J Plant Growth Regul (2009) 28:367–380
DOI 10.1007/s00344-009-9105-8

Molecular Cloning and Characterization of a Guanylyl Cyclase, *PnGC-1*, Involved in Light Signaling in *Pharbitis nil*

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Received: 20 April 2009 / Accepted: 12 May 2009 / Published online: 22 June 2009

Abstract Guanylyl cyclases (GCs) are enzymes involved in the biosynthesis of cyclic guanosine monophosphate (cGMP). Here we report the cloning and characterization of a new guanylyl cyclase, designated *PnGC-1*, from *Pharbitis nil*. This gene encodes a protein of 286 amino acids, with a calculated molecular mass of 32 kDa. The predicted amino acid sequence contains all typical features and shows high identity with known plant GCs. The GST-PnGC-1 was catalytically active in *E. coli* cells and the purified, recombinant PnGC-1 was able to convert GTP to cGMP in the presence of Mn^{2+}. Moreover, the enzyme activity was strongly inhibited by a specific sGC inhibitor, NS2028, whereas in the case of nitric oxide, an animal sGC stimulator, no positive effect was observed. Besides the identification of the PnGC-1 as a guanylyl cyclase, it was shown that a transcript of *PnGC-1* was present in every tested organ of the light- or dark-grown plants; however, the highest expression level was found in dark-treated plants. The *PnGC-1* mRNA level in the cotyledons exhibited diurnal oscillations under short-day conditions (8/16-h photoperiod). Meanwhile, monitoring of transcript levels in cotyledons exposed to a special photoperiodic regime (24 h light of low intensity then 24 h long night with or without far-red light before the night) revealed that a stabile phytochrome is involved in this process. These data unequivocally identify the product of the *PnGC-1* gene as a guanylyl cyclase and emphasize the potential that soluble GC can be an element of light signal transduction.

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Keywords Guanylyl cyclase · cGMP · Light signaling · *Pharbitis nil*

Introduction

The intracellular messenger 3′,5′-cyclic guanosine monophosphate (cGMP) plays an important role both in animal and plant cells. The enzymes responsible for cGMP synthesis are guanylyl cyclases that are a set of cytosolic and membrane proteins (Murad 1994; Lucas and others 2002). Guanylyl cyclase catalyzes the conversion of GTP to cGMP in response to various extra- and intracellular stimuli, thereby providing an important second messenger for the regulation of protein kinases, phosphodiesterases, and ion channels (Newton and others 1999).

Although cGMP is supposed to be a putative intermediary involved in signal transduction, its specific position in signal transduction and the mechanism of its action in plant cells remains unknown. Since the discovery of cGMP in plant cells, it has become clear that an enzyme responsible for its synthesis must also be present (Newton and Smith 2004). However, in plants, the knowledge about the enzymes involved in cGMP metabolism is insufficient. Only a few studies on plant GC have been conducted. For example, in intact chloroplasts from *Phaseolus vulgaris* the specific GC activity was analyzed using the conversion of labeled [^{32}P]-GTP into [^{32}P]-cGMP (Newton and others 1984). Volotovsky and others (2003) revealed the activity of guanylyl cyclase in plasma membranes in *Avena sativa* cells. In *Pharbitis nil* an enzyme with GC activity was localized in cytoplasm and its activity changed in various light conditions (Szmidt-Jaworska and others 2008b, 2009).

Recently, important advances using molecular and genetic approaches have been made in plant GC research. A small gene family of GCs encoding different isoforms of the enzyme has been identified. There are three reports of cloning of putative cyclase from higher plants (Ludidi and Gehring 2003; Kwezi and others 2007; Yuan and others 2008). A motif search of the *Arabidopsis* genome based on conserved and functionally assigned amino acids in the catalytic domain of GCs returned one candidate (*AtGC-1*) that contained the glycine-rich motif typical for GCs. When *AtGC-1* was expressed in *E. coli*, the cell extract contained 2.5 times more cGMP than the control one. The amino acid sequence of protein indicates that it is soluble-type cyclase, unaffected by NO (Ludidi and Gehring 2003). Using the same strategy, Kwezi and others (2007) cloned and expressed a recombinant protein (AtBRI1-GC). This brassinosteroid receptor harbors the putative catalytic domain and can convert GTP to cGMP in vitro. It seems that AtBRI1-GC may belong to a novel class of cyclases that contains a GC and cytosolic kinase domain. Recently, a guanylyl cyclase-like gene from *Zea mays* has been cloned and characterized (Yuan and others 2008). Multiple copies of this gene have been mapped and it was shown that gene expression is associated with *Fusarium graminearum* resistance.

Cyclic GMP as a second messenger plays an essential role in many important cellular processes. In animal and plant cells, transient changes in cGMP level in the cytosol have been observed during growth, development, and under stress conditions (Cousson 2001; Maathuis 2006). Significant changes in cGMP levels have been reported in response to light treatment, in phytochrome-dependent gene expression required for chloroplast development and anthocyanin biosynthesis (Bowler and others 1994), gibberellic acid-dependent α-amylase synthesis (Penson and others 1996), and photoperiodic flower induction (Szmidt-Jaworska and others 2004, 2008a).

It is known that the endogenous level of cGMP can be modulated by either controlling the activity of GCs, of cyclic nucleotide phosphodiesterase, or of both. Moreover, cGMP formation may be regulated also indirectly at the transcriptional and post-transcriptional levels (Jiang and Stojilkovic 2006). The mechanisms by which plant GC can be regulated are important questions which touch upon molecular cloning of the GC gene as well as analysis of the expression of GC coding gene(s), especially since expression of the plant GC was not tested by any of the constructs reported in the earlier works. Studies focusing on such a problem and identification of the GC gene in *P. nil* are the subject of this report. Moreover, the biochemical characterization of the recombinant protein is described and confirms that the analyzed enzyme belongs to a class of plant GCs.

Materials and Methods

Plant Material and Light Treatments

The investigations were conducted on 5-day-old seedlings of morning glory (*Pharbitis nil* L. Chois), the Japanese variety Violet (Marutane Seed Co., Kyoto, Japan). Seeds were soaked in concentrated sulfuric acid for 50 min and then washed in running tap water for 3 h. They were left in distilled water at 25°C overnight. The swollen seeds were planted on a mixture of vermiculite and sand (2:1 w/w) and grown at 25°C in various light conditions.

For expression analysis in vegetative organs, plants were grown in darkness or under continuous light (130 μmol m^{-2} s^{-1}, white light fluorescent tubes, Polam) for 5 days. The cotyledons, hypocotyls, and roots were picked, immediately frozen in liquid nitrogen, and stored at −80°C.

For expression analysis in various light/dark regimes in variant I, plants were growing in continuous light for 5 days. A portion of 5-day-old plants was left in such conditions. The rest of the plants were exposed to a 16-h-long darkness, but for some of them the long night was disrupted by a 5-min-long pulse of red light (R, 1.5 μmol m^{-2} s^{-1}, fluorescent tubes TLD 15R/18 W, Philips) at the 8th hour or R 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-bandwidth = 9 nm). In variant II, plants were grown for 4 days in darkness and then cultivated for 24 h in low-intensity white light (40 μmol m^{-2} s^{-1}, cool white fluorescent tubes, Philips). A portion of these plants was left in such conditions. The rest were moved to darkness for 24 h (long inductive night). Some of the seedlings were irradiated with a 10-min-long pulse of FR light at the end of the 24-h white-light period or FR light followed by R light.

Afterward, for both variants, cotyledons were harvested every hour, immediately frozen in liquid nitrogen, and stored at −80°C.

Molecular Cloning of *PnGC-1*

Molecular cloning of *PnGC-1* consisted of three steps: *PnGC-1* fragment production by PCR, 3′ RACE (rapid amplification of cDNA ends), and full-length *PnGC-1* cDNA amplification.

PnGC-1 fragment production by PCR. Total RNA was isolated from green cotyledons of *P. nil* seedlings and then the mRNA was purified using an Oligotex mRNA Mini Kit (Qiagen). The first-strand cDNA for RT-PCR was synthesized with RevertAid M-MuLV RT (Fermentas) following the manufacturer's instructions. Degenerate oligonucleotide primers 5′-TGG GA(C/T) TGC GG(C/T) CT(C/T) GCT-3′ and 5′-ACA TAT (C/T)AC (A/G)AC ATA (A/G)TG (A/C/T)CC-3′, corresponding to the conserved

amino acid sequences WDCGLA and GHYVVIC, respectively, were designed for two conserved regions located in the regulatory domain of previously cloned putative plant GCs. The database accession numbers are as follows: *Arabidopsis thaliana* (AY118140, AAM51559), *Hordeum vulgare* (ABD18447), *Zea mays* (DQ372067), and *Triticum aestivum* (DQ372070). PCR was performed with 50 ng of cDNA and 100 ng of degenerate primers. PCR parameters consisted of 50 s at 95°C for denaturing, 50 s at 53°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 1.3% (w/v) agarose gel, eluted, and sequenced.

3′ RACE of PnGC-1. 3′-RACE-ready cDNA synthesis was performed with the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech). The mRNA from total green cotyledons' RNA was purified and then reverse-transcribed with the 3′-RACE CDS Primer A [5′-AAG CAG TGG TAT CAA CGC AGA GTA $C(T)_{30}VN$-3′, N = A, C, G, or T and V = A, G, or C]. 3′ RACE was performed with the Advantage 2 Polymerase Mix (Clontech). The PCR reaction was performed by using a GSP (gene-specific primer) 1 (5′-TTG AGG AAT TGG CAC AGT CCT GCT CT-3′) and Universal Primer A Mix (UPM; Long: 5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3′; Short: 5′-CTA ATA CGA CTC ACT ATA GGG C-3′) under the following conditions: 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min for 35 cycles. The PCR product was purified and cloned into a pTZ57R vector (Fermentas) for sequencing.

Full-length PnGC-1 cDNA amplification. An alignment of sequences obtained from degenerate GSP1 primers and a search of the NCBI EST database was used to predict a full-length *PnGC-1* cDNA. The sequence of 5′ cDNA end was found in the dbEST of *Ipomoea nil* (synonym *Pharbitis nil*) 8-day-old seedling shoots (gi74391850). Thus, two PCR primers *PnGC-1* (5′-GGT CCC AGT TTT GCA ACT TT -3′) and *PnGC-2* (5′-CAA AAT CAG TCA ACC CAG CA -3′) were designed for the amplification of full-length cDNA. The mRNA from green cotyledons was purified and reverse transcribed with the SuperScript III Reverse Transcriptase (Invitrogen) to obtain a PCR template. PCR was performed in a total volume of 50 µl reaction solution containing 5 µl 10 × buffer A (minus Mg^{2+}), 2.5 µl 50 mM $MgCl_2$, 1 µl 5 mmol/µl each of dNTPs, 1µl 10 µmol/µl *PnGC-1*, 1µl 10 µmol/µl *PnGC-2*, 1% (v/v) DMSO, 2 µl cDNA, and 1 unit Yellow PfuPlus DNA polymerase (Eurx) using the following protocol: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 1 min, with a final extension of 7 min at 72°C. The nucleotide sequence of *PnGC-1* reported here is available in GenBank under the accession number DQ672602.

RNA Extraction and Reverse Transcription

Samples were quick-frozen in liquid nitrogen and ground to powder by mortar and pestle. Total RNA was isolated from various organs (cotyledons, roots, hypocotyls) with the GeneMATRIX Universal RNA Purification Kit (Eurx) according to the manufacturer's instructions. Prior to reverse transcription, RNA samples were treated with RNase-free DNaseI (Fermentas) and then reverse transcribed with the MMLV RT enzyme (Epicentre) in 20 µl at 37°C for 1 h.

Semiquantitative PCR

RT-PCR analysis was performed to analyze the expression of the *PnGC-1* gene. PCR was conducted at the linearity phase of the exponential reaction for each gene. The gene-specific primer pairs were as follows: for *PnGC-1*, forward primer: 5′-TGA ACG TTC GTC TCA ACT GC -3′ and reverse primer: 5′-ACC GAC CAA AGC AAA CTC A-3′; and for *actin4* gene (*ACT*), forward primer: 5′-GAA TTC GAT ATC CGA AAA GAC TTG TAT GG-3′ and reverse primer: 5′-GAA TTC CAT ACT CTG CCT TGG CAA TC-3′. Amplifications were performed in a thermocycler programmed for 30 cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C. The *actin4* expression level was used as a quantitative control.

Expression and Purification of GST-PnGC-1 Fusion Protein

The full-length *PnGC-1* cDNA was amplified by PCR. The PCR product was verified by DNA sequencing and introduced into the plasmid pTZ57R/T (Fermentas). For expression of the *GST-PnGC-1* in bacteria, the PnGC-1 ORF (open reading frame) was cut from pTZ57R/T and inserted into the pGEX-6P2 (GE Healthcare) expression vector at *Not*1 and *Sal*1 restriction sites. The *E. coli* BL21 strain, transformed with the resulting plasmid, was used to produce the GST-tagged protein. The expression of fusion protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and incubation 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) and 150 mM NaCl, the GST-PnGC-1 complex was either eluted with 10 mM glutathione in 50 mM Tris–HCl (pH 8.0) or PnGC-1

was released from the fusion protein by proteolytic cleavage of the protein with PreScission protease (GE Healthcare) following the manufacturer's instructions. For a control expression, the pGEX-6P2 vector was used or GST alone was purified as described above.

Electrophoresis and Western Blotting Analysis

The homogeneity and purity of eluted protein fractions were analyzed by 12% (v/v) SDS–PAGE and gels were stained with Coomassie Blue. For Western blotting analysis, proteins resolved by SDS–PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Hybond-P, GE Healthcare) by 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 polyclonal anti-GST antibodies (1:10,000) (GE Healthcare). The Western blots were visualized with horseradish peroxidase-conjugated secondary anti-goat IgG antibodies (1:30,000) (Sigma-Aldrich) and the blots were detected using a chemiluminescence kit (ECL plus) following the manufacturer's instructions (GE Healthcare).

Determination of cGMP and cAMP Concentration

The concentration of cGMP or cAMP was measured with a specific [^{3}H]-radioimmunoassay kit (Amersham Pharmacia Biochem). Analyzed samples were added to scintillation vials containing scintillation cocktail and counted in a liquid scintillation counter (Wallac 1407). Data are expressed as pmol cGMP g^{-1} fresh tissue or pmol cyclic nucleotide (cGMP or cAMP) min^{-1} mg^{-1} protein. All measurements were performed in duplicates and repeated three times.

Guanylyl Cyclase Activity

Guanylyl cyclase activity was determined as described previously by Kamisaki and others (1986) and Szmidt-Jaworska and others (2008b) by estimating the rate of cGMP formation. The analyzed fractions (containing PnGC-1) were assayed for guanylate cyclase activity in a final volume of 100 µl at 30°C for 10 min with gentle shaking. The incubation buffer contained 50 mM Tris–HCl (pH 7.5), 4 mM $MnCl_2$, 1 mM GTP, 1 mM DTT, and 0.5 mM isobutylmethylxanthine. The reaction was terminated by the addition of 96% (v/v) ethanol. The samples were shaken for 5 min, incubated on ice for 10 min, and then centrifuged at 13,000 *g* for 10 min. Supernatants were collected and lyophilized. The concentration of the formed cyclic GMP was determined by radioimmunoassay as described above. The enzyme activity was defined as the amount of cGMP produced by 1 mg of protein per minute.

The effect of temperature on the activity was evaluated with the standard activity assay at pH 7.5 and different temperatures in the 20–37°C range.

Kinetic parameters (K_m and V_{max}) were determined from rate measurements (1–20 min) using GTP concentrations from 0.1 to 3.0 mM. The data were processed following the classical Lineweaver-Burk-type plots. All the measurements were performed in three replicates. Moreover, ATP (0.1–3 mM) was used to analyze the specificity of the identified enzyme.

The inhibitor's and activators' effects were determined by comparing the enzymatic reaction rates obtained when 1 mM SNP and 1 mM NS 2028 were added in the presence or absence of divalent cations (Mn^{2+} or Mg^{2+}).

Results

Molecular Cloning of *PnGC-1*

The *PnGC-1* gene was isolated using degenerated PCR primers that were derived from conserved sequences of previously cloned GCs of other plant species. RT-PCR amplification was performed on mRNA from cotyledons. A DNA fragment of the expected size was recovered from the agarose gel, TA-cloned into the plasmid vector, and sequenced. The complete coding sequence of the GC gene, designated as *PnGC-1*, was obtained using RACE-PCR. The full-length *PnGC-1* cDNA contains start and stop codons and consists of a 861-bp ORF which encodes a 286-amino-acid peptide (Fig. 1) with a theoretical molecular mass of 32.3 kDa and a pI of 5.7. The full-length sequence of *PnGC-1* has been placed into GenBank under accession number DQ 672602.

A search of the protein database using BLASTP revealed that the deduced amino acid sequence of *PnGC-1* exhibited homology to other GCs, including *S. lycopersicum* (61% identity), *A. thaliana* AtGC-1 (56% identity), *T. aestivum* (52% identity), and *O. sativa* (57% identity) (Fig. 2). *PnGC-1* contains all typical features of other plant GC (Fig. 3). It contains a variable catalytic motif in its N-terminal region and highly conserved C-terminal region.

The catalytic domain exhibited high conservation of glycines (G, positions 32 and 39) and arginine (R, position 21), all responsible for interaction with the phosphate group in GTP via a water molecule. Moreover, it possesses cysteine (C, position 13) responsible for recognition of the guanine. The glutamic acid and/or aspartic acids (E and D, position 25 and/or 51) are believed to bind essential metal ions (Mn^{2+} or Mg^{2+}). The asparagine (N, position 33) and lysine (K, position 18) are likely responsible for transition-

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  1 atgcatcagatcagttggtcacacgtgtggcctttctgtattctc
    M  H  Q  I  S  W  S  H  V  W  P  F  C  I  L
 46 ctaagcaagttcatgagatcagacgacgaagatgctcagcaacca
    L  S  K  F  M  R  S  D  D  E  D  A  Q  Q  P
 91 gcggggaatgagtttgctttggtcggttcgttccccttaaacag
    A  G  N  E  F  A  L  V  G  S  F  P  F  K  Q
136 tcactggatagagtggaaagcaaagccgttctgtcctccaaatca
    S  L  D  R  V  E  S  K  A  V  L  S  S  K  S
181 ttctcggttgaagtgccacatgttaagcaactatatacatgggat
    F  S  V  E  V  P  H  V  K  Q  L  Y  T  W  D
226 tgtggtcttgcttgtgttgtaatggttttgagaactctcgggatt
    C  G  L  A  C  V  V  M  V  L  R  T  L  G  I
271 tacaatagtaatattgaggaattggcacagtcctgctctaccaca
    Y  N  S  N  I  E  E  L  A  Q  S  C  S  T  T
316 agcatttggacagtagacctagcatacatgttgcggaaattttca
    S  I  W  T  V  D  L  A  Y  M  L  R  K  F  S
361 gttaacttttcctactttacgatcacgataggagccaatccaaac
    V  N  F  S  Y  F  T  I  T  I  G  A  N  P  N
406 ttttgcgtcgagacattttacaaggagcagttgcctagtgatctt
    F  C  V  E  T  F  Y  K  E  Q  L  P  S  D  L
451 gcccgagtgaatacgctatttcagaaggcacgagaggcgggaatt
    A  R  V  N  T  L  F  Q  K  A  R  E  A  G  I
496 aacattgagtgcaggtcaattagcggagaagaaatgtgtatgtta
    N  I  E  C  R  S  I  S  G  E  E  M  C  M  L
541 atattgtccgggaattgcattgcaatagcgttagttgatcatcac
    I  L  S  G  N  C  I  A  I  A  L  V  D  H  H
586 aagctaagtcattattggtcggaggatggttgtattcaaaacttc
    K  L  S  H  Y  W  S  E  D  G  C  I  Q  N  F
631 taccccaagagcccgggttatactggtcactatgttgtcatctgt
    Y  P  K  S  P  G  Y  T  G  H  Y  V  V  I  C
676 ggctacgatgcagccatggatgaattcgagatacgagatccagca
    G  Y  D  A  A  M  D  E  F  E  I  R  D  P  A
721 agctcgaggaaacatgaaaaggttacttcaaggcggctagcagag
    S  S  R  K  H  E  K  V  T  S  R  R  L  A  E
766 gcgcgcaaatcctttggcaccgacgaggatcttcttctgatccat
    A  R  K  S  F  G  T  D  E  D  L  L  L  I  H
811 ttggagaagggagtcgatctaaatcgcccgttgtcatctctatct
    L  E  K  G  V  D  L  N  R  P  L  S  S  L  S
856 tcatag 861
    S  *
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Fig. 1 Nucleotide and deduced amino acid sequences of guanylyl cyclase from *Pharbitis nil*. Numbering starts at the putative translation initiation codon for nucleotide (left margin). Stop codon is marked by an asterisk

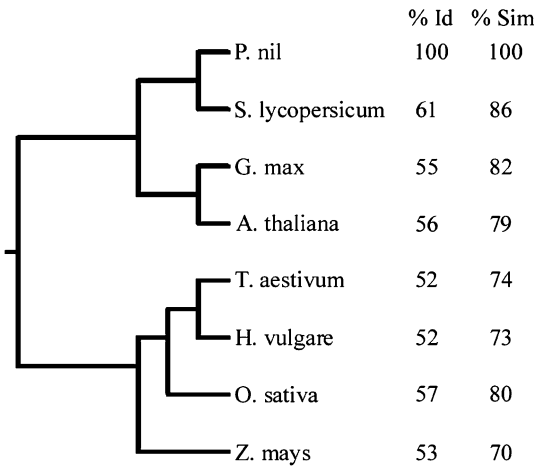

Fig. 2 A rooted phylogenetic tree based on the nucleotide sequences illustrates the relationship between the cloned plant GC. The percentage of identical (% Id) and similar (% Sim) residues is presented. The phylogenetic tree was constructed using ClustalW in GenBank. The EMBL database accession numbers are as follows: *P. nil* (ABG67691), *A. thaliana* (AAM51559), *Z. mays* (DQ372067), *S. lycopersicum* (ABK15531), *G. max* (ABK15530), *H. vulgare* (ABD18447), *T. aestivum* (ABD18449), and *O. sativa* (ABD18448)

state stabilization. It is possible to find putative myristoylation sites in both N and C terminals of the predicted GC protein.

To assess the properties of *PnGC-1*, the full-length gene was expressed in *E. coli* BL21 as a glutathione S-transferase (GST)-fusion protein. The *PnGC-1* was cloned into pGEX-6P2 and the recombinant *PnGC-1* was expressed in *E. coli* using the GST-fusion system. When the protein expression was induced by addition of IPTG, GST-PnGC-1 emerged as a clear main band with a molecular mass of 56 kDa, which was not observed in control fractions (empty pGEX vector or pGEX-PnGC-1 without IPTG application) (Fig. 4a). When a sample of this purified protein was digested with PreScission protease, one main 32-kDa band appeared, corresponding to PnGC-1 (Fig. 4a). Western blot analysis showed that an anti-GST antibody reacted with the 56-kDa peptide as well as with GST (26 kDa) (Fig. 4b), demonstrating that the induced protein was GST-PnGC-1.

The possibility that the expression of pGEX-PnGC-1 forms an active enzyme was analyzed by in vivo and in vitro analyses of enzyme activity. When an equal amount of cells was extracted and assayed for cGMP, a 2.5-fold increase was observed in extracts from transformed, induced bacteria compared with extracts from a noninduced cell and the control (empty pGEX vector) (Fig. 5).

Enzymatic analysis performed with the supernatant also revealed a positive reaction. The supernatant after IPTG treatment had a GC activity of about 0.18 pmol min^{-1} mg^{-1} protein. Cyclic GMP, as a product of PnGC-1 activity, was undetectable when IPTG was not applied (Fig. 6a).

To analyze the kinetic properties of purified PnGC-1, cGMP formation was determined in the presence of increasing GTP concentration (Fig. 6b). A Lineweaver-Burk plot of the data revealed a K_m value of 0.87 mM GTP and V_{max} of 78.1 pmol min^{-1} mg^{-1} protein, suggesting proper folding of the catalytic domain. Moreover, the possibility that a recombinant protein might act as both an adenylyl and guanylyl cyclase was examined directly. However, in the case of PnGC-1, no associated adenylyl cyclase activity toward ATP was detected (Fig. 6b).

The effect of temperature on PnGC-1 activity was determined within a range of 20–37°C, and the optimum temperature was found to be approximately 30°C (Fig. 6c).

As was shown in Fig. 6d, PnGC-1 essentially requires divalent metal ions for its enzymatic activity and Mn^{2+} is

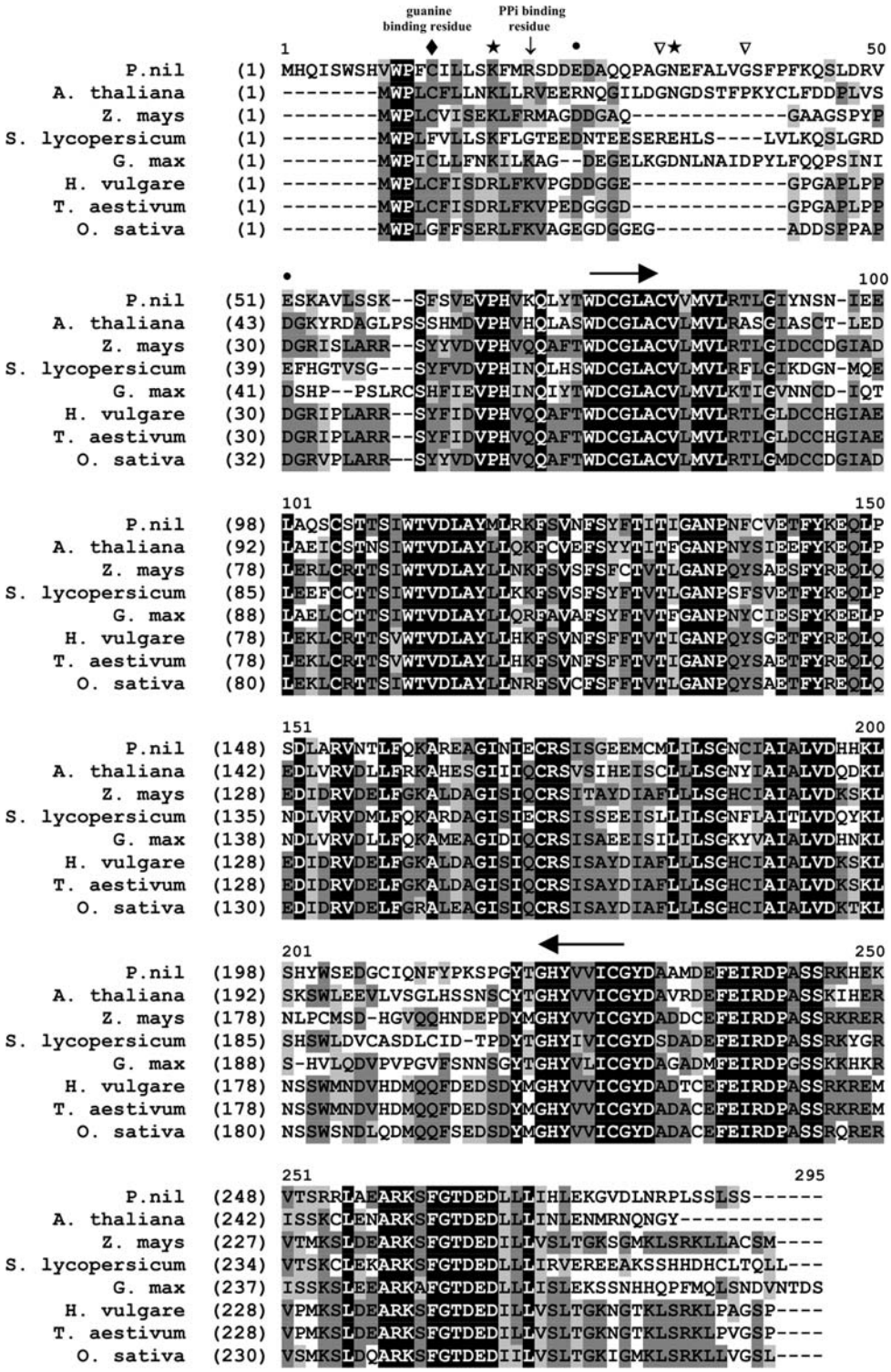

Fig. 3 A comparison of the amino acid sequences of putative guanylyl cyclases from various plants. Black and shaded backgrounds indicate regions of identity and consensus, respectively, between eight GC from various plants. The alignment was generated using the VECTOR program. Dashes represent sequence gaps to allow for maximum alignment. In the catalytic domains the glycine-rich motifs are indicated as ∇; the PP_i-binding residue is indicated as ↓; the guanine-binding residue is indicated as ◆; the metal ion-binding residues are indicated as ●; the amino acids responsible for transition state stabilization are indicated as ★. The arrows indicate the amino acid residues corresponding to the degenerate oligonucleotides. The accession numbers of the GC sequences are indicated in Fig. 2

the most favorable. Apart from metal ions, the influence of SNP (as a NO donor and animal sGC stimulator) and NS 2028 (as a GC inhibitor) on the activity of recombinant PnGC-1 was tested. Application of SNP did not alter the enzyme activity, whereas NS 2028 caused a significant (4.5-fold) reduction in the production of cGMP.

Expression Analysis in Vegetative Organs

To investigate changes in the *PnGC-1* expression level, plant organs' specificity was analyzed first. A semiquantitative RT-PCR technique was used to monitor the tissue-specific transcript levels of *PnGC-1*. Total RNA was

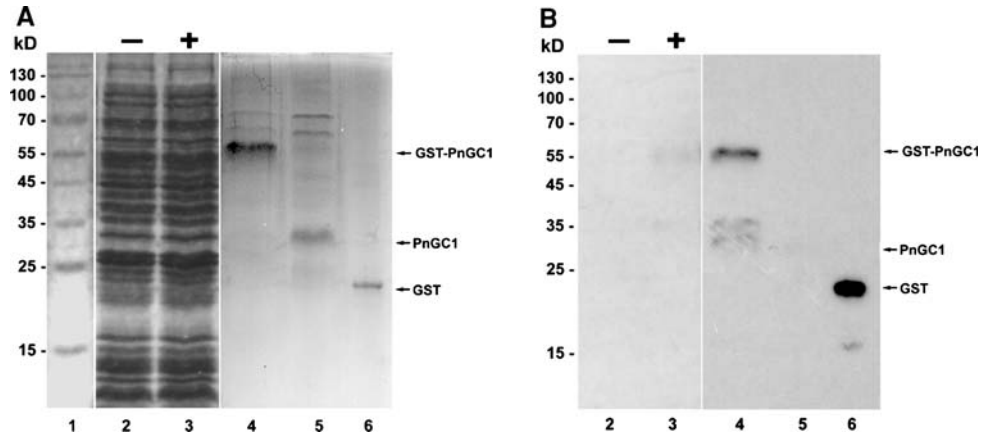

Fig. 4 Purification and characterization of recombinant *PnGC-1*. The full-length *PnGC-1* reading frame was expressed in *E. coli* as a GST-fusion protein. Purification was monitored by SDS-PAGE followed by Coomassie Brillant Blue staining (**a**) or Western blot analysis with anti-GST antibody (**b**). **a** SDS-PAGE. Lane 1 is protein molecular mass marker (kDa). Lane 2 is the supernatant from noninduced *E. coli* cells. Lane 3 is the supernatant from IPTG-induced *E. coli* cells. Lane 4 is the recombinant GST-PnGC-1 purified using a glutathione affinity chromatography. Lane 5 is a PnGC-1 after digestion with PreScission protease. Lane 6 is GST protein. **b** Western blot. Lanes 2 and 3 are supernatants from noninduced (−) and IPTG-induced (+) culture of *E. coli* transformed with pGEX-PnGC-1. Lane 4 is a purified GST-PnGC-1. Lane 5 is a recombinant PnGC-1. Lane 6 is a GST protein

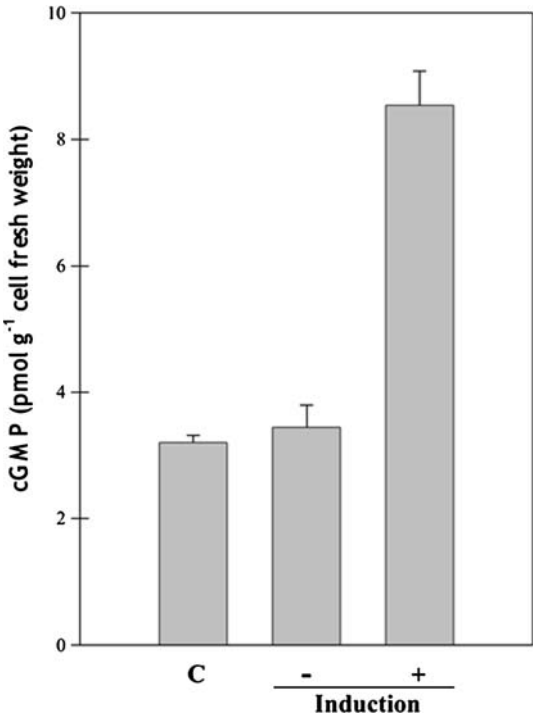

Fig. 5 Content of endogenous cGMP in *E.coli* cells determined with [^{3}H] cGMP radioimmunoassay system. The control (C) is the concentration of cGMP in the whole-cell *E. coli* extract containing empty pGEX 6P2 after IPTG addition. + and − show the value of cGMP in IPTG-induced or noninduced extracts of *E. coli* containing the construct (pGEX-PnGC-1). All assays were performed in duplicate and each experiment was repeated three times

isolated from cotyledons, stems, and roots of *P. nil* seedlings grown either in continuous white light or treated with darkness. As shown in Fig. 7, *PnGC-1* mRNA was detected in all organs, but the highest expression pattern was observed when plants were grown in darkness. In these plants the expression level was higher in hypocotyls and roots than in cotyledons. The transcript levels of *PnGC-1* were found to be downregulated by light. The most pronounced decrease (2- and 2.5-fold) was found in cotyledons and hypocotyls, respectively. The amplification of the *actin4* gene in the corresponding samples was used to determine the availability of relatively intact RNA samples and to normalize the quantification of the target (*PnGC-1*) transcript. The level of *actin4* mRNA did not show any statistically significant variation, thereby validating the results obtained.

Expression Analysis Under Photoperiodic Conditions

To determine the relationship between *PnGC-1* expression and the light/dark treatment, we followed changes in *PnGC-1* expression during two different light/dark regimes.

To analyze the expression pattern of *PnGC-1* during short-day conditions (8/16 h photoperiod), we compared mRNA populations extracted from cotyledons that either had or had not been exposed to a 16-h-long night. Total RNA was isolated from cotyledons at 1-h intervals, and the levels of mRNA were determined by RT-PCR analysis (Fig. 8a). The normalized *PnGC-1* to *actin4* transcript ratio changed during the long night. The transcript level reached three peaks at the 3rd, 7, and 11th hour after moving plants to the darkness and then fell during the subsequent dark period. In contrast, in the seedlings that were kept in light, the level of GC mRNA remained at a basal level.

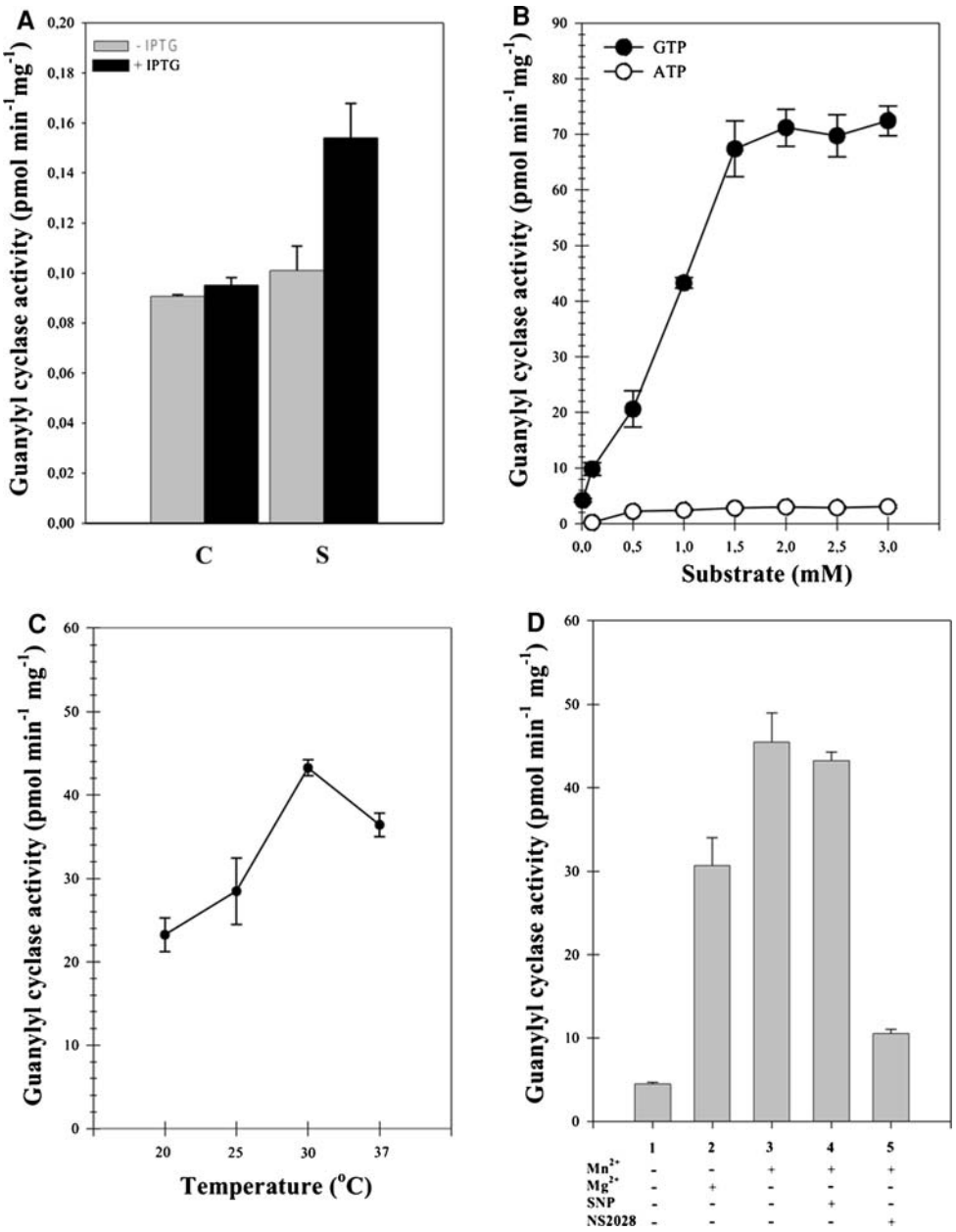

Fig. 6 Biochemical properties of recombinant *PnGC-1*. Values are the means of three replicates. Bars represent SE. **a** Guanylyl cyclase activity toward Mn-GTP. C is the enzyme activity in the supernatant from noninduced and IPTG-induced *E. coli* cells containing empty pGEX 6P2. S is the enzyme activity in the supernatant from noninduced and IPTG-induced *E. coli* cells transformed with pGEX-PnGC-1. **b** Determination of recombinant *PnGC-1* cyclase activity in response to increasing concentration of substrate (GTP or ATP, in the range of 0.1-3 mM). **c** Effect of temperature on PnGC-1 activity. **d** Comparison of enzyme activity after application of various substances (divalent cations: Mn^{2+} and Mg^{2+}; SNP as a NO donor and potent soluble GC stimulator; NS2028 as a potent soluble GC inhibitor)

In addition, an examination was undertaken to discover whether light also affects the mRNA level in cotyledons already exposed to the darkness. Seedlings of *P. nil* exposed to a 16-h-long night were treated with R or FR in the middle of the night. The interruption of the darkness by R reduced the level of *PnGC-1* mRNA rapidly, and then a renewed increase was observed (Fig. 8b). FR light alone did not reduce the *PnGC-1* mRNA level (data not shown). To determine whether FR 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, respectively. 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.

Experiments conducted in traditional conditions for cultivation of *P. nil* seedlings did not distinguish between the phytochrome types involved. This was the reason why special photoperiodic conditions for cultivation of seedlings were used (72-h-long darkness, 24-h-long white light of low intensity, and 24-h-long night with or without FR before the night). In such conditions, moving plants to the darkness changed the amount of the transcript, reaching a maximal level at 3, 7, and 12 h of the night and decreasing during the subsequent dark period (Fig. 9). When FR treatment preceded the night, the oscillations did not occur. However, when FR and subsequent R were given prior to the 24-h-long night, the *PnGC-1* expression pattern was restored (data not shown).

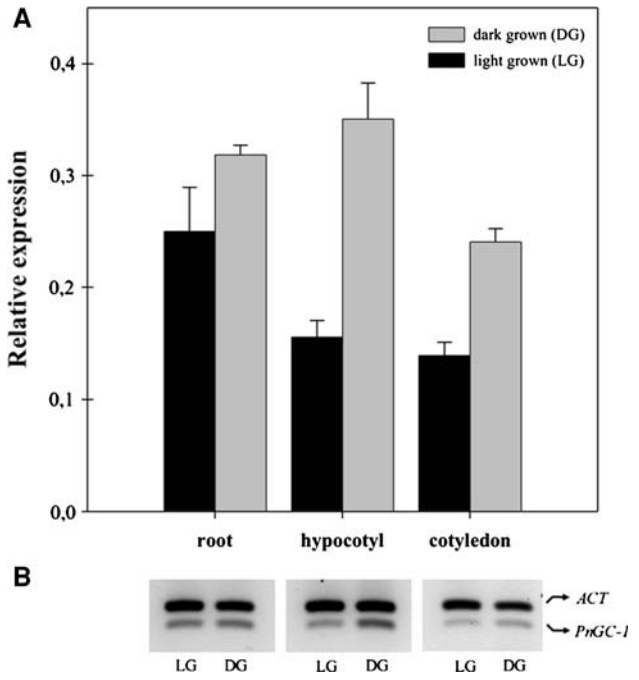

Fig. 7 Expression analysis of *PnGC-1* in light- or dark-grown *P. nil* cotyledons, hypocotyls, and roots. The transcript level was determined by RT-PCR using specific primers and total RNA as the template. The data represent three independent experiments showing similar results. **a** Calculated values of *PnGC-1* mRNA levels in *P. nil* organs. **b** Changes in the transcript levels of *PnGC-1* on 1.7% (w/v) agarose gel. As an internal control the expression of *actin4* gene (*ACT*) in roots, hypocotyls and cotyledons was assessed

Discussion

The cellular processes that are regulated by cGMP are central to many aspects of growth and development. Despite the widely recognized importance of cGMP, little is known about the mechanism of its synthesis, and the limited data concerning GC in plants were treated with skepticism because of the lack of the GC gene described. Recently, three cDNA encoding putative genes were isolated and opened the way for further analyses.

In light of our interest in studying the role of cGMP and the intracellular signaling processes in photoperiodic flower induction of *Pharbitis nil*, we cloned cDNA that represents a putative member of the GC gene family in plants. Using RACE-PCR, we isolated and characterized *PnGC-1*, which encoded a 286-amino-acid peptide with a theoretical molecular mass of 32.3 kDa. This predicted molecular mass is smaller than that previously described for the native *Pharbitis nil*, which migrated on SDS-PAGE with a mobility corresponding to 40 kDa (Szmidt-Jaworska and others 2008b).

Predicted amino acid sequence alignment indicated that the identified cDNA shared great similarity with other plant GC coding sequences, which for *Arabidopsis* GC is 79% (56% identity) and for *Zea mays* is 70% (53% identity). The highest percentage of identity was observed between *PnGC-1* and GC from *Solanum lycopersicum* (86%) (GenBank accession No. ABK15531). It must be noted that *PnGC-1* from *P. nil* is of closest proximity to other dicots in the constructed phylogenetic tree. Such strong similarity with regard to their sequence may suggest that GC function is conserved in various species, but the gene evolution shows divergence of monocots and dicots of GC.

As was already mentioned (Ludidi and Gehring 2003; Schaap 2005), plant-soluble GCs are significantly distinct from known animal and prokaryotic GCs. Also, *PnGC-1* from *Pharbitis nil* contains a number of features that are unusual in animal GCs and similar to currently annotated plant GCs. First, analysis of the deduced amino acid sequence shows that PnGC-1 contains all-important residues responsible for substrate specificity for GTP, transition-state stabilization, and binding of the essential metal ions (Tucker and others 1998; McCue and others 2000). Second, the catalytic center is at the N-terminal, which is usually a characteristic feature of only a particulate type of guanylyl cyclase. As mentioned above, there are three experimentally confirmed GCs reported in higher plants. The first two, from *Arabidopsis* and *Zea* (Ludidi and Gehring 2003; Yuan and others 2008), are soluble. The third one, from *Arabidopsis*, is a part of a brassinosteroid receptor (AtBRI1) (Kwezi and others 2007). In *PnGC-1* neither the sequence for direction to the membrane nor the dimerization domain, extracellular ligand-binding domain, transmembrane, and intracellular kinase-like domains were found. However, *PnGC-1* does contain a putative *N*-myristoylation site. *N*-myristoylation plays a vital role in membrane targeting and protein-protein interactions (Farazi and others 2001). However, this process is also involved in signal transduction, for example, in plant responses to environmental stress (deVries and others 2006), and myristoylation is required for some function other than membrane affinity.

Animal-soluble GCs function as heme sensors that selectively bind nitric oxide (NO) (Lawson and others 2000; Boon and others 2005), triggering reactions essential for animal physiology. sGCs were placed in the H-NOX (heme nitric oxide and/or oxygen-binding domain) family that also includes bacterial proteins from aerobic and anaerobic organisms (Boon and Marletta 2005). H-NOX proteins possess either a distal pocket tyrosine required for O_2 binding or a very conserved C-X-X-H motif in the heme-binding domain that is responsible for NO binding. Similar to *AtGC-1* and *ZnGC-1*, in *PnGC-1* there is a lack of residues responsible for heme binding and further analysis revealed that NO had no effect on recombinant protein activity. The participation of NO in cGMP signaling is still an unresolved but very important problem, especially because in physiological experiments NO is able

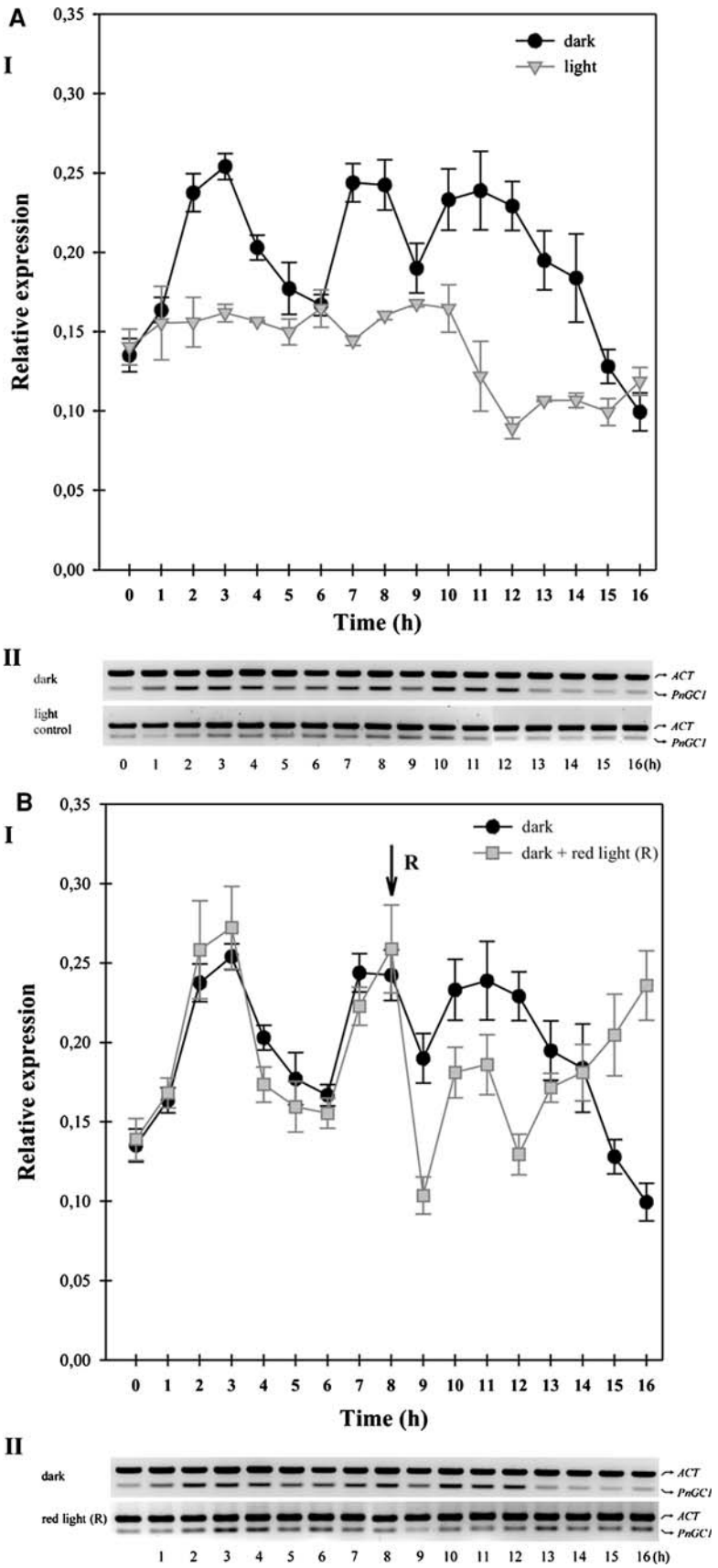

Fig. 8 Dependence of *PnGC-1* expression on light/dark conditions in cotyledons of *Pharbitis nil*. **a** Effect of white light and darkness on *PnGC-1* mRNA levels. Five-day-old seedlings grown under continuous light were placed in darkness for 16 h. RNA was isolated at the indicated times and analyzed by RT-PCR. Results from cotyledons without dark treatment are included for comparison. Section I shows selective expression levels of *PnGC-1* (average from three independent experiments), 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. **b** Effect of red light on *PnGC-1* gene expression. Plants exposed to a 16-h-long night were irradiated for 5 min with red light in the middle of the night. RNA samples for analysis were taken at the times indicated

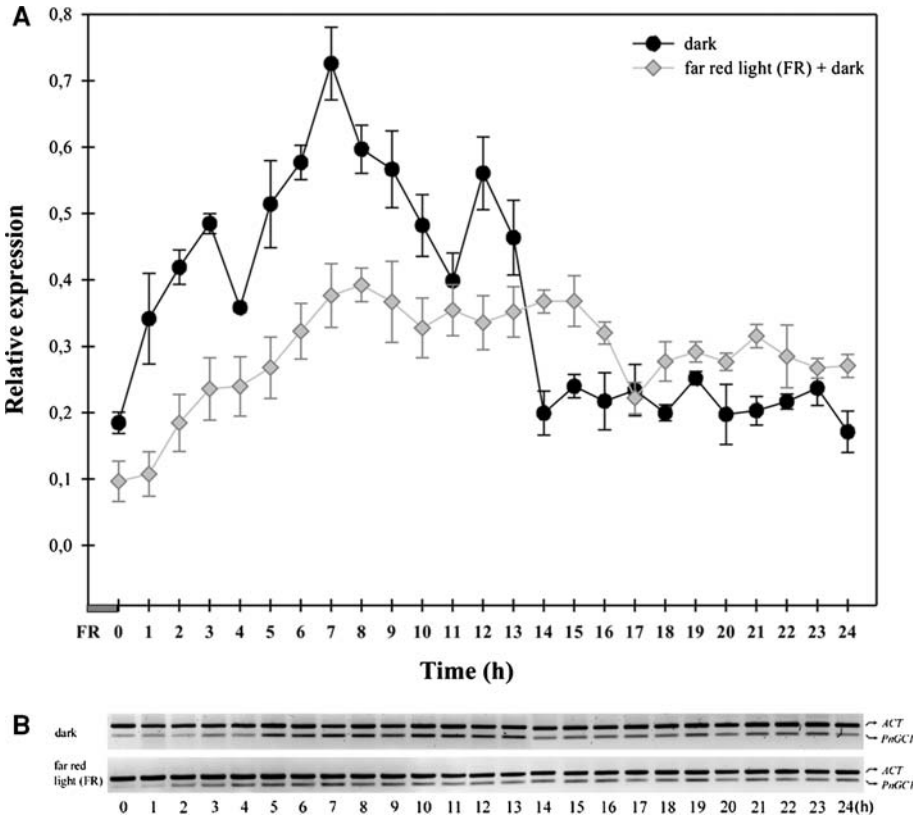

Fig. 9 Dependence of GC gene expression on light/dark conditions in cotyledons of *Pharbitis nil*. Four-day-old seedlings grown under continuous darkness were placed for 24 h in white light of low intensity. Subsequently, seedlings were moved for 24 h to the darkness with or without exposure to far-red light (FR) before the night. RNA was isolated at the indicated times and analyzed by RT-PCR. **a** Selective expression levels of *PnGC-1* (average from three independent experiments). **b** Changes in the transcript levels on 1.7% agarose gel. *Actin4* (*ACT*) was used as a control of equal loading of RNA

to elevate the cGMP level (Pfeiffer and others 1994; Durner and others 1998).

The possibility that the expression of pGEX-PnGC-1 forms an active enzyme was analyzed. Guanylyl cyclase activity in bacterial extract was about 0.18 pmol min^{-1} mg^{-1} protein. The level of activity compares favorably with the activity of the enzyme in supernatant from *P. nil* cotyledons (Szmidt-Jaworska and others 2008b) and is higher than that described by Ludidi and Gehring (2003) for *AtGC-1* from *A. thaliana*. Additional analysis performed with the purified enzyme revealed positive kinetic behavior as a function of Mn-GTP. These findings agree with earlier results concerning animal sGC (Koesling and others 1991; Winger and Marletta 2005), where the enzymes were more active in the presence of Mn^{2+} compared with the physiological cofactor Mg^{2+} and were different from data received for *A. thaliana*, where AtGC-1 was stimulated by Mg^{2+}-GTP only (Ludidi and Gehring 2003).

It is known from animal and prokaryotic systems that under certain conditions the soluble form of GC can produce cAMP (Mittal and Murad 1977; Thorpe and Morkin 1990). The active sites of guanylyl and adenylyl cyclases are closely related. The specificity of ATP or GTP is dictated in part by a few amino acid residues invariant in each family (Sunahara and others 1998), which interact with the purin ring of the substrate. For PnGC-1, no associated adenylyl cyclase activity toward ATP was detected; therefore, PnGC-1 activity retained the enzymatic specificity for GTP.

All these results not only confirm the presence of active GC in *Pharbitis nil* tissue but also show that its catalytic subunit is sufficient to catalyze the formation of cGMP. Wedel and others (1995) provided the first evidence that the catalytic region of the one subunit of the GC heterodimer contains sufficient information for basal enzymatic activity. Therefore, it is not surprising that PnGC-1, the same as AtGC-1 from *A. thaliana*, acts as a monomer.

Some genes are expressed constitutively, but some display an expression pattern that is not only organ- or tissue-specific but also dependent on growth conditions. Previous studies conducted on animal tissue have shown that the transcription of GC and the presence of mRNA are regulated in a manner specific to cell type (Kummer and others 1996) and development (Bloch and others 1997). In contrast, it was reported that rat sGC mRNA is widely expressed in different tissues (Behrends and others 1995). Previous analysis of *P. nil* cyclase activity revealed that the enzyme is present in all organs, with a high level in roots and cotyledons (Szmidt-Jaworska and others 2008b). In this study we showed that the *PnGC-1* transcript is present in variable levels in all analyzed organs. It was higher in dark-grown roots followed by hypocotyls and cotyledons. Exposure to light was found to

downregulate *PnGC-1* transcript levels in cotyledons and hypocotyls, unlike in root tissue where a weak inhibitory effect was observed.

Light is certainly one of the major factors that control growth and development of plants. It is perceived by a sophisticated system of photoreceptors that allows them to detect light wavelengths within a wide spectral range. Red and far-red wavelengths are perceived by the photoreversible phytochrome family of photoreceptors, while the detection of blue and ultraviolet light is conferred by the phototropin and cryptochromes (Thomas 2006).

For a few years it has been known that cGMP is one of the compounds of the light signal transduction pathway in plants. Direct evidence that cGMP is involved in the action of phytochrome has been obtained from single-cell assays in a phytochrome-deficient tomato mutant (Bowler and others 1994). In *Spinacia*, the increase of chloroplast cGMP in response to light was described (Brown and others 1989). Illumination of *Lemna paucicostata* 381 with FR light caused circadian oscillations in the concentration of cAMP and cGMP, which stimulated flowering (Hasunuma 1998; Hasunuma and others 1988). In addition to the GC-cGMP role in mediating the above responses, we have provided evidence that in *P. nil* cotyledons growing in various light conditions, the total amount of cGMP underwent fluctuations, which seems to be connected with the photoperiodic flower induction and transition of a plant from the vegetative to the generative phase (Szmidt-Jaworska and others 2004). These data were supported by the observation of GC activity in vitro (Szmidt-Jaworska and others 2008b).

It is known that cGMP formation by GC may be regulated via not only modulation of enzyme activity, but also indirectly at transcriptional and post-transcriptional levels, affecting the total amount of functional enzyme (Klöss and others 2003; Jiang and Stojilkovic 2006). It will be important to see which of the various mechanisms that regulate GC levels in mammalian cells are operating in plants under physiological conditions, especially as there were no previous attempts to analyze the expression pattern of plant GC. Moreover, it would be interesting to determine if the light conditions affect the abundance of the *PnGC-1* transcript in *Pharbitis nil* cotyledons.

In this study the mRNA abundance was examined in response to a light/dark treatment for plants entrained to the 8-h/16-h light/dark regime. Interestingly, there are no significant changes in the *PnGC-1* expression pattern in cotyledons grown in continuous light, but moving plants to the darkness significantly changed the expression profile. The mRNA level of the examined gene was higher during the dark phase and three oscillations of *PnGC-1* expression were noted.

If *PnGC-1* expression is associated with light/dark conditions, mRNA levels should change after modifying the light regime. It was noted that *PnGC-1* expression decreased markedly after red light irradiation and 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 (Smith and Whitelam 1997; 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, 2008a). Failure to reverse the effect of the R pulse by the FR pulse may be due to already induced irreversible responses by R. These results suggest that red light is the dominant factor in the modulation of *PnGC-1* mRNA levels and that a red light receptor is involved in the light/dark induction of the *PnGC-1* gene. On the other hand, it indicates that *PnGC-1* expression is also highly regulated at the level of mRNA turnover and suggests that the photoresponse of GC activity is regulated primarily at a transcriptional level, then at the enzymatic level. It is worth noting that in the case of a 16-h-long night, the level of mRNA *PnGC-1* at the moment of transferring the plants into light is low, whereas disrupting the night with a pulse of red light caused the plants to end the night with a high level of the transcript. The significance of this observation is not understood, but these results point to the existence of a timing mechanism that by monitoring the night-length regulates the transcript level of *PnGC-1*.

Once the oscillating nature of expression for *PnGC-1* in cotyledon tissue in darkness was unequivocally established and the role of the red light receptor confirmed, we wanted to know what kind of phytochrome was involved. This was the reason that special photoperiodic conditions were used in the cultivation of plants. The exposure of plants to low-intensity white light resulted in light-labile phytochrome degradation. A pulse of far-red light applied before the night caused inactivation of the remaining light-stable phytochrome. The P_{fr}-to-P_r phototransformation can act as a switch-off, inhibiting a given response. Such a model was described and used previously (Heide and others 1986; Maciejewska and others 2004; Szmidt-Jaworska and others 2008a).

In the present experiments, in response to darkness *PnGC-1* mRNA abundance increased and three main peaks appeared after the light was switched off (Fig. 9). Subsequently, transcript levels declined slowly and *PnGC-1* mRNA remained at a basal level for the next hours. When plants were treated with far-red light before the night, such

a high increase was not observed. In the case of far-red and subsequent red light irradiation given prior to the 24-h-long night, the *PnGC-1* expression pattern was restored. The results of our gene expression experiments confirm the hypothesis that GC is involved in light/dark-dependent processes and show that stabile phytochrome is involved in this process.

In summary, based on *PnGC-1* structure and enzyme activity it was shown that *PnGC-1* has features and properties typical of soluble guanylyl cyclases and can be clearly identified as a new member of a class of plant guanylyl cyclase genes. Moreover, results from expression analysis support the concept that changes in *PnGC-1* dark expression may be involved in the mechanism of photoperiodically controlling processes, including phytochrome-controlled flower induction.

Acknowledgment This work was supported by the Ministry of Science and Higher Education (Poland) grant No. N N303 0704 33.

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