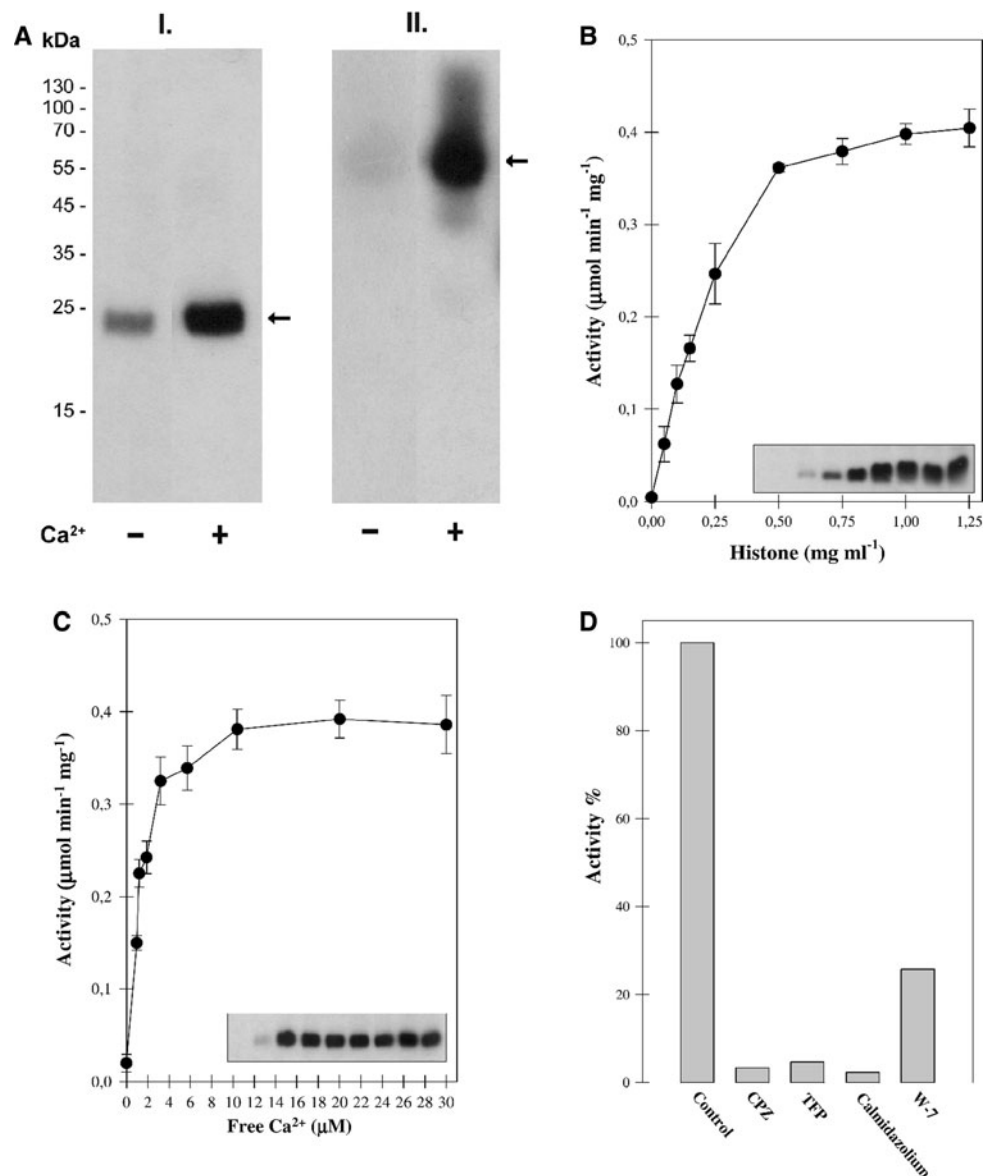
chlorpromazine, trifluoperazine, and calmidozolium causing the strongest inhibition.

Expression Analysis of *PnCDPK1* in *Pharbitis nil* Seedlings Grown under Various Light Conditions

To investigate the role of light conditions in the *PnCDPK1* expression level during seed germination and seedling growth, two different light regimes were analyzed. We examined the *PnCDPK1* transcript in entire seeds and during seedling growth in continuous light or darkness. In addition, *PnCDPK1* mRNA concentration was analyzed in excised organs: cotyledons, hypocotyls, and roots. A part of the 3′ UTR of *PnCDPK1* cDNA was used to ensure gene-specific expression analysis.

The expression of *PnCDPK1* during seedling development was examined at 24-h intervals (Fig. 5). There were
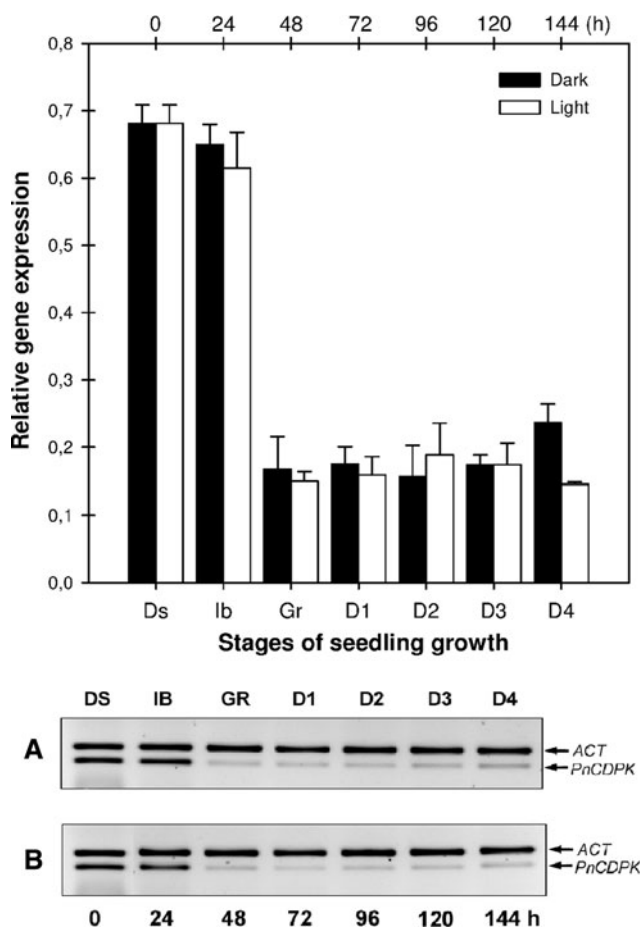
no differences in *PnCDPK1* mRNA levels in plants grown in light or dark conditions. The *PnCDPK1* transcript was high in dry and imbibed seeds; then, during germination a significant decrease was noted, reaching a low steady-state level during the next steps of seedling growth. The levels of the *actin4* transcript (internal control) did not show any statistically significant variation, thereby validating the results obtained.

Having established the variation in *PnCDPK1* mRNA level during germination and seedling growth, we next examined the expression level in 5-day-old seedling organs (Fig. 6). The results revealed that a band of the expected size (173 pb) was detected in all analyzed samples. With the exception of hypocotyls, a reduction in the *PnCDPK1* mRNA level in the light-grown seedlings compared to that in dark-grown seedlings was observed.

Expression Analysis under Photoperiodic Conditions

To answer the question of whether the expression of this CDPK isoform changes in photoperiodic flower induction conditions, *PnCDPK1* mRNA accumulation was measured before and after a 16-h-long inductive night in entire seedlings and cotyledons. In contrast to the seedlings and cotyledons that were kept in light, the level of *PnCDPK1* mRNA increased after the dark treatment (Fig. 7).
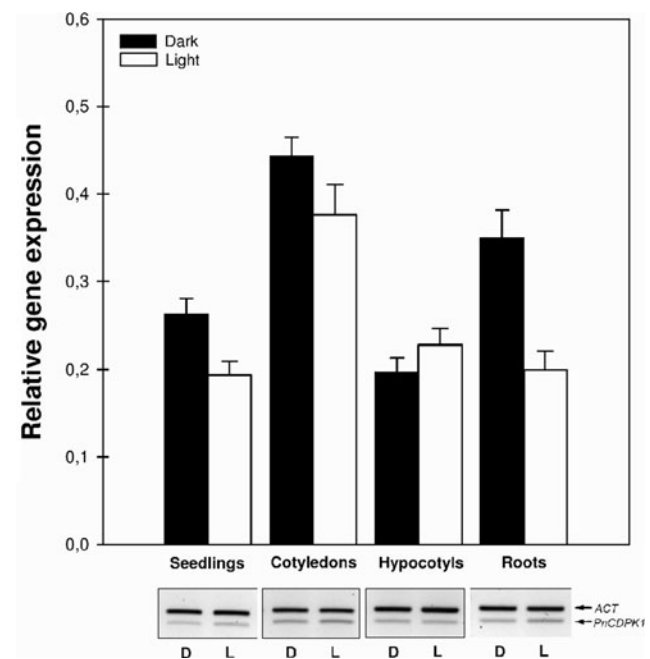

Fig. 5 RT-PCR analyses of *PnCDPK1* expression in different stages of seedling growth. Total RNA was extracted from dry seeds (Ds, 0 h), imbibed seeds (Ib, 24 h), germination stage (Gr, 48 h), and stages of seedling growth (D1-D4, 72-144 h) in darkness (**a**) and in light (**b**). The *actin4* transcript was amplified as a RT-PCR control. The histograms show relative intensity of mRNA levels. Results are the mean values of two independent experiments (each done in triplicate assays); *bars* indicate standard deviation

Fig. 6 RT-PCR analyses for *PnCDPK1* expression in vegetative organs and entire 5-day-old seedlings. Total RNA was extracted from plants grown in dark (D) and light (L). The *actin4* transcript was amplified as a RT-PCR control. The histograms show relative intensity of mRNA levels. Results are the mean values of two independent experiments (each done in triplicate assays); *bars* indicate standard deviation

Fig. 7 RT-PCR analyses for *PnCDPK1* expression in vegetative organs of 5-day-old seedlings. Total RNA was extracted from light-grown (L) and induced (16-h-long darkness) (IN) plants. The *actin4* transcript was amplified as a RT-PCR control. The histograms show relative intensity of mRNA levels. Results are the mean values of two independent experiments (each done in triplicate assays); *bars* indicate standard deviation
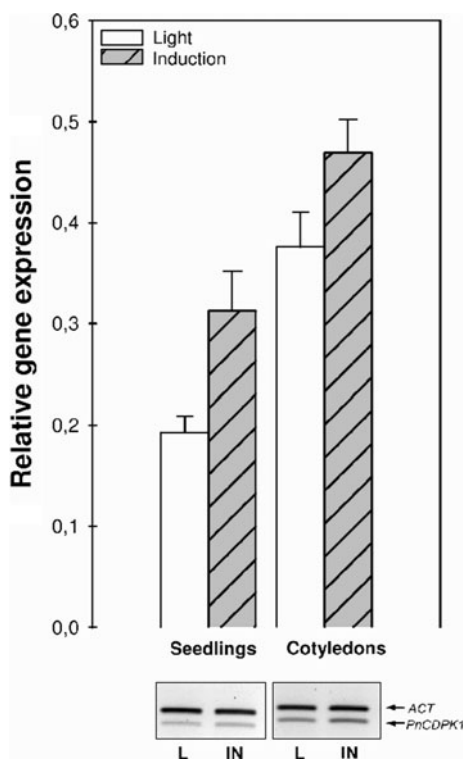
To determine the relationship between *PnCDPK1* expression and the length of the light/dark period, we followed changes in *PnCDPK1* mRNA during three different light/dark regimes: (1) continuous light, (2) 16 h of darkness and 8 h of light, and (3) 16 h of darkness disrupted with red or red/far-red light in the middle of the night and 8 h of light. Total RNA was isolated from cotyledons at 1-h intervals and the levels of mRNA were determined by RT-PCR analysis (Fig. 8). The normalized *PnCDPK1* to *actin4* transcript ratio remained at a basal level in light-grown cotyledons and changed during the night. In such conditions, moving plants to the 16-h-long night changed the amount of the transcript, reaching a maximal level at the second and eighth hour of the night, and then falling when plants were exposed to white light (Fig. 8b). In control plants, which were grown in continuous white light, the *PnCDPK1* mRNA concentration remained at the same low level (Fig. 8a).

In addition, an examination was undertaken to discover whether light also affects the mRNA level in cotyledons already exposed to the darkness and to assess the possible involvement of a specific light receptor—phytochrome.
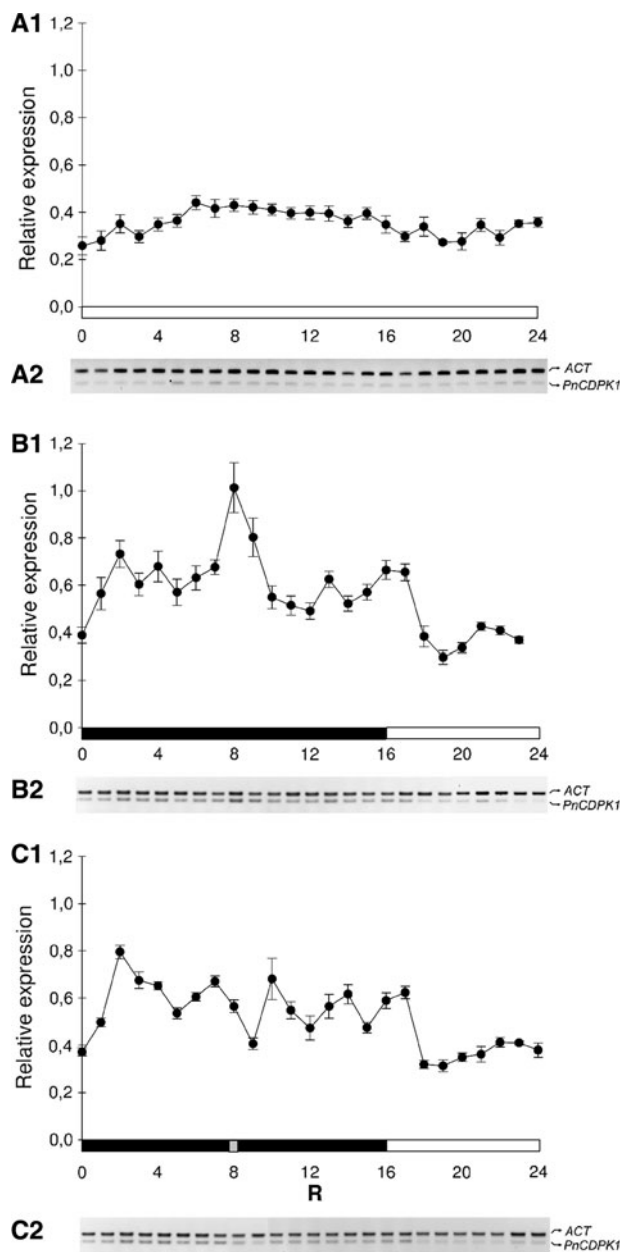

Fig. 8 Dependence of *PnCDPK1* expression on light/dark conditions in cotyledons of *Pharbitis nil*. RNA was isolated at the indicated times and analyzed by RT-PCR. *Section I* shows selective expression levels of *PnCDPK1* and *section II* depicts changes in the transcript levels on 1.7% agarose gel. *Actin4* (*ACT*) was used as a control of equal loading of RNA. **a** Effect of white light on *PnCDPK1* mRNA level. **b** Effect of darkness on *PnCDPK1* mRNA level. Five-day-old seedlings grown under continuous light were placed in darkness for 16 h and then moved to the light. **c** Effect of red light on *PnCDPK1* gene expression. Plants exposed to a 16-h-long night were irradiated for 5 min with red light in the middle of the night. Results are the mean values of two independent experiments (each done in triplicate assays); *bars* indicate standard deviation

Seedlings of *P. nil* exposed to a 16-h-long night were treated with red (R) light in the middle of the night. The interruption of the darkness by R significantly changed the

level of *PnCDPK1* mRNA (Fig. 8c). To determine whether FR (far-red light) could reverse the effect of R, the same set of experiments were performed and R and FR were added in the middle of the 16-h-long night. The effectiveness of R followed by FR was still very high (data not shown); therefore, FR was not able to reverse the effect caused by R.

Discussion

The physiological processes where Ca^{2+} signaling has been shown or inferred to play a role are numerous. As a consequence, for a couple of years attention has been focused on calcium effectors, of which CDPK seems to be the most ubiquitous. Calcium-dependent signaling operates at three levels. First, different stimuli can induce specific calcium signatures (Sathyanarayanan and Poovaiah 2004; Mazars and others 2009; McAinsh and Pittman 2009). Second, variations in the Ca^{2+} level will activate specific calcium decoders, for example, CDPK isoforms, which can be differentially expressed upon internal or external stimuli. Depending on the calcium signature, the extent and duration of CDPK enzyme activation will vary and have a direct effect on the phosphorylation of downstream targets (Hrabak and others 2003; Klimecka and Muszyńska 2007; Tuteja and Mahajan 2007). Third, CDPK participates in cross-talk between signaling pathways (Ludwig and others 2004, 2005).

In light of our interest in studying the role of calcium-dependent protein kinase in light signaling, we have cloned cDNA that represents a member of the CDPK family. *PnCDPK1* sequence analysis revealed high homology to other known plant CDPKs and showed the characteristic structural features of the CDPK family, including an N-terminal variable domain with potential myristoylation and palmitoylation sites, a conserved catalytic kinase domain, an autoinhibitory junction domain, and a CaM-like domain with four putative Ca^{2+}-binding EF hands. The motifs for myristoylation and palmitoylation suggest possible membrane-associated proteins. Such proteins are OsCDPK2 from *Avena sativa*, CpCPK from *Cucurbita pepo*, and LeCPK1 from *Lycopersicon esculentum* (Ellard-Ivey and others 1999; Martin and Busconi 2000; Rutsch-mann and others 2002). A rice isoform (OsCDPK14) lacking this motif has been localized in the cytoplasm (Zhang and others 2005). The *PnCDPK1* gene encodes protein with a predicted molecular mass of 57.9 kDa. Amino acid sequence alignment showed that the deduced PnCDPK1 protein had high homology to other known plant CDPK proteins, especially CsCDPK5 from *Cucumis sativus*, AtCDPK6 from *Arabidopsis thaliana*, and CrCDPK from *Capsella rubella*.

PnCDPK1 was expressed in *E. coli* as a GST fusion protein and was purified by affinity chromatography on glutathione-Sepharose. The recombinant peptide was cleaved from GST with a protease from the GST fusion protein and assayed by SDS-PAGE and Western blot as a single polypeptide, consistent with the predicted molecular mass of 56.5 kDa (Fig. 3). This weight is similar to the native mature PnCDPK, which migrated on SDS-PAGE with a mobility corresponding to 54 kDa (Jaworski and others 2003). The purified protein displays higher phosphorylation of histone III-S in the presence of Ca^{2+} than in the presence of EGTA, suggesting that PnCDPK1 is a calcium-dependent protein kinase. Activity was stimulated by the increasing concentration of Ca^{2+} with a $K_{0.5}$ of 1.57 μM and V_{max} of 0.5 μmol min^{-1} mg^{-1} protein and was decreased by a calmodulin antagonist, what indicates that this PnCDPK1 has a calmodulin-like domain (Li and Kamatsu 2000). These results confirm that PnCDPK1 is similar to some plant CDPKs with respect to enzymatic properties (Lee and others 1998; Anil and others 2000; Szczegielniak and others 2005; Zhang and others 2005).

In plants from which CDPK genes have been cloned, these kinases are encoded by a multigene family. Most CDPK isoforms are expressed constitutively and neither organ nor tissue specificity was observed. However, for some of them a unique, very restricted expression pattern and enzyme activity in different organs or tissues at different stages of growth and development were noted (Anil and others 2000; Hrabak 2000). For example in maize, a specific CDPK isoform is expressed only in the late stages of pollen development (Estruch and others 1994) and another isoform is expressed in very rapidly growing tissue (Abbasi and others 2004). In *Nicotiana tabaccum*, *NtCDPK4* is present in rapidly growing tissues such as root tip and lateral root primordia (Lee and others 2003). A rice calcium-dependent protein kinase called SPK is expressed uniquely in the endosperm of developing seeds (Asano and others 2002).

The *PnCDPK1* mRNA level significantly changed in seeds and during seedling growth. It was high in dry and imbibed seeds as well as during germination and dropped during the next stages of seedling growth. These observations imply that this gene encodes protein that is preferentially expressed in dry and imbibed seeds. It probably is involved in the mechanism of germination and high accumulation of its transcript is necessary for fast CDPK synthesis at that time. *PnCDPK1* may also be involved in mobilization of starch during imbibition and early stages of seedling growth. Earlier reports already suggested that some isoforms of CDPK are implicated in the regulation of starch synthesis and/or breakdown (Huber and others 1996; Iwata and others 1998; Harmon and others 2000).

Some CDPKs exhibit differences in expression and activity as a consequence of changes in light growth

conditions (Klimecka and Muszyńska 2007). The ubiquitous expression of *A. thaliana* isoforms *AtCDPK3*, *AtCDPK9*, and *AtCDPK12* has been shown to occur in roots, stems, leaves, and flowers; however, only expression of *AtCDPK9* was downregulated by light (Hong and others 1996). The differences in expression were also observed for maize *ZmCDPK7* and *ZmCDPK9* and the transcript level was much higher in etiolated leaves than in green ones, suggesting that some CDPK isoforms may be regulated by light (Saijo and others 1997). The *Cucumis sativus* *CsCDPK3* mRNA level was high in dark-grown organs, whereas exposure to light caused downregulation of transcript levels in hypocotyls and roots, unlike in cotyledon tissue where light had an upregulatory effect (Ullanat and Javabaskaran 2002). Ellard-Ivey and others (1999) reported on the organ-specific and light-regulated expression of the *Cucurbita pepo* *CpCDPK1* gene, with high levels of *CpCDPK1* mRNA apparent in etiolated hypocotyls and hooks but low levels in cotyledons. In rice, Frattini and others (1999) have demonstrated the differential expression of *OsCDPK11* and *OsCDPK2* in response to light exposure. Although mRNA of *OsCDPK11* was unaffected by light, *OsCDPK2* was closely associated with light perception. The level of *OsCDPK2* mRNA was low in green leaves exposed to light and increased dramatically during the 2 h when plants were shifted to darkness (Morello and others 2000). As shown in Fig. 6, the *PnCDPK1* transcript is present in variable levels in all analyzed organs. Whereas in hypocotyls light/dark-induced differences are barely seen, in roots and cotyledons the level of mRNA in light- and dark-grown seedlings is different and light was found to downregulate the *PnCDPK1* transcript level. Such distribution patterns of mRNA *PnCDPK1* may suggest a role for this isoform in different tissues in regulating cellular function.

In this article we also addressed the question of whether photoperiodic conditions influence expression of *PnCDPK1* in *P. nil* cotyledons. To get the answer the abundance of its mRNA was tested under short-day conditions (8 h light/16 h dark). In cotyledons, the *PnCDPK1* mRNA level showed oscillations during the night so that its concentration started to rise just after moving plants to the dark and reached its maximal level at the second and eighth hour of darkness. By contrast, the transcript of this gene was on a steady-state level in plants grown under continuous light. If *PnCDPK1* expression is associated with light/dark conditions, mRNA levels should change after modifying the light regime. It was noted that *PnCDPK1* expression decreased markedly after red-light irradiation but the reaction was not reversed by subsequent far-red-light treatment. It is known that far-red light reverses the effects induced by red light if it is used after red-light illumination and that the reaction is dependent on phytochromes (Thomas 2006). Such reactions were found not only for the flowering of short-day plants (Furuya and Schäfer 1996) but also for the phytochrome-dependent swelling and Ca^{2+} accumulation in wheat protoplasts (Bossen and others 1990) and oat (Sokolovsky and others 1996). Also, the R/FR reversibility of the phytochrome-dependent increase in the cGMP level was revealed in oat (Volotovsky and others 2003) and *Pharbitis nil* (Szmidt-Jaworska and others 2004; Szmidt-Jaworska and others 2008). Failure to reverse the effect of the R pulse by the FR pulse may be due to already R-induced irreversible responses. These results suggest that red light is the dominant factor in the modulation of the *PnCDPK1* mRNA level and that a red-light receptor is involved in the light/dark induction of the *PnCDPK1* gene. On the other hand, it indicates that *PnCDPK1* expression is also highly regulated at the level of mRNA turnover and suggests that the photoresponse of CDPK activity is regulated primarily at a transcriptional level rather than at the enzymatic level.

The flowering behavior of such treated plants was already measured (Szmidt-Jaworska and others 2004) by scoring the number of flower buds. The control plants that were not exposed to night break produced about six flower buds, whereas plants exposed to night break produced no flower buds. At the moment there is no direct evidence to support the hypothesis that the transcript level of *PnCDPK1* plays a direct role in flowering responses of *P. nil*. However, we can say that a light/dark-sensitive mechanism accelerates or reduces transcription of this gene. We are currently in the process of elucidating the biological role of PnCDPK1, with particular emphasis on Ca^{2+}/CDPK-dependent promotion of flowering.

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