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RESEARCH PAPER

Identification and functional analysis of *PCNA1* and *PCNA-like1* genes of *Phaseolus coccineus*

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Abstract

Proliferating cell nuclear antigen (PCNA) is an essential factor in DNA replication and in many other processes in eukaryotic cells. Genetic analysis of *Phaseolus coccineus* showed the presence of at least two *PCNA*-like genes in the runner bean genome. Two *PCNA* genes have previously been found in a few plant species including *Arabidopsis*, tobacco, and maize. In these species, genes were nearly identical. Two cDNAs of *P. coccineus* PCNA (*Pc*PCNA1 and *Pc*PCNA-like1) have been identified that differ distinctly from each other. Interestingly, both the genetic organization of *PcPCNA1* and *PcPCNA-like1* genes and their expression patterns were similar, but these were the only similarities between these genes and their products. The identity between *Pc*PCNA1 and *Pc*PCNA-like1 at the amino acid level was only 54%, with *Pc*PCNA-like1 lacking motifs that are crucial for the activity typical of PCNA. Consequently, these two proteins showed different properties. *Pc*PCNA1 behaved like a typical PCNA protein: it formed a homotrimer and stimulated the activity of human DNA polymerase delta. In addition, *Pc*PCNA1 interacted with a p21 peptide and was recognized by an anti-human PCNA monoclonal antibody PC10. By contrast, *Pc*PCNA-like1 was detected as a monomer and was unable to stimulate the DNA polymerase delta activity. *Pc*PCNA-like1 also could not interact with p21 and was not recognized by the PC10 antibody. Our results suggest that *Pc*PCNA-like1 either is unable to function alone and therefore might be a component of the heterotrimeric PCNA ring or may have other, yet unknown functions. Alternatively, the *PcPCNA-like1* gene may represent a pseudogene.

Key words: DNA polymerase delta, PCNA, *Phaseolus coccineus*, PRINS, RACE.

Introduction

Proliferating cell nuclear antigen (PCNA) was first identified as a factor recognized by an autoantibody present in the sera of patients with autoimmune disorder called systemic lupus erythematosus (Miyachi *et al.*, 1978). It is a homologue of a beta subunit of *Escherichia coli* DNA polymerase III and a product of bacteriophage T_4 gene-45 (Kelman, 1997). The function attributed for PCNA was a processivity factor of DNA polymerase delta required for the synthesis of a new DNA strand (Tan *et al.*, 1986; Bravo *et al.*, 1987; Prelich *et al.*, 1987). It was shown that PCNA with the help of a replication factor C (RF-C) is loaded on DNA, where it forms a trimeric ring structure encircling DNA (Mossi and Hubscher, 1998, Moldvan *et al.*, 2007). These findings were supported by the results of structural studies of yeast and human PCNA (Krishna *et al.*, 1994; Schurtenberger *et al.*, 1998). Afterwards, PCNA was shown to be involved not only in DNA replication but also in DNA repair (Kelman, 1997). In addition, interaction of PCNA with proteins that are involved in many other cellular processes indicates its potential role in chromatin assembly, sister-chromatid cohesion, transcription, and cell cycle regulation (Maga and Hubscher, 2003; Naryzhny,

Abbreviations: cpm, counts per minute; PCNA, proliferating cell nuclear antigen; PRINS, primed *in situ* DNA labelling; RACE, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TLS, translesion synthesis.

2008; Stoimenov and Helleday, 2009). Analysis of all known PCNAs suggests that during evolution of eukaryotic organisms, PCNA remained conserved in function, structure, and sequence. Yeast and *Drosophila* PCNAs were shown to be able to substitute for mammalian PCNA in DNA replication assays (Bauer and Burgers, 1988; Ng *et al.*, 1990). Hashimoto's group demonstrated that recombinant rice PCNA stimulated the enzymatic activity of DNA polymerase delta from human cells (Matsumoto *et al.*, 1994). In other studies, mammalian PCNA stimulated the activity and processivity of two wheat delta-like polymerases (Laquel *et al.*, 1993). Moreover, the formation of a stable complex of purified pea PCNA and human p21/WAF-1 (a p53-dependent protein involved in cell cycle regulation and stress response) was observed (Ball and Lane, 1996). PCNA homologues have been cloned from several groups of eukaryotic organisms such as yeast: budding yeast (Bauer and Burgers, 1990) and fission yeast (Waseem *et al.*, 1992); animals: human (Almendral *et al.*, 1987), rat (Matsumoto *et al.*, 1987), mouse (Yamaguchi *et al.*, 1991), *Drosophila* (Yamaguchi *et al.*, 1990), and *Xenopus* (Leibovici *et al.*, 1990) as well as plants: carrot (Hata *et al.*, 1992), maize (Lopez *et al.*, 1995, 1997), periwinkle (Kodama *et al.*, 1991), rice (Suzuka *et al.*, 1991), oilseed rape (Markley *et al.*, 1993), pea (Shimizu and Mori, 1998*a*), and common bean (Strzalka and Ziemienowicz, 2007).

More detailed studies concerning plant PCNA have been conducted only with a few organisms such as rice and tobacco and concentrated mainly on regulatory elements of *PCNA* gene expression. Upstream sequences of the rice *PCNA* gene were shown to mediate expression of the *PCNA-GUS* chimeric gene in meristems of transgenic tobacco plants (Kosugi *et al.*, 1991). Moreover, two *PCNA* gene promoter elements essential for meristematic tissue-specific expression were identified (Kosugi *et al.*, 1995). Continuation of this work resulted in the identification of two proteins, PCF1 and PCF2, which specifically bind to *cis* elements in the rice *PCNA* gene (Kosugi and Ohashi, 1997). E2F-like sites of the rice and tobacco *PCNA* promoter were shown to be required for meristematic tissue-specific expression of this gene in actively dividing cells (Kosugi and Ohashi, 2002). Engagement of the E2F site of the tobacco *PCNA* gene promoter was presented by Hanley–Bowdoin's group who found that the E2F1 + 2 sites contribute to repression of the *PCNA* promoter in mature tissues, whereas the E2F1 site with transcription activators positively regulates *PCNA* gene expression in young leaves (Egelkrout *et al.*, 2002).

Most recently, the first analyses of plant PCNA proteins have been reported. *Arabidopsis* PCNA1 and PCNA2 proteins show very high levels of amino acid sequence similarity and share some common features. Both proteins were shown to be able to form a homotrimeric ring structure while interacting with the C-terminal segment of human p21 (Strzalka *et al.*, 2009). Moreover, protein–protein interaction analysis using yeast two hybrid system revealed that *At*PCNA1 and *At*PCNA2 could interact with the TLS DNA polymerase eta (Anderson *et al.*, 2008).

In previous studies, an open reading frame (ORF) of the *Phaseolus vulgaris PCNA* gene was identified (Strzalka and Ziemienowicz, 2007). Here for the first time, the isolation and analysis of two different PCNA cDNAs of *Phaseolus coccineus, Pc*PCNA1 and *Pc*PCNA-like1 is reported.

Materials and methods

Plant material and growth condition

Seeds of runner bean (*Phaseolus coccineus* L. cultivar KONTRA) were purchased from Plantico Golebiew HiNO Sp. z o.o Poland. The seeds were germinated in darkness at 20 °C in a Petri dish containing water. Samples of embryonic axes were collected from germinating seeds every 24 h, frozen in liquid nitrogen, and stored at –80 °C. In addition, the seeds were germinated and grown in a greenhouse under natural summer light conditions. Ten days after germination, the samples of root, stem, and leaf tissues were collected, frozen in liquid nitrogen, and stored at –80 °C. Moreover, the segments containing the micropylar region of 3–5 mm long seeds (containing micropylum and a part of the embryonic sac including the developing embryo at an early stage of maturation) were collected after pollination and stored as described above.

Cloning of Pc*PCNA1 and* Pc*PCNA-like1 cDNA using 5′ and 3′ RACE*

5′ RACE (rapid amplification of cDNA ends) was carried out using FirstChoice RLM-RACE (Ambion) following the protocol provided by the supplier. Ten μg of total RNA isolated using the Trizol reagent (Invitrogen) from the micropylar region of the seed were treated with calf intestinal alkaline phosphatase. Next, the sample was treated with tobacco acid phyrophosphatase and, subsequently, with RNA ligase to ligate the RNA adapter to the 5′ end of full-length mRNAs. The RNA was reverse transcribed, and two-step PCR amplification was performed. Amplification of *Pc*PCNA1 and *Pc*PCNA-like1 was done using specific reverse primers 5′-*Pc*PCNA1R (5′-TAATATCCTAACCCAACATTCA-ATAGTG-3′) and 5′-*Pc*PCNAL1R (5′-AC(G/T)GAAAGAA(A/C)AAT(C/T)CTA(A/G)TATCCTAACCC-3′), respectively. Subsequently, nested specific reverse primers for reamplification of the obtained PCR products, 5′-*Pc*PCNA1NR (5′-TTACACTTGA-GGTTTCTCTTCTTC-3′) and 5′-*Pc*PCNAL1NR (5′-CTATGA-TGTAGGGGATGTAATGGG-3′), were used.

3′ RACE was performed similarly using 1 μg of total RNA and specific forward primers: 3′-*Pc*PCNA1F (5′-AACCCTAACCA-TTTCTAAACGAAACCC-3′) and 3′-*Pc*PCNAL1F (5′-TA-TTGCTTCCAGACCTCAAAACCCCAAC-3′) for the first PCR amplification, followed by PCR with specific nested primers: 3′-*Pc*PCNA1NF (5′-GGA(C/T)ATTGATAG(C/T)GA(A/G)CA(C/T)CTTGG-3′) and 3′-*Pc*PCNAL1NF (5′-CCCCAACCATTCC-TAAACCGCTATC-3′).

The PCR reactions were done in 50 μl volume containing: 1× PCR buffer (10 mM TRIS-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.0), 200 μM dNTPs, 1.25 units of Super*Taq* DNA polymerase (Ambion) and 2 μM of each primer. The amplification reactions consisted of a preliminary denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and an incubation at 72 °C for 7 min were performed in a (Biometra) termocycler. The resulting PCR products were purified and cloned into the pTZ57R\T vector (Fermentas) followed by sequencing. The nucleotide sequence data have been deposited in the NCBI GenBank under accession numbers: EF602032 (*Pc*PCNA1) and EF602034 (*Pc*PCNA-like1).

Cloning of Pc*PCNA1 and* Pc*PCNA-like1 genomic sequences*

Amplification of genomic fragments encoding *Pc*PCNA1 and *Pc*PCNA-like1 was performed using genomic DNA extracted using the Genomic Maxi AX Kit (A&A Biotechnology). Gene-specific primers: 3′-*Pc*PCNA1F (5′-AACCCTAACCATTTCTAAACG-AAACCC-3′) and g*Pc*PCNA1R (5′-AACTGAATTCCAAAT-TCGTTGCTCACAG-3′) were used for *PcPCNA1* amplification. Amplification of genomic *PcPCNA-like1* was done using 3′-*Pc*PCNAL1F (5′-TATTGCTTCCAGACCTCAAAACCCCAAC-3′) and 5′-*Pc*PCNAL1R (5′-AC(G/T)GAAAGAA(A/C)AAT(C/T)CTA(A/G)TATCCTAACC C-3′) primers. The reaction was done in 50 μl volume containing: 1× PCR buffer, 200 μM dNTPs, 1.25 units of Super*Taq* DNA polymerase (Ambion), and 2 μM of each primer.

The samples were heated at 94 °C for 5 min and then subjected to 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. Then they were incubated at 72 °C for 7 min in a (Biometra) termocycler. The resulting amplified PCR products were purified and cloned into pTZ57R\T vector (Fermentas) followed by sequencing. The nucleotide sequence data have been deposited in the NCBI GenBank under accession numbers: EF602033 (g*Pc*PCNA1) and EF602035 (g*Pc*PCNA-like1).

Real-time RT-PCR

For real-time RT-PCR, total RNA was isolated using the Trizol reagent (Invitrogen). cDNA synthesis was carried out on 1 μg of total RNA using the QuantiTect Reverse Transcription Kit with genomic DNA wipe-out buffer (Qiagen). Real-time PCR reactions were performed in mixtures containing: 1× of SYBR Green PCR Master Mix (SYBR Green qPCR Kit, Finnzymes), 0.5 μM of each primer and 200 ng of cDNA in a final volume of 15 μl. The reactions were performed using control *18S RNA* gene-specific primers: 3′-18SRNA_RTPCRF (5′-CCAGGTCCAGACATAG-TAAG-3′) and 5′-18SRNA_RTPCRR (5′-GTACAAAGGGCA-GGGACGTA-3′) (Duval *et al.*, 2002), *PcPCNA1* gene-specific primers 3′-*Pc*PCNA1_RTPCRF (5′-GATATTGGATCTGCA-AATATAG-3′) and 5′-*Pc*PCNA1R (5′-TAATATCCTAACC-CAACATTCAATAGTG-3′) or *PcPCNA-like1* gene-specific primers 3′-*Pc*PCNAL1_RTPCRF (5′-AGAATAAGAAATGGA-GGGAC-3′) and 5′-*Pc*PCNAL1_RTPCRR (5′-ATGATGTA-GGGGATGTAATG-3′) with annealing temperatures 56 °C, 47 °C, and 55 °C, respectively. All reactions were performed in a PCR machine (Corbett-Research, Rotor-Gene, RG-3000) using the following cycling conditions: 95 °C for 10 min and 40 three-step cycles of 30 s at 95 °C, 45 s of annealing, and 45 s at 72 °C. All PCR reactions were carried out in triplicate. Relative quantification of gene expression was calculated based on the comparative C_t (threshold cycle value) method ($\Delta C_t = C_{t\ \text{gene of interest}} - C_{t\ 18S\ RNA}$). Comparison of gene expression between tested samples was derived by subtracting the leaf ΔC_t values from the tested sample ΔC_t values to give a $\Delta\Delta C_t$ value. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$.

DNA isolation and Southern blot analysis

Genomic DNA was extracted from 96 h old seedlings using the Genomic Maxi AX Kit (A&A Biotechnology). The purified DNA (30 μg) was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII or *Xba*I, separated in 0.8% agarose gel, blotted on a positively charged nylon membrane (Roche), following the standard hybridization protocol (Sambrook and Russell, 2001). Hybridization was performed for 16 h at 65 °C with the *Pc*PCNA1 798 bp long DIG-labelled probe generated by PCR. After autoradiography, the probe was stripped off and the blot was hybridized with the 816 bp long *Pc*PCNA-like1 DIG-labelled probe (under the same conditions as used for the *Pc*PCNA1 probe).

PCR

PCR reactions were performed in mixtures containing: 1× PCR buffer (Takara) with 2 mM $MgCl_2$, 200 μM dNTPs, 2 μM of each primer, 1 unit of Takara *Taq* polymerase, and 50 ng of genomic DNA isolated from *P. coccineus* seedlings or plasmid pTZ57R\T DNA containing genomic sequence of the *PcPCNA1* or *PcPCNA-like1* genes in a final volume of 25 μl. The reactions were performed using degenerated primers: *Pc*PCNAF (5′-GT-GCAAGGTTC(T/C/A)(C/G)T(T/C)CTGAAGAAGG-3′) and *Pc*-PCNAR (5′-C(C/A)(G/A)TCTC(A/T)GCAAT(T/C)TTGTA(T/C)TC-3′). All reactions were performed in a PCR machine (Biometra) using the following cycling conditions: 95 °C for 5 min and 30 three-step cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by 5 min at 72 °C.

PRINS (Primed in situ *DNA labelling)*

Seeds of runner bean were imbibed for 5 h in distilled water at 25 °C with aeration, and then germinated on moistened filter paper in Petri dishes (25 °C) for 16 h. Then they were treated with Hoagland's solution (1.6 g l^{-1}, Sigma-Aldrich) for 5 h (Dolezel *et al.*, 1999). Next, 1–2 cm long roots were collected in iced water and incubated at 0 °C for 24 h, fixed in Carnoy's solution (ethanol and glacial acetic acid, 3:1 v/v) and stored at 4 °C. Permanent squash preparations were made from root meristems as described previously (Schwarzacher and Heslop-Harrison, 2000), with some modifications developed for *Lupinus* (Naganowska *et al.*, 2003). The slides were stored at –20 °C until used for PRINS. Before performing a PRINS reaction, the slides were dried overnight at 37 °C. Frame-Seal Chambers (MJ Research, Inc.) were stuck to the slides. Gene specific primers: 3′-*Pc*PCNA1F (5′-AACCCTAAC-CATTTCTAAACGAAACCC-3′) and g*Pc*PCNA1R (5′-AACT-GAATTCCAAATTCGTTGCTCACAG-3′) were used for *PcPCNA1* amplification. Amplification of *PcPCNA-like1* was performed using 3′-*Pc*PCNAL1F (5′-TATTGCTTCCAGACCT-CAAAACCCCAAC-3′) and 5′-*Pc*PCNAL1R (5′-C(G/T)GAAA-GAA(A/C)AAT(C/T)CTA(A/G)TATCCTAACCC-3′) primers. Reaction mixtures contained a DIG DNA labelling mixture (0.1 mM dATP, dCTP, dGTP, and 0.035 mM DIG-12-dUTP with 0.065 mM dTTP) (Roche), 3 mM $MgCl_2$, 3 units of *Taq* polymerase (Invitrogen), and 2 μM of each primer. 25 μl of the mixture were put into each frame, and the frames were covered with polyester coverslips. The PRINS reaction mixtures were heated at 91 °C for 5 min and then incubated at 55 °C for 15 min. In the third stage, primer extension reactions were performed at 72 °C for 30 min (MJ Thermal Cycler PTC-200 with a *Slide Griddle* plate). The reactions were stopped by adding stop buffer (500 mM NaCl, 50 mM EDTA, pH 8.0) followed by incubation at 70 °C for 2 min. Next, the slides were incubated in blocking buffer [0.5% blocking reagent (Roche), 100 mM maleic acid, 150 mM NaCl, pH 7.5] at 37 °C for 30 min, and then in anti-DIG-fluorescein antibody solution (20 μg ml^{-1}) (Roche) for 60 min. Then the slides were placed in washing buffer (100 mM maleic acid, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) at room temperature for 5 min and counterstained with DAPI in Vectashield antifade solution (Vector). The preparations were examined with the OLYMPUS BX60 Research System Microscope. The images were acquired with a black and white CCD camera, interfaced to a PC running the analySIS 3.0 software (Soft Imaging System).

Purification of recombinant Pc*PCNA1 and* Pc*PCNA-like1 proteins*

The open reading frames of *Pc*PCNA1 and *Pc*PCNA-like1 were amplified with specific sets of primers: *Pc*PCNA1ORFf (5′-GG-AATTCCATATGTTGGAATTACGTCTCGTGCAAG-3′) and *Pc*PCNA1ORFr (5′-CGGGATCCTTACACTTGAGGTTTCTC-TTCTTC-3′), or *Pc*PCNAL1ORFf (5′-GGAATTCCATATGT-TGGAAGTCCGTTTCGTGCAAG-3′) and *Pc*PCNAL1ORFr

(5′-CGGGATCCCTATGATGTAGGGGATGTAATGGG-3′), respectively. Next, the amplified products were cloned into *Nde*I/*Bam*HI sites of pET15b expression vector and sequenced. Constructs were introduced into *E. coli* BL21(DE3) strain. Bacteria were grown at 37 °C in 2.0 l LB medium containing ampicillin (100 μg ml^{-1}) until OD_{595} 0.6 was reached, and production of *Pc*PCNA proteins was induced with 1 mM IPTG at 37 °C. After 4 h of induction, cells were harvested by centrifugation (5 000 *g* for 15 min at 4 °C) and resuspended in 50 ml of lysis buffer A [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 1 mM PMSF (phenylmethylsulphonyl fluoride), 0.05% Tween 20, pH 8.0] containing lysozyme (1 mg ml^{-1}), RNase A (10 μg ml^{-1}), DNase I (5 μg ml^{-1}), and sonicated (5 pulses for 30 s). All the following procedures were performed at 4 °C. The cells were centrifuged at 40 000 *g* for 30 min, and the cell lysate was loaded onto a 2 ml Ni-NTA Superflow (Qiagen) column. The unbound proteins were washed with 10 vols of buffer A containing 20 mM imidazole. The bound proteins were eluted with buffer A containing 250 mM imidazole and then dialysed against buffer B (50 mM TRIS-HCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 0.05% Tween 20, pH 7.6). The dialysed fraction was loaded onto a 2 ml Heparin Sepharose 6 Fast Flow (Amersham) column, and the flow-through was collected and loaded onto 1 ml HiTrap Q HP Sepharose (Amersham). The unbound proteins were removed with 10 ml of buffer B. The bound proteins were eluted with a 30 ml linear gradient of 0 to 1 M NaCl in buffer B. The fractions containing the recombinant protein were dialysed against buffer C (50 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 10% glycerol, pH 7.6), then frozen in liquid nitrogen and stored at –80 °C until use. Protein concentration was determined using the Bio-Rad Protein Assay.

Recombinant PCNA2 protein of *Arabidopsis thaliana* (*At*PCNA2) was purified as described previously (Strzalka *et al.*, 2009).

Gel filtration

All the following procedures were performed at 4 °C. The purified proteins (*Pc*PCNA1, *Pc*PCNA-like1, and *At*PCNA2) were dialysed against 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The protein sample (0.5 ml; 250 μg) was loaded onto 24 ml Superdex 200 column with the flow rate 0.5 ml min^{-1}. The recorded chromatogram was used for molecular mass calculation of the tested protein, based on previously separated standard proteins: tyroglobulin (670 kDa), γ calf globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

Complex formation with p21 peptide

Biotinylated synthetic peptide (KRRQTSMTDFYHSKRRLIFS, 2 μg; synthesized by the Protein Analysis service unit at FMI, Basel) was dissolved in DMSO (a final concentration of 0.5 mg ml^{-1}), diluted in 100 μl PBS and incubated with 20 μl of streptavidin-agarose beads (Pierce) for 1 h at room temperature. Unbound peptide was removed by three washing steps with 1 ml of PBS each. The beads with bound peptide were incubated with 1 μg of the recombinant protein (*Pc*PCNA1, *Pc*PCNA-like1, or *Hs*PCNA) at 4 °C for 1 h. Unbound protein was removed by three washings with 1 ml of PBS, and the beads were heated at 94 °C for 5 min in 1× protein sample loading buffer containing SDS and DTT. The samples were separated in 12% polyacrylamide gel during SDS-PAGE (Laemmli, 1970), followed by Commassie staining.

Western blotting and immunodetection

One μg of recombinant *Pc*PCNA1, *Pc*PCNA-like1, and *Hs*PCNA was separated in 12% polyacrylamide gel (SDS-PAGE; Laemmli, 1970) and electrotransferred onto a PVDF (0.2 μm) membrane (Millipore) as described previously (Towbin *et al.*, 1979). After the transfer was completed, the membrane was washed three times for 5 min in 1× PBS supplemented with 0.5% Tween 20 (PBS-T) and blocked with PBS-T containing 5% fat-free milk (PBS-TB) for 30 min. The membrane was incubated overnight at 4 °C with an anti-human PCNA monoclonal antibody (PC10, Sigma, dilution 1:2 000). After washing in PBS-TB, the membrane was incubated for 1 h at room temperature with goat anti-mouse IgG alkaline phosphatase-conjugate (Sigma, dilution 1:10 000). After several washes in PBS-T, immunodetection was performed using the BCIP/NBT substrate (ImmunO, MP Biomedicals) at room temperature.

The DNA polymerase assay

The reaction was carried out according to the previously published protocol (Weiser *et al.*, 1991). A 25 μl volume mixture contained the following components: 50 mM BIS-TRIS, pH 6.5, 1 mM DTT, 0.25 mg ml^{-1} BSA, 6 mM $MgCl_2$, 20 μM [^{3}H]dTTP 500 (cpm $pmol^{-1}$), 0.5 μg poly(dA)/oligo(dT) template (10:1), and 40 ng of human polymerase delta (0.54 units), in the absence or presence of 5 μg of the tested protein (BSA, human PCNA, *Pc*PCNA1 or *Pc*PCNA-like1). Reaction mixtures were incubated at 37 °C for 30 min, precipitated with TCA, and the radioactivity of insoluble material was determined in a scintillation counter using CytoScint (ICN) scintillation solution. One unit was defined as 1 pmol of dTMP incorporated into acid-precipitable material during 30 min at 37 °C.

Results

Cloning of cDNA and genomic DNA coding for Pc*PCNA1 and* Pc*PCNA-like1*

Rapid amplification of 5′ and 3′ cDNA ends (RACE) techniques were employed for the amplification of full-length cDNAs encoding *Pc*PCNA1 and *Pc*PCNA-like1. The primers used for the identification of *Pc*PCNA1 and *Pc*PCNA-like1 cDNAs were designed based on the analysis of *Phaseolus coccineus* EST fragments deposited in the National Centre for Biotechnology Information (NCBI). The micropylar region of seeds containing an active suspensor was used as a source of total RNA. The identified cDNA sequence of *Pc*PCNA1 contained a 798 bp open reading frame encoding a polypeptide of 265 amino acids. A calculated molecular mass of the polypeptide was 29.45 kDa and p*I*=4.69. *Pc*PCNA-like1 cDNA consisted of a 816 bp open reading frame encoding a polypeptide of 271 amino acids with a molecular mass of 30.73 kDa and p*I*=5.0. An alignment of *P. coccineus Pc*PCNA1 and *Pc*PCNA-like1 showed that the identity between these two amino acid sequences was 54.5% (Fig. 1). Alignment analysis of *Pc*PCNA1 against human PCNA (accession number: CAC27344) and pea PCNA (accession number: CAA76392) at the amino acid level demonstrated an identity of 64.5% and 92.9%, respectively, whereas alignment analysis of *Pc*PCNA-like1 against human and pea PCNAs showed an identity of 38.3% and 52.3%, respectively. Analogous evolutionary divergences and similarities of PCNA proteins from various species can also be confirmed, based on the analysis of a PCNA phylogenic tree (see Discussion and Fig. 10).

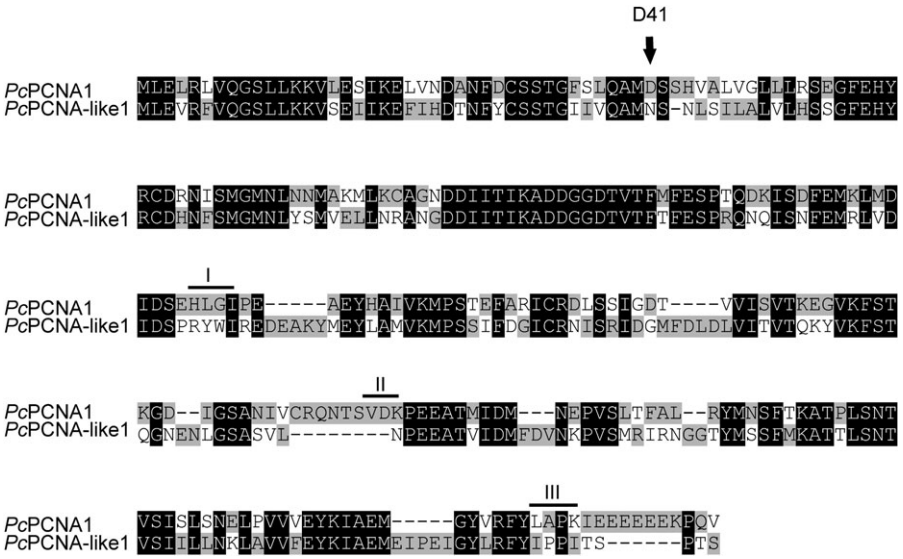

Fig. 1. Alignment of amino acid sequences of *Pc*PCNA1 (accession number: ABQ96591) and *Pc*PCNA-like1 (accession number: ABQ96593) proteins. The analysis was done using StretcherP and the graphical representation was made using GeneDoc software. Identical amino acids are denoted as white letters on a black background. The black letters on grey and white backgrounds represent different amino acids. The residue D^{41} and motifs I ($H^{125}L^{126}G^{127}I^{128}$), II ($V^{188}D^{189}K^{190}$), and III ($L^{251}A^{252}P^{253}K^{254}$) are marked. Dashes represent gaps introduced to maximize identities.

Genomic sequences encoding *Pc*PCNA1 and *Pc*PCNA-like1 have been amplified, cloned, and sequenced. Sequence analysis revealed that both genes contained two introns (Fig. 2A). The *PcPCNA1* intron 1 was 633 bp in length, and intron 2 was 137 bp, whereas introns of the *PcPCNA-like1* gene were 97 bp and 83 bp. Highly conserved (5′-GT/AG-3′) intron termini were identified in all introns of both *PcPCNA1* and *PcPCNA-like1* genes (Fig. 2A).

Genomic organization and localization of PcPCNA *genes*

The copy number of *PCNA*-like sequences and their chromosomal localization in the *P. coccineus* genome was investigated by Southern blot and PRINS analyses. For Southern blot analysis, two different probes were used; one probe was complementary to the *Pc*PCNA1 open reading frame (ORF) and the second one was complementary to *Pc*PCNA-like1 ORF. At least two bands for both probes were detected during analysis performed with *P. coccineus* genomic DNA digested (separately) with five restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Xba*I) (Fig. 2B). The pattern obtained with the *Pc*PCNA-like1 probe was different from and more complex than the pattern obtained with the *Pc*PCNA1 probe. According to the number of bands detected with these probes, at least two different sequences similar to the sequence of the probes used were present in the *P. coccineus* genome.

Next, PCR analysis of *P. coccineus* genomic DNA using degenerated primers (designed based on the sequence of *PcPCNA1* and *PcPCNA-like1* genes) was performed. PCR reaction resulted in the amplification of three DNA fragments with molecular sizes of around 1.5 kb, 1.1 kb, and 0.95 kb (Fig. 2C). Two of these PCR products matched those identified in this work *PcPCNA1* and *PcPCNA-like1* genes, whereas the third product most likely corresponds to another *PcPCNA-like* gene (*PcPCNA-like2?*). Similarity between coding sequences of *PcPCNA1* and *PcPCNA-like2?* genes is close to that between *PcPCNA-like1* and *PcPCNA-like2?* (60% and 57% identity, respectively), whereas *PcPCNA1* and *PcPCNA-like1* genes share 70.5% of their coding sequences. Thus, PCR analysis revealed the presence of at least one additional *PCNA-like* gene in the genome of *P. coccineus*.

The genomic localization of the *PcPCNA1* and *PcPCNA-like1* genes was analysed using chromosomes of *P. coccineus* by primed *in situ* DNA labelling (PRINS) reactions. PRINS is a method of molecular cytogenetics for detecting DNA sequences in chromosomes of a species studied. It was first described by Bolund's group and, in subsequent years, important applications in human and plants cytogenetics were found (Koch *et al.*, 1989; Abbo *et al.*, 1993; Kubalakova *et al.*, 2001; Kaczmarek *et al.*, 2007). In our experiments, most of the signals after the PRINS reaction were visible as single dots but some of them were visible as double dots (on both chromatids). The signals of *PcPCNA1* were observed on two chromatides in one locus, in the centromeric region of a submetacentric chromosome (Fig. 3A). The signals of *PcPCNA-like1* were also shown to be localized at one locus, on a short arm of the medium submetacentric chromosome near the centromere (Fig. 3B).

Purification and biochemical characterization of Pc*PCNA proteins*

To compare biochemical characteristics of *Pc*PCNA1 and *Pc*PCNA-like1, recombinant proteins were produced in *E. coli* with short N-terminal His-tags which were shown

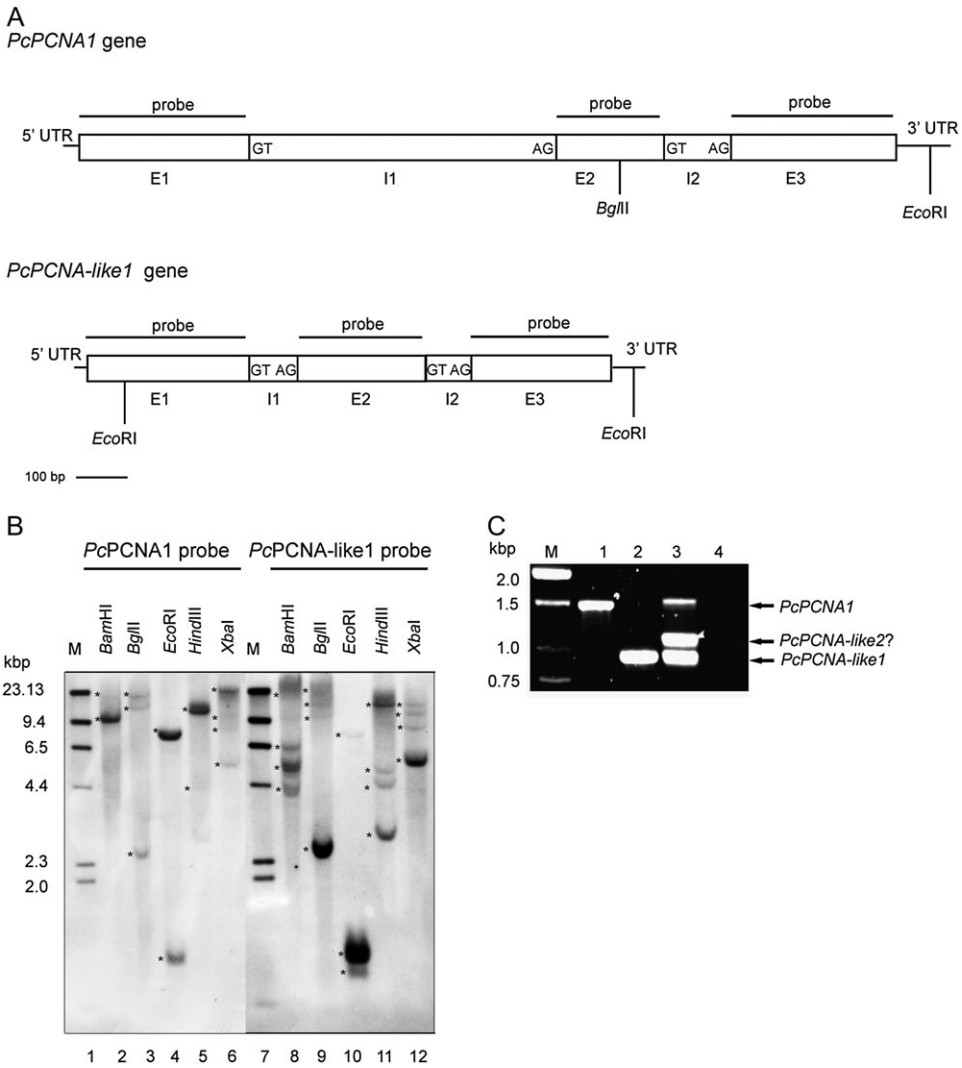

Fig. 2. Southern blot and PCR analysis of *P. coccineus* genomic DNA. (A) Structure of *PcPCNA1* and *PcPCNA-like1* genes. The nucleotide sequences were analysed using WebGene software. Exons (E), introns (I), and 5′-UTR and 3′-UTR regions are marked. The border sequences of introns termini are placed in the boxes. The positions of internal sites recognized by restriction enzymes used in the Southern blotting analysis are marked. (B) Southern blotting results. 30 μg of the genomic DNA isolated from *P. coccineus* seedlings were digested with *Bam*HI (lanes 2 and 8), *Bgl*II (lanes 3 and 9), *Eco*RI (lanes 4 and 10), *Hin*dIII (lanes 5 and 11) or *Xba*I (lanes 6 and 12), separated in 0.8% agarose gel and subjected to Southern blot procedure with the *Pc*PCNA1 (lanes 2–6) or *Pc*PCNA-like1 (lanes 8–12) probe. Lanes 1 and 7: DNA molecular weight marker II Digoxigenin-labelled (Roche). Stars indicate position of DNA fragments detected with *Pc*PCNA probes. (C) PCR results. PCR was performed using degenerate primers and gDNA isolated from *P. coccineus* seedlings (lane 3), or plasmid pTZ57R\T DNA containing genomic sequence of the *PcPCNA1* gene (lane 1) or of the *PcPCNA-like1* gene (lane 2). In negative control, DNA template was omitted (lane 4). Lane M: DNA size standards (1 kb DNA ladder).

earlier not to disrupt the PCNA activity (Kimura *et al.*, 2001). These recombinant proteins were purified using a three-step chromatography procedure. First, affinity chromatography on a nickel column was performed, followed by chromatography on heparin and Q-Sepharose columns (see Materials and methods for details). Both proteins were purified to 90% homogeneity (Fig. 4A, B).

Biochemical characterization of the purified recombinant *Pc*PCNA1 and *Pc*PCNA-like1 proteins included analysis of their native structure, stimulation of the DNA polymerase delta activity, ability to interact with the p21 peptide, and reactivity with anti-PCNA antibody.

For analysing the native structure of the *Pc*PCNA proteins, gel filtration chromatography on Superdex 200 was employed. This analysis demonstrated that the *Pc*PCNA1 protein was present in solution mainly as a homo-oligomer (most likely a homotrimer), as its native molecular mass was estimated to be around 118 kDa (Fig. 5). The estimated mass is close to a theoretically calculated molecular mass of 93 kDa for three *Pc*PCNA1 molecules and similar to the estimated native molecular mass of *Arabidopsis* PCNA2 (~124 kDa; Fig. 5) which has been shown to form a homotrimer (Strzalka *et al.*, 2009). However, for *Pc*PCNA-like1, the formation of the trimeric

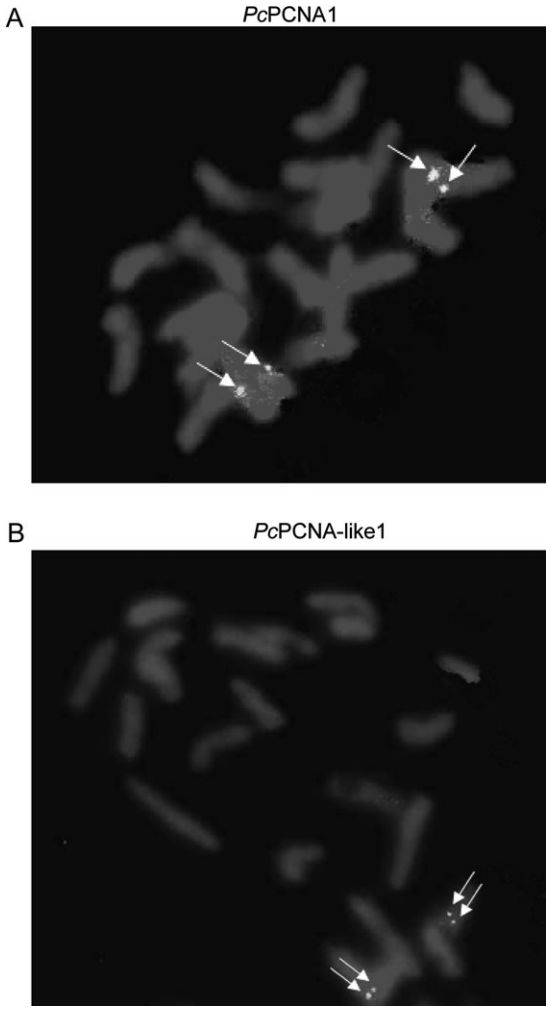

Fig. 3. PRINS analysis performed on metaphase chromosomes of *P. coccineus*. The reactions were performed with *Pc*PCNA1 specific primers (A) and with *Pc*PCNA-like1 specific primers (B) in the presence of DIG-12-dUTP and 3 units of *Taq* polymerase. After the reaction, slides were incubated with anti-DIG-fluorescein antibody and preparations were examined with the OLYMPUS BX60 Research System Microscope. The observed signals are denoted by white arrows. Each pictures shows a single diploid cell ($2n=22$).

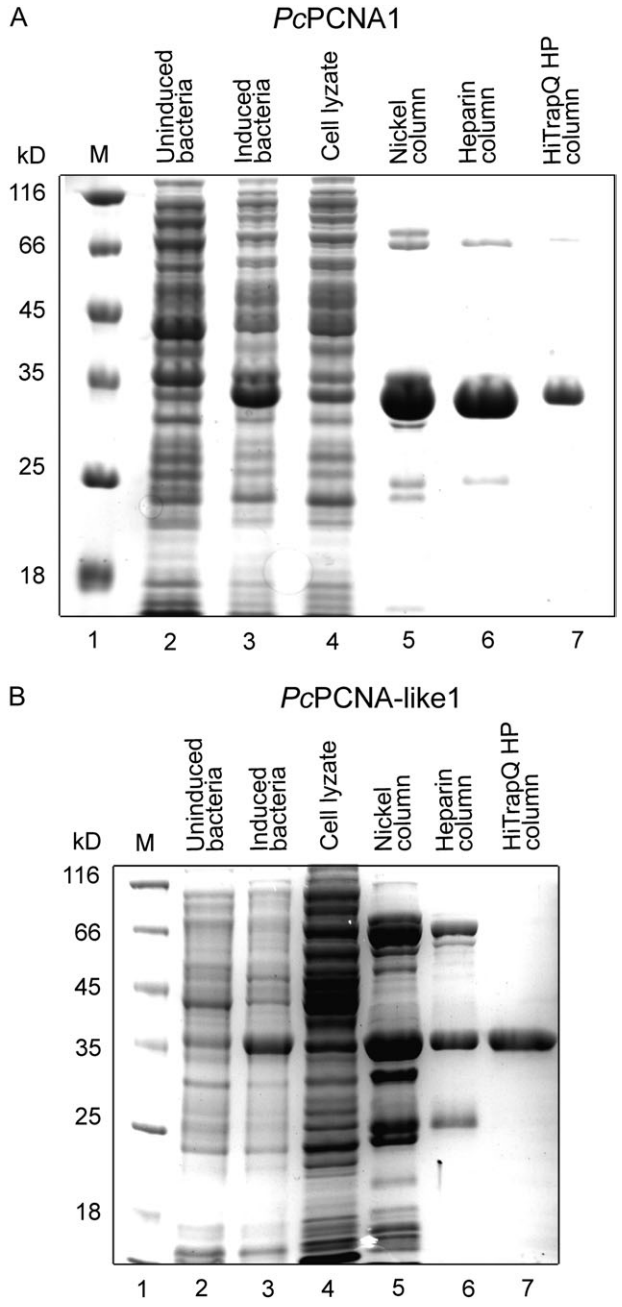

Fig. 4. Purification of the recombinant *Pc*PCNA1 (A) and *Pc*PCNA-like1 (B) proteins. The protein samples were separated on 12% SDS-PAGE gels stained with Coommassie dye. The level of production of *Pc*PCNA1 and *Pc*PCNA-like1 and the subsequent purification steps are shown. Lane 1: molecular mass marker; lanes 2 and 3: non-induced and induced BL21(DE3)[pET15b*Pc*PCNA1] and BL21(DE3)[pET15b*Pc*PCNA-like1]; lane 4: cell lyzates; lane 5: elution from nickel column; lane 6: heparin column flow-through; lane 7: elution from HiTrap Q HP sepharose (1 μg of purified protein).

structure was not observed, as it migrated with a molecular mass of around 25 kDa, suggesting that this protein was in a monomeric form (Fig. 5).

To assess biological function of recombinant *Pc*PCNAs in the DNA replication process, an *in vitro* polymerase activity assay was used. Analysis of the stimulatory effect on the activity of polymerase delta was performed using human DNA polymerase delta with human PCNA as a positive control, BSA as a negative control, and *Pc*PCNA1 and *Pc*PCNA-like1 as tested proteins. *Pc*PCNA1 exhibited a distinct ability to stimulate the processivity of human polymerase delta, similarly to human PCNA (*Hs*PCNA; Fig. 6). By contrast, the *Pc*PCNA-like1 protein was not able to stimulate the activity of polymerase delta. A similar effect was also observed for the negative control (BSA), as expected. No endogenous polymerase activity was detected in the proteins tested (Fig. 6). The observed stimulatory effect was dose-dependent and could also be observed if low amounts of the tested proteins were used (data not shown).

Moreover, *Pc*PCNA proteins were tested for their ability to interact with the p21 peptide, as well as to be recognized

by the anti-PCNA antibody. To study the interaction of *Pc*PCNA proteins with a fragment of the human p21 protein, an affinity-precipitation assay was applied, using p21-streptavidin–agarose beads. Analysis of *Pc*PCNA1 and *Pc*PCNA-like1 interactions with the p21 peptide showed that only *Pc*PCNA1 was able to bind to the p21 peptide specifically, similarly to human PCNA protein (Fig. 7). By contrast, no binding of *Pc*PCNA-like1 to the p21 peptide was observed (Fig. 7). In addition, analysis of the *Pc*PCNAs recognition by an anti-human PCNA monoclonal antibody (PC10) was performed. This antibody had been shown previously to be able to recognize plant (pea) PCNA (Ball and Lane, 1996). Our experiment performed with runner bean and human PCNA proteins showed that *Hs*PCNA and *Pc*PCNA1, but not *Pc*PCNA-like1, was recognized by the PC10 antibody (Fig. 8).

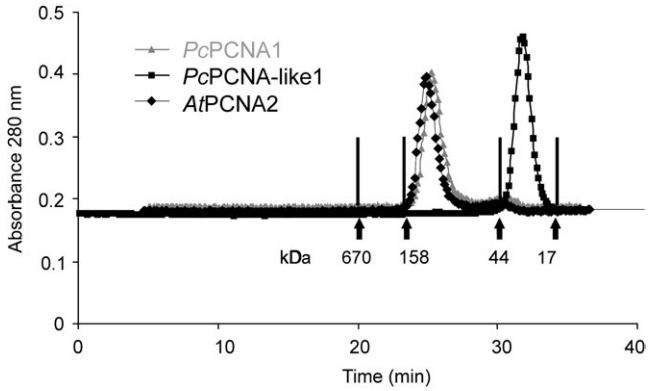

Fig. 5. Analysis of native structures of the *Pc*PCNA1 and *Pc*PCNA-like1 proteins. 250 μg of *Pc*PCNA1, *Pc*PCNA-like1, and *At*PCNA2 protein were separately filtered through Superdex 200. The arrows indicate the retention time of proteins used as molecular mass standards: thyroglobulin (670 kDa), γ calf globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

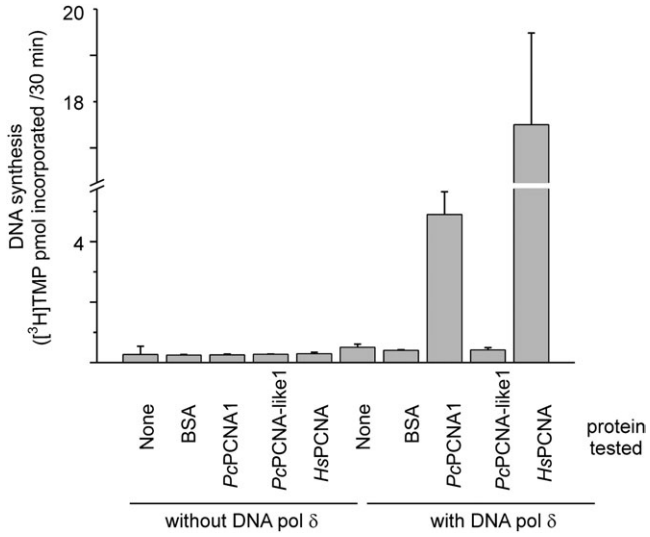

Fig. 6. Test for the stimulatory effect of PCNA proteins on DNA polymerase delta activity. The polymerase activity assay was performed using poly(dA)/oligo(dT) as a template, [^{3}H]TTP and 0.54 units of human DNA polymerase delta either alone or in the presence of 5 μg of the tested proteins: human PCNA (*Hs*PCNA), *Pc*PCNA1, *Pc*PCNA-like1 or BSA (negative control). In addition, these proteins were assayed in the absence of human DNA polymerase delta. The radioactivity of the acid-precipitable material, measured in cmp, was then converted to pmol of [^{3}H]TMP incorporated during 30 min of the reaction.

Expression of PcPCNA *genes at early stages of plant development*

A real-time RT-PCR technique was used to evaluate the relative levels of *Pc*PCNA1 and *Pc*PCNA-like1 transcripts in germinating embryos (embryonic axes) and plant organs: roots, stems, leaves, and the micropylar region of seeds. These tissues were chosen for the analysis of *PcPCNA* gene expression since intensive cell proliferation accompanied by DNA replication is expected to occur in germinating embryos as well as in the micropylar region of developing seeds which contains the developing embryo at the early stages of maturation, whereas mature plant organs predominantly contain differentiated, non-dividing cells.

To test changes in the levels of these transcripts in germinating embryos, a time-course experiment was employed (Fig. 9A). The level of the *Pc*PCNA1 transcript was low at time 0 (dry embryo). During the 24 h of germination, it rapidly increased by a factor of several hundred to reach the maximum level after 48 h. Then, between 72 h and 96 h after the start of germination, the level of the transcript decreased to the level that had been

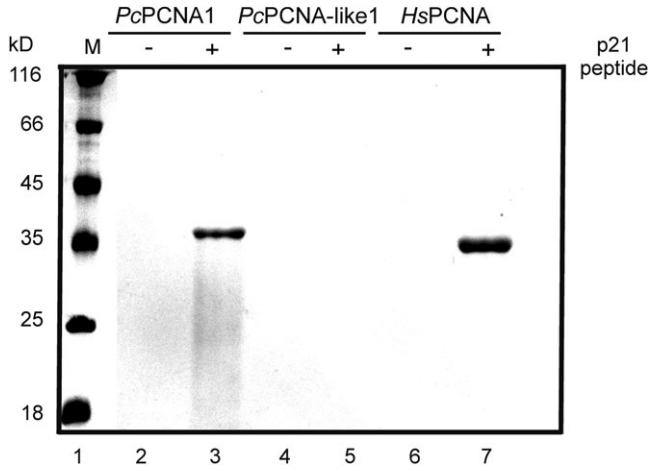

Fig. 7. Analysis of *Pc*PCNA1 and *Pc*PCNA-like1 binding to p21 peptide. Biotinylated p21 peptide was attached to streptavidin–agarose beads and incubated with 1 μg of the recombinant protein followed by extensive washing. The samples were then separated in 12% SDS-PAGE and stained with Coommassie dye. Lane 1: molecular mass marker; lane 2: streptavidin–agarose beads without p21, reacted with *Pc*PCNA1; lane 3: beads with p21, reacted with *Pc*PCNA1; lane 4: beads without p21, reacted with *Pc*PCNA-like1; lane 5: beads with p21 reacted with *Pc*PCNA-like1; lane 6: beads without p21, reacted with *Hs*PCNA; lane 7: beads with p21, reacted with *Hs*PCNA.

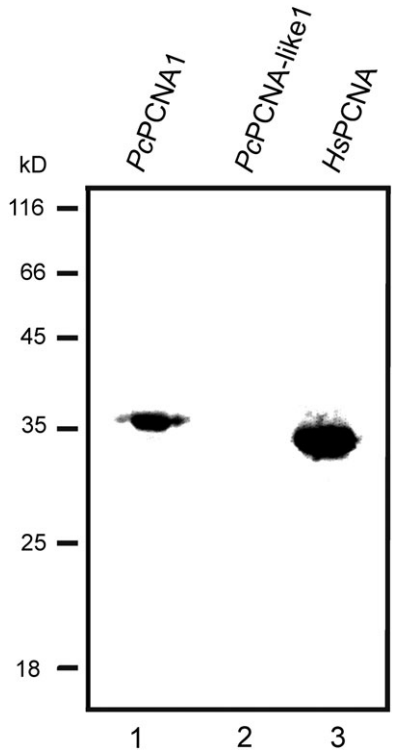

Fig. 8. Western blot analysis of *Pc*PCNA proteins with anti-human PCNA monoclonal antibody (PC10). One microgram of *Pc*PCNA1, *Pc*PCNA-like1, and *Hs*PCNA was separated in 12% polyacrylamide gel and subjected to Western blot analysis using PC10 antibodies. Lane 1: *Pc*PCNA1; lane 2: *Pc*PCNA-like1; lane 3: *Hs*PCNA. The positions of molecular mass markers are indicated.

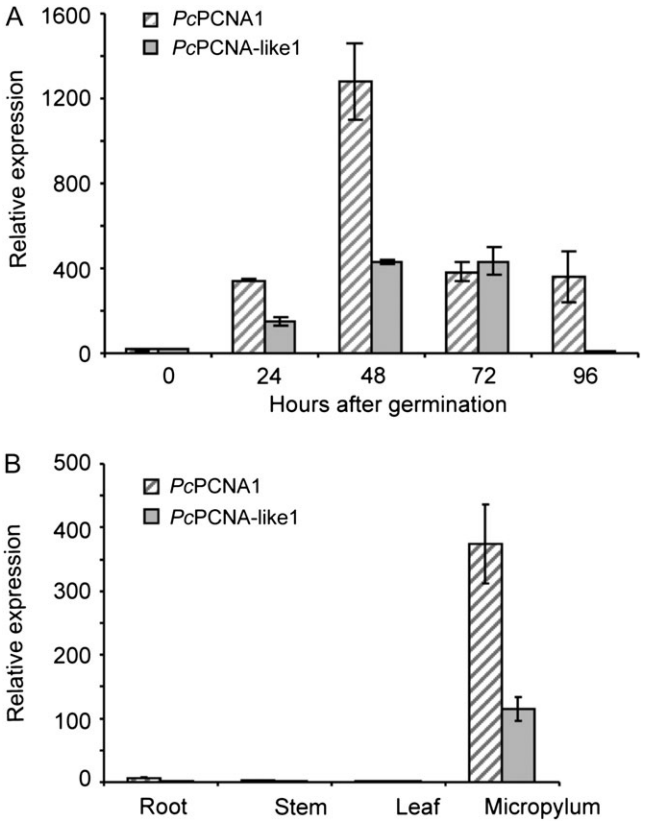

Fig. 9. Real-time RT-PCR analysis of *PcPCNA* genes expression. (A) Relative changes in the *Pc*PCNA1 and *Pc*PCNA-like1 transcript levels during the early stages of germination from 0 h to 96 h. (B) Relative expression of *PcPCNA1* and *PcPCNA-like1* genes in *P. coccineus* root, stem, leaf, and micropylum. The figure presents data obtained in one of three independent experiments, and is representative for the observed changes.

observed after 24 h. Next, the *Pc*PCNA1 transcript level was evaluated for *P. coccineus* plant organs. The expression of this gene in root, stem, and leaf tissues was at a very low level (Fig. 9B). Analysis of the *PcPCNA1* gene expression in the micropylar region of the seed showed that its transcript was present at a level comparable to the levels observed in germinating embryos at 24, 72, and 96 h of germination (Fig. 9A, B).

Analysis of the *PcPCNA-like1* gene expression revealed that the transcript was present at a very low level in the dry embryo (time 0; Fig. 9A). After 24 h of seed germination, the transcript level increased by several hundred fold and reached a stable level at 48 h and 72 h of germination, followed by a decrease to a lower level at 96 h of germination. Analysis of root, stem, and leaf tissues showed that expression of the *PcPCNA-like1* gene was at a very low level, whereas the transcript level in the micropylar region was comparable to the one observed in 24 h germinating embryos (Fig. 9A, B).

Discussion

The purpose of this work was to characterize PCNA coding genes of *Phaseolus coccineus*. PCNA is an important factor involved in many cellular processes: DNA replication, DNA repair, and cell cycle regulation. However, most data published on PCNA originate from studies on animal organisms (including human) and yeasts. Although the quantity of new experimental data that broaden our knowledge about the role of PCNA in plant cells has increased during recent years, many aspects of its function in plants still remains obscure.

During the course of this study, two putative PCNA coding cDNAs were identified. The degree of identity at the amino acid level was over 50%. The theoretically calculated molecular mass and the isoelectric point of both proteins were similar (the molecular mass was around 30 kDa and the p*I* value ~5), corresponding to PCNA from other organisms (human, accession number: CAC27344; mouse, accession number: P17918; rat, accession number: NP_071776; pea, accession number: CAA76392; *Arabidopsis*, accession number: Q9M7Q7). It is interesting that the level of identity between amino acid sequences of identified *Pc*PCNA1 and *Pc*PCNA-like1 was lower than that between *Pc*PCNA1 and human PCNA. In this context, it is especially striking that if identified *Pc*PCNA1 and *Pc*PCNA-like1 act as eukaryotic PCNA proteins, one could expect higher identity between these two proteins than the identity between PCNA proteins originating from evolutionary distant organisms.

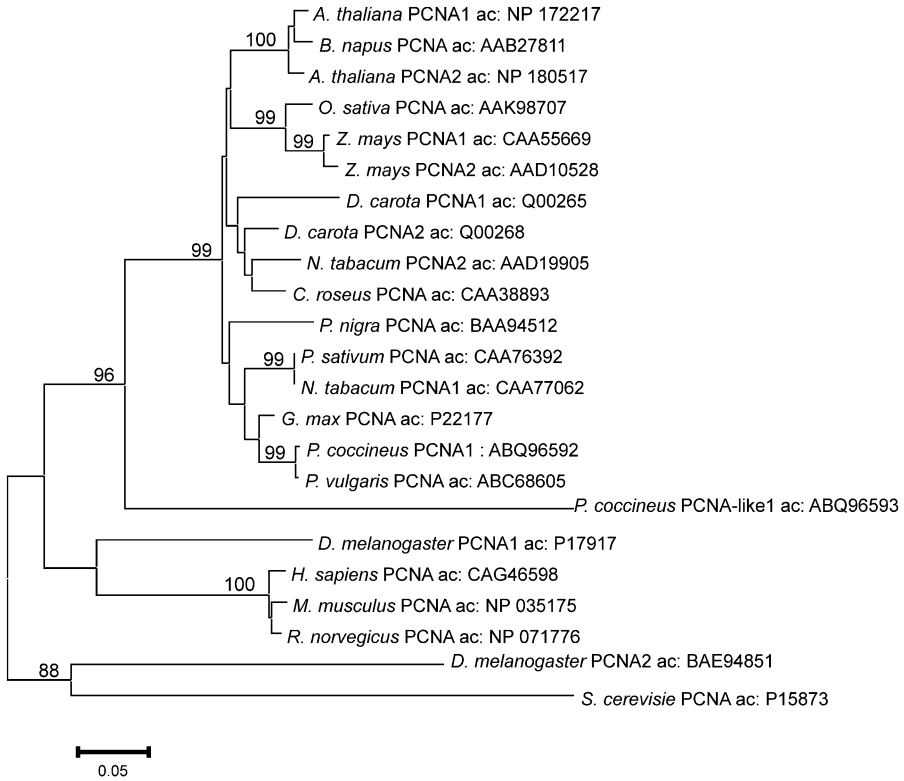

Fig. 10. Phylogenetic tree of PCNA constructed by the Neighbor–Joining method based on amino acid (aa) sequences from *P. coccineus* and other selected eukaryotic organisms was created using MEGA 3.1 software (Kumar *et al.*, 2004). The scale bar represents 0.05 substitutions per site, and the numbers next to the nodes are bootstrap values from 100 000 replicates. Values equal to or higher than 80% are shown.

PCNA, as an important replication factor and cell cycle regulator, was shown to have several conserved motifs and residues, such as the residue D^{41} responsible for the stimulation of DNA polymerase delta and the efficient stimulation of the RF-C ATPase activity (Ayyagari *et al.*, 1995; Fukuda *et al.*, 1995), a motif I (mammals: $Q^{125}L^{126}G^{127}I^{128}$, plants: $H^{125}L^{126}G^{127}I^{128}$) that is essential for binding of p21 and polymerase delta (Gulbis *et al.*, 1996; Jonsson *et al.*, 1998; Zhang *et al.*, 1998), a motif II ($V^{188}D^{189}K^{190}$) conserved within plants and vertebrates (Jonsson *et al.*, 1998), and a motif III ($L^{251}A^{252}P^{253}K^{254}$) responsible for proper folding of PCNA (Jonsson *et al.*, 1998). By analysing the linear amino acid sequence of both *Pc*PCNA1 and *Pc*PCNA-like1 proteins, all the above listed motifs characteristic for PCNA were identified in the *Pc*PCNA1 protein (Fig. 1). In contrast to *Pc*PCNA1, none of the motifs was found in *Pc*PCNA-like1. The lack of these motifs in *Pc*PCNA-like1 may be responsible for different biochemical properties of this protein compared with *Pc*PCNA1.

Interestingly, it could be shown that human PCNA and *Pc*PCNA1 effectively stimulated the activity of human DNA polymerase delta, whereas *Pc*PCNA-like1 did not exhibit any stimulatory effect on this enzyme. Although human PCNA used in the same dose as *Pc*PCNA1 had a greater stimulating impact on the activity of human DNA polymerase delta as compared to *Pc*PCNA1, such a phenomenon is not surprising, a similar observation was also reported when the biological activity of the yeast proliferating cell nuclear antigen was tested using human DNA polymerase delta (Bauer and Burgers, 1988). PCNA function is much conserved among eukaryotes; however, there might be some subtle differences in human/plant/yeast PCNA and human polymerase delta interactions resulting from slight differences in the protein surface charge and structure.

Gel filtration analysis of the purified recombinant *Pc*PCNA1 and *Pc*PCNA-like1 proteins performed under native conditions clearly showed that *Pc*PCNA1 formed a homotrimer, and this feature is known to be necessary for its biological activity. Contrary to *Pc*PCNA1, *Pc*PCNA-like1 could only be detected as a monomer. This finding indicates that this protein is unlikely to function as a sliding clamp itself. However, it cannot be excluded that, although *Pc*PCNA-like1 was not able to form a homotrimeric ring, it might be involved in the formation of a heterotrimeric ring around DNA. Such a phenomenon was described previously for archaeons *Sulfolobus solfataricus* and *Aeropyrum pernix*, in which three different PCNA proteins were found (Dionne *et al.*, 2003; Imamura *et al.*, 2007). Despite the low sequence similarities (less than 25% identity), PCNAs from *S. solfataricus* and *A. pernix* showed some analogous features. In both species, PCNAs formed a heterotrimeric ring structure. However, in the case of *S. solfataricus*, none

of these proteins could itself form a homotrimer (trimer formation occurred only in the presence of three different PCNA proteins; Dionne *et al.*, 2003), whereas *A. pernix* PCNA2 could form a trimeric structure both by itself (a homotrimer) and with PCNA1 and PCNA3 proteins (a heterotrimer), while neither PCNA1 nor PCNA3 of *A. pernix* could form a homotrimer (Imamura *et al.*, 2007). Moreover, it was shown that archaeal PCNA monomers exhibited different substrate interaction specificities, indicating that each PCNA is responsible for attracting different replication-related proteins to the replication fork (Dionne *et al.*, 2003; Imamura *et al.*, 2007). Similar features may characterize *Phaseolus coccineus* PCNAs as well. On the other hand, Sakaguchi's group identified *Drosophila melanogaster Dm*PCNA2 showing 51.7% identity to *Dm*PCNA1 (Ruike *et al.*, 2006); and such a low similarity was also observed for *Pc*PCNA1 and *Pc*PCNA-like1. *Dm*PCNA1 showed all features typical of PCNA, similarly to *Pc*PCNA1 (Henderson *et al.*, 1994). *Dm*PCNA2 contains D^{41} and motif III, but its motifs I and II are incomplete. However, *Dm*PCNA2, in contrast to *Pc*PCNA-like1, was capable of forming a homotrimer and stimulating the DNA pol delta activity. Differences in the expression pattern of *DmPCNA1* and *DmPCNA2* genes in response to UV treatment suggested that *Dm*PCNA2 may function as an independent sliding clamp of *Dm*PCNA1 during DNA repair (Ruike *et al.*, 2006). In another organism containing two *PCNA* genes, *Toxoplasma gondii*, both gene products also contain D^{41} and motif III and are able to form homotrimers (Guerini *et al.*, 2000). However, only *Tg*PCNA1 probably serves as the major replisomal PCNA, whereas *Tg*PCNA2 probably exhibits a different function (Guerini *et al.*, 2005). In fact, no actual (specific) function could be shown for *TgPCNA2*, since disruption of its gene did not influence the DNA polymerase activity, the response to chemical mutagens or the recombination frequency (Guerini *et al.*, 2000). Recent studies on *Arabidopsis* PCNA1 and PCNA2 revealed that these proteins showed very high similarity in their amino acid sequence as well as their ability to interact with *Arabidopsis* DNA polymerase eta and human p21 (Anderson *et al.*, 2008; Strzalka *et al.*, 2009). However, only *AtPCNA2*, but not the *AtPCNA1* gene, was able to trigger restoration of normal UV resistance and mutation kinetics in the yeast *rad30* mutant expressing the *Arabidopsis POLH* gene (yeast *Rad30* and *Arabidopsis POLH* genes encode DNA polymerase eta; Anderson *et al.*, 2008). In addition, *AtPCNA1* and *AtPCNA2* genes showed slightly different expression patterns in response to the exposure of *Arabidopsis* plants to heavy metal cadmium ions which cause genotoxic effects (Liu *et al.*, 2009). These findings indicate that in eukaryotic cells the second PCNA protein may indeed exert functions different from those of PCNA1.

In addition to a stimulatory function that PCNA exerted on DNA polymerase delta during DNA replication and repair, this protein is also involved in the regulation of the cell cycle through its interaction with the p21/WAF1 protein. p21 is known to function as a p53-dependent cyclin-dependent kinase inhibitor, thus enabling cells to survive exposure to DNA damaging factors such as UV radiation (Maeda *et al.*, 2002). Following the previously published results of Ball and Lane (1990) who demonstrated that the p21 peptide was able to precipitate pea PCNA from a crude extract, p21 interactions were analysed with *Pc*PCNA proteins and showed that *Pc*PCNA1 was co-precipitated with the p21 peptide, thus confirming their interaction, whereas the *Pc*PCNA-like1 protein did not interact with the peptide most likely due to the lack of motif I in *Pc*PCNA-like1. Another piece of evidence confirming differences between *Pc*PCNA1 and *Pc*PCNA-like1 in the structure and function was provided by Western blot analysis with the anti-human PCNA monoclonal antibody (PC10). This antibody can not only be used for the detection of human PCNA, it was also shown to recognize PCNA proteins originating from other animal organisms such as mouse and rat and from plants (e.g. pea; Ball and Lane, 1996). An epitope recognized by the PC10 antibody can be found in PCNA isolated from mammalian organisms and in PCNA from plant species, including soybean, *Arabidopsis thaliana*, and *P. coccineus* (*Pc*PCNA1; Table 1). Although sequences of this epitope are not identical, they differ only in 1 or 2 amino acids. On the contrary, the sequence of the *Pc*PCNA-like1 epitope is only similar but not identical to sequences of other PCNA epitopes, and a degree of differentiation is obviously too high for *Pc*PCNA-like1 to be recognized by the PC10 antibody. Since *Pc*PCNA-like1 does not exhibit any features required for its function as the typical PCNA, it is likely that the *Pc*PCNA-like1 protein possesses another as yet unknown function in *P. coccineus* cells. Further experiments need to be done to shed more light on the functions of *Pc*PCNA1 and *Pc*PCNA-like1 by analysing their localization in the plant cell. These proteins (or at least *Pc*PCNA1) are expected to function in the plant cell nucleus, although no obvious conserved nuclear localization signals (NLS) could be found in the amino acid sequence of these proteins.

Table 1. PC10 epitope sequences of PCNA proteins from selected eukaryotic organisms

Sequence of PC10 epitope	PCNA proteins[a]
SDYEMKLMDL	*Homo sapiens* PCNA
SDYEMKLMDL	*Mus musculus* PCNA
SDYEMKLMDL	*Rattus norvegicus* PCNA
SDFEMKLMDI	*Pisum sativum* PCNA
SDFEMKLMDI	*Glycine max* PCNA
ADFEMKLMDI	*Arabidopsis thaliana* PCNA1 and PCNA2
SDFEMKLMDI	*Phaseolus. coccineus* PCNA1
SNFEMRLVDI	*Phaseolus coccineus* PCNA-like1

[a] PC10 epitopes of human (accession number: CAC27344), mouse (accession number: P17918), rat (accession number: P04961), pea (accession number: CAA76392), soybean (accession number: P22177), *Arabidopsis* (accession numbers: NP172217 and NP180517), and runner bean PCNA1 (accession number: ABQ96591) proteins as well as PC10-like epitope of runner bean PCNA-like1 (accession number: ABQ96593) protein were compared.

Analysis of the relative expression of *PcPCNA1* and *PcPCNA-like1* genes using real-time RT-PCR gave us the opportunity to study expression patterns of both genes in *P. coccineus* plants at the early stages of plant development and in mature plant organs. It was observed that these patterns were generally similar; analogous observations have been reported for other plant species. The data from the AtGenExpress atlas show that in non-stressed *A. thaliana* plants both *AtPCNA1* and *AtPCNA2* genes have a similar expression pattern (Schmid *et al.*, 2005). No significant differences in the expression pattern of maize *ZmPCNA1* and *ZmPCNA2* genes could be observed by Hussey's group, although the level of each transcript between the samples tested was slightly varied (Lopez *et al.*, 1997), similar to our observations for *PcPCNA1* and *PcPCNA-like1*. It was found that at the beginning of germination, *Pc*PCNA1 and *Pc*PCNA-like1 transcripts were present at low levels, whereas the expression of both genes was up-regulated during the first stage of germination and then down-regulated during the late phase of germination. Studying the expression pattern of *PcPCNA1* and *PcPCNA-like1* in plant organs, it was noticed that in root, stem, and leaf tissues, the level of both transcripts was very low, contrary to their level in the micropylar region where these genes were actively expressed. The observed increase in *PcPCNA1* and *PcPCNA-like1* expression in the embryonic axis during seed germination and in the developing embryo from the micropylar region of developing seeds was related to intensive cell proliferation. As cell proliferation is accompanied by DNA replication, an increase in *PcPCNA*s expression is most likely due to the resumption of DNA replication. The observed decrease in the level of *PcPCNA* transcripts at the later stages of germination when young seedlings are formed is most likely due to the shift in the ratio between dividing (meristematic) and non-dividing cells towards the latter ones. Low expression levels of *PcPCNA* genes in mature plant organs confirm a correlation between *PCNA* expression and cell proliferation/DNA replication as these organs predominantly contain non-dividing cells. *PCNA* expression at the early stages of seed germination could also be related to DNA repair that occurs throughout the nearly entire period of germination and decreases significantly before cell proliferation begins. However, *PcPCNA*s expression due to DNA repair may be low and comparable to the level in dry embryos because of a small number of cells in the embryo at this stage.

A correlation between *PCNA* gene expression and cell proliferation was also observed in other plant species. *ZmPCNA1* and *ZmPCNA2* genes were expressed in root and shoot tips as well as in young spikelets and cobs but not in leaves, old spikelets, and pollen. These results were confirmed by the analysis of *PCNA* expression in rice. It has been shown that the transcript was intensively produced in roots and in root tips but not in mature leaves where it was undetectable (Kimura *et al.*, 2001). Also, the data presented by Shimizu and Mori who studied levels of PCNA transcripts in dormant auxiliary buds confirmed the correlation between *PCNA* gene expression and cell proliferation. They demonstrated that, before decapitation, the level of the transcript in dormant auxiliary buds was very low, whereas after decapitation, *PCNA* gene expression in pea was remarkably up-regulated, which correlated with bud growth and thus, with cell proliferation (Shimizu and Mori, 1998*a*, *b*).

Many attempts were undertaken in order to estimate the number of *PCNA* genes in plant genomes. However, due to plant genome complexity, the clearest results were obtained for *Arabidopsis thaliana* and *Oryza sativa,* and they originated as a result of the completion of the genome sequencing projects. In our studies by employing a PCR technique using degenerated primers, the presence of at least three *PCNA-like* genes in the genome of *P. coccineus* was demonstrated. In addition, Southern blot analysis revealed that, in the genome of *P. coccineus,* there are at least two sequences that are highly similar to *Pc*PCNA1 as well as to *Pc*PCNA-like1 cDNAs. However, high similarity between nucleotide fragments coding for the ORF of *Pc*PCNA1 and *Pc*PCNA-like1 could cause recognition of the *Pc*PCNA-like1 sequence by the *Pc*PCNA1 probe and vice versa. Based on these results, it is suggested that more than two sequences or genes similar to the *PcPCNA1* or *PcPCNA-like1* genes are present in the genome of *P. coccineus*. As gene duplication is a common mechanism and source of the evolutionary variability of eukaryotic genomes, it cannot be excluded that if *PcPCNA* gene duplication occurred, the *PcPCNA-like1* gene evolved separately from the *PcPCNA1* gene. As result, the *Pc*PCNA-like1 protein might have lost some functions of the ancestral *Pc*PCNA but retained and even gained other functions that are still unknown. It is theoretically possible that, after duplication, some functions of the ancestral *Pc*PCNA were split into presently existing *Pc*PCNA1 and *Pc*PCNA-like1. The mechanisms responsible for the preservation of duplicate genes have been debated for more than 70 years. Recently, Lynch and Force (2000) have proposed a new explanation: subfunctionalization–suggesting that, after duplication, two gene copies specialize to perform complementary functions. Two *PCNA* genes are present in the genomes of some but not all plant species, for example, in the genome of *Arabidopsis thaliana* (Fig. 10). The *AtPCNA1* gene located on chromosome 1 and the *AtPCNA2* gene localized on chromosome 2 encode almost identical proteins. Moreover, both proteins have motifs characteristic for PCNA. Studies conducted on maize and carrot showed the presence of two *PCNA* genes and did not exclude the presence of more than two *PCNA* genes (Hata *et al.*, 1992; Lopez *et al.*, 1997). In all the cases known so far of plants containing two *PCNA* genes, high levels of the PCNA proteins amino acid sequence identity were observed: *A. thaliana*, 96.6%; *N. tabacum*, 97.0%; and *Z. mays*, 98.5%. Lower identity was observed only for *D. carota*, 63.0%, but this is mainly due to the presence of a >100 amino-acid-long C-terminal tail in *Dc*PCNA2; the identity level between the first 264 aa of *Dc*PCNA1 and *Dc*PCNA2 being 87.6%. In animals, one copy of the *PCNA* gene was found in the rat genome (Matsumoto *et al.*, 1987),

whereas one *PCNA* gene and several pseudogenes are present in mouse and human genomes (Almendral *et al.*, 1987; Ku *et al.*, 1989; Travali *et al.*, 1989; Yamaguchi *et al.*, 1991). Most pseudogenes are not functional and expressed, although a few exceptions from this rule are known, for example the *Makorni1-p1* pseudogene that is expressed in mouse cells and regulates the expression of the functional *Makorin1* gene by increasing the stability of a gene transcript (Hirotsune *et al.*, 2003). It cannot be excluded that in the genome of *P. coccineus* there are *PCNA* pseudogenes and *PcPCNA-like1* might be such a pseudogene. It is presumed that *PcPCNA-like1* is a functional gene because its cDNA contains a full ORF, by contrast to *Makorni1-p1* that has premature stop codons (Hirotsune *et al.*, 2003). Based on its genetic structure, *PcPCNA-like1* may definitely be excluded as a processed pseudogene. Analysis of *PcPCNA1* and *PcPCNA-like1* genomic sequences showed that both genes contain two introns and three exons. It would be interesting to study in the future whether the *PcPCNA1* or *PcPCNA-like1* introns play a role in the regulation of the expression of *PcPCNA*s genes. Such a phenomenon occurred in the human *PCNA* gene and, in this case, introns 1 and 4 were shown to regulate *HsPCNA* gene expression (Ottavio *et al.*, 1990; Alder *et al.*, 1992). The possibility cannot be exclude that *PcPCNA-like1* gene is expressed only at the transcript level, as some, although few, untranslated transcript containing ORF have been found in eukaryotic cells, i.e. *Sry* in the testes of adult mice (Capel *et al.*, 1993) and 22k48 cDNA of the *HIRA* gene in human cells (Pizzuti *et al.*, 1999). However, in contrast to the linear expressed form, unexpressed *Sry* transcript exists in a circular form, whereas 22k48 is composed of several tandemly arranged repeat elements. Such features have not been found for *PcPCNA-like1*. On the other hand, a few functionally transcribed and translated pseudogenes are known, for example, PsiCx43 and CRIPTO3 (Kandouz *et al.*, 2004; Sun *et al.*, 2008). If *PcPCNA-like1* is an expressed pseudogene, it encodes protein exerting functions different from those typical for PCNA. Alternatively, *PcPCNA-like1* may represent a pseudogene that encodes non-functional PCNA protein. It would also be the first *PCNA* pseudogene ever discovered in plants.

Finally, the PRINS technique was used to study the chromosomal localization of *PcPCNA1* and *PcPCNA-like1* genes. Using gene-specific starters (to eliminate the cross-reactivity between these two investigated sequences), it was demonstrated that both *PcPCNA1* and *PcPCNA-like1* localize in the submetacentric chromosomal region(s). However, due to the PRINS resolution, the copy number of *PcPCNA1* and *PcPCNA-like1* genes cannot be estimated. Even considering the data from both Southern blot and PRINS analyses, it is impossible to discriminate between three possibilities: (i) only one copy of the *PcPCNA1* and *PcPCNA-like1* gene is present in the genome of *P. coccineus*; or (ii) additional members of PCNA-like sequences are also present besides the identified *PcPCNA1* and *PcPCNA-like1* genes; or (iii) several copies of both *PCNA* genes are present in the genome of *P. coccineus.* If the latter possibility is true, these additional copies of the particular *PcPCNA* gene localize within the region of detected spots on chromosomes.

To conclude: two PCNA-like genes have been identified in the genome of *Phaseolus coccineus*. Although these genes show a number of common structural features and their expression patterns analysed at the transcript level are relatively similar, they encode two distinct proteins. The recombinant *Pc*PCNA1 protein showed biochemical features typical for all known PCNA proteins, allowing it to function in the DNA replication/repair and cell cycle regulation processes. None of these features was observed in *Pc*PCNA-like1. Since the *PcPCNA-like1* gene most probably encodes a functional protein, the *Pc*PCNA-like1 protein must exert as yet unknown functions, different from those of *Pc*PCNA1.

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