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 (PcPCNA1 and PcPCNA-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 PcPCNA1 and PcPCNA-like1 at the amino acid level was only 54%, with PcPCNA-like1 lacking motifs that are crucial for the activity typical of PCNA. Consequently, these two proteins showed different properties. PcPCNA1 behaved like a typical PCNA protein: it formed a homotrimer and stimulated the activity of human DNA polymerase delta. In addition, PcPCNA1 interacted with a p21 peptide and was recognized by an anti-human PCNA monoclonal antibody PC10. By contrast, PcPCNA-like1 was detected as a monomer and was unable to stimulate the DNA polymerase delta activity. PcPCNA-like1 also could not interact with p21 and was not recognized by the PC10 antibody. Our results suggest that PcPCNA-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 T4 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, 2009). This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
et al. (1987), rat (Matsumoto 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 (Suzuka et al., 1993). Moreover, the segments containing the microsporadic region of 3–5 mm long seeds (containing microsporium 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.

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 AtPCNA1 and AtPCNA2 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, PrPCNA1 and PcPCNA-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 embryos containing the microsporadic region of 3–5 mm long seeds (containing microsporium 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 PpPCNA1 and PcPCNA-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 microsporadic region of the seed were treated with calf intestinal alkaline phosphatase. Next, the sample was treated with tobacco acid phosphatase 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 PpPCNA1 and PcPCNA-like1 was done using specific reverse primers 5’-PpPCNA1R (5’-TGATTCCTAACCACACATTTTGGA(C/T)ATTGATAG(C/T)GA(A/G)CA(C/T)CATTGGG-3’) and 5’-PrPCNA1F (5’-AC(G/T)GAAAGAAA/ C)AATT(C/T)CTTA(A/G)ATACCTTAAACC-3’), respectively. Subsequently, nested specific reverse primers for reamplification of the obtained PCR products, 5’-PpPCNA1NR (5’-TTACCCTTGAGGTTCTCTTCTTC-3’) and 5’-PpPCNA1NR (5’-CTATGAGTGAGGTGGGTGAATGGG-3’), were used.

3’ RACE was performed similarly using 1 μg of total RNA and specific forward primers: 3’-PpPCNA1F (5’-AACCTTAACATTCTAACACGAAACC-3’) and 3’-PrPCNA1F (5’-TAATATCTAAACCACCACTTCTTGGG-3’), respectively. The PCR reactions were done in 50 μl volume containing: 1× PCR buffer (10 mM TRIS-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.0), 200 μM dNTPs, 1.25 units of SuperTag 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) thermocycler. 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 (PrPCNA1) and EF602034 (PpPCNA-like1).
Cloning of PcPCNA1 and PcPCNA-like1 genomic sequences

Amplification of genomic fragments encoding PcPCNA1 and PcPCNA-like1 was performed using genomic DNA extracted using the Genomic Maxi AX Kit (A&A Biotechnology). Gene-specific primers: 5'-PCNA1F (5'-AACCCTAACCCTTTGAAAGC- AAACC-3') and gPCNAIR (5'-AAGTATTCAATTTCGAC- TCCTGTCCTCAG-3') were used for PcPCNA1 amplification. Amplification of genomic PcPCNA-like1 was done using 3'- PCNA1F (5'-TATGGCTTCCAGACCTTAACACCC-3') and 5'-PCNAL1R (5'-AC(G/T)GAAAGAA(A/C)AAATC(T)C(TA(A/G)TTATCACCAA-3') primers. The reaction was done in 50 µl volume containing: 1× PCR buffer, 200 µM dNTPs, 1.25 units of SuperTaq 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 pTZ57/R/T vector (Fermentas) followed by sequencing. The nucleotide sequence data have been deposited in the NCBI GenBank under accession numbers: EF602033 (gPC-PcPCNA1) and EF602035 (gPC-PcPCNA-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, Finzymes), 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: 5'-18SRNA_RTPCRF (5'-CCAGGTCTCACAG-3') and 5'-18SRNA_RTPCR (5'-GAACCAATGGCACC-3') (Duval et al., 2002). PcPCNA1 gene-specific primers 3'-PCNA1F_RTPCR (5'-CATATGTGGATCTGCAAATTG-3') and 5'-PCNA1R (5'-TAATTCAATCTGAGCCT-3') were used. The reactions were performed using control 18S RNA gene-specific primers: 5'-18SRNA_RTPCRF (5'-CCAGGTCTCACAG-3') and 5'-18SRNA_RTPCR (5'-GAACCAATGGCACC-3'). The reactions were performed using control 18S RNA gene-specific primers: 5'-18SRNA_RTPCRF (5'-CCAGGTCTCACAG-3') and 5'-18SRNA_RTPCR (5'-GAACCAATGGCACC-3').

DNA isolation and Southern blot analysis

Genomic DNA was isolated from 96 h old seedlings using the Genomic Maxi AX Kit (A&A Biotechnology). The purified DNA (30 µg) was digested with BamHI, BgIII, EcoRI, HindIII or XbaI, 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 PcPCNA1 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 PcPCNA-like1 DIG-labelled probe (under the same conditions as used for the PcPCNA1 probe).

PCR

PCR reactions were performed in mixtures containing: 1× PCR buffer (Takara) with 2 mM MgCl2, 200 µM dNTPs, 2 µM of each primer, 1 unit of Takara Taq polymerase, and 50 ng of genomic DNA isolated from Phaseolus coccineus seedlings or plasmid pHZ7/RT 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: PcPCNAF (5'-GGCGAGTGTGCT(T/C/A/G)T(T/U)CCTGAAGAGG-3') and PcPCNAIR (5'-CC(A/G)A(T/C)CTGAAAT(T/C)TTGATF/ GTC-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 ice 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 in overnight at 37 °C. Frame-Seal Chambers (MJ Research, Inc.) were stuck to the slides. Gen specific primers: 3'-PCNA1F (5'-AACCCTAACC- CATTCTTAAACGAAACC-3') and gPCNAIR (5'-AAGTATTCAATCTGACCT- GGAACC-3') (Duvai et al., 2002), 2 µM each. The reactions were performed using control 18S RNA gene-specific primers: 5'-18SRNA_RTPCRF (5'-CCAGGTCTCACAG-3') and 5'-18SRNA_RTPCR (5'-GAACCAATGGCACC-3').

DNA labelling

DNA was isolated from 96 h old seedlings using the Genomic Maxi AX Kit (A&A Biotechnology). The purified DNA (30 µg) was digested with BamHI, BgIII, EcoRI, HindIII or XbaI, 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 PcPCNA1 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 PcPCNA-like1 DIG-labelled probe (under the same conditions as used for the PcPCNA1 probe).

Purification of recombinant PcPCNA1 and PcPCNA-like1 proteins

The open reading frames of PcPCNA1 and PcPCNA-like1 were amplified with specific sets of primers: PcPCNA1ORFr (5'-GGGAAATCCATATGCCATAGTCTGTCACAG-3') and PcPCNA1ORIr (5'-CGGGATCCATATGCCATATGTCTGTCACAG-3').
(5'-CGGGATCCCTATGATGGGATATGGG-3'), respectively. Next, the amplified products were cloned into *NdeI* BamHI 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 medium containing ampicillin (100 μg ml⁻¹) until OD₆₀₀ 0.6 was reached, and production of 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 Na₂HPO₄, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 1 mM PMSF (phenylmethylsulphonyl fluoride)], 0.05% Tween 20, pH 7.0 containing lysozyme (1 mg ml⁻¹), RNase A (10 μg ml⁻¹), DNase I (5 μg ml⁻¹), and sonicated (5 pulsés 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 NaCl in buffer B). The fractions containing the recombinant protein 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* (AtPCNA2)** was purified as described previously (Strzalka *et al.*, 2009).

**Gel filtration**

All the following procedures were performed at 4 °C. The purified proteins (PcPCNA1, PcPCNA-like1, and HsPCNA2) 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 a 2 ml HiTrap Q HP Sepharose 6 Fast Flow (Amersham) column, and the flow-through was collected and loaded onto a 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 TRIS-HCl, 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.

**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 washings with 1 ml of PBS each. The beads with bound peptide were incubated with 1 μg of the recombinant protein (PcPCNA1, PcPCNA-like1, or HsPCNA) 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 Comassie staining.

**Western blotting and immunodetection**

One μg of recombinant PcPCNA1, PcPCNA-like1, and HsPCNA 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 (Immunon, 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 MgCl₂, 20 μM [³H]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, PcPCNA1 or PcPCNA-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 PcPCNA1 and PcPCNA-like1**

Rapid amplification of 5' and 3' cDNA ends (RACE) techniques were employed for the amplification of full-length cDNAs encoding PcPCNA1 and PcPCNA-like1. The primers used for the identification of PcPCNA1 and PcPCNA-like1 cDNAs were designed based on the analysis of *Phaseolus coccineus* EST fragments deposited in the National Centre for Biotechnology Information (NCBI). The microsomal region of seeds containing an active suspensor was used as a source of total RNA. The identified cDNA sequence of PcPCNA1 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 pl = 4.69. PcPCNA-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 pl = 5.0. An alignment of *P. coccineus* PcPCNA1 and PcPCNA-like1 showed that the identity between these two amino acid sequences was 54.5% (Fig. 1). Alignment analysis of PcPCNA1 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 PcPCNA-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 phylogenetic tree (see Discussion and Fig. 10).
Genomic sequences encoding *PcPCNA1* and *PcPCNA-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 *PcPCNA1* open reading frame (ORF) and the second one was complementary to *PcPCNA-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*II, *Bgl*II, *Eco*RI, *Hin*dIII, *Xba*I) (Fig. 2B).

The pattern obtained with the *PcPCNA-like1* probe was different from and more complex than the pattern obtained with the *PcPCNA1* 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 *PCNA-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 *PcPCNA* proteins**

To compare biochemical characteristics of *PcPCNA1* and *PcPCNA-like1*, recombinant proteins were produced in *E. coli* with short N-terminal His-tags which were shown...
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 
PcPCNA1 and 
PcPCNA-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 
PcPCNA proteins, gel filtration chromatography on Superdex 200 was employed. This analysis demonstrated that the 
PcPCNA1 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 
PcPCNA1 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 
PcPCNA-like1, the formation of the trimeric
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*PCNA 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 PcPCNA proteins with a fragment of the human p21 protein, an affinity-precipitation assay was applied, using p21-streptavidin-agarose beads. Analysis of PcPCNA1 and PcPCNA-like1 interactions with the p21 peptide showed that only PcPCNA1 was able to bind to the p21 peptide specifically, similarly to human PCNA protein (Fig. 7). By contrast, no binding of PcPCNA-like1 to the p21 peptide was observed (Fig. 7). In addition, analysis of the PcPCNAs 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 HsPCNA and PcPCNA1, but not PcPCNA-like1, was recognized by the PC10 antibody (Fig. 8).

Expression of PcPCNA genes at early stages of plant development

A real-time RT-PCR technique was used to evaluate the relative levels of PcPCNA1 and PcPCNA-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 PcPCNA1 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
observed after 24 h. Next, the $Pc$ 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 pI 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 $PcPCNA1$ and $PcPCNA-like1$ was lower than that between $PcPCNA1$ and human PCNA. In this context, it is especially striking that if identified $PcPCNA1$ and $PcPCNA-like1$ 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.
PCNA, as an important replication factor and cell cycle regulator, was shown to have several conserved motifs and residues, such as the residue D41 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: Q125L126G127I128, plants: H125L126G127I128) 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 (V188D189K190) conserved within plants and vertebrates (Jonsson et al., 1998), and a motif III (L251A252P253K254) responsible for proper folding of PCNA (Jonsson et al., 1998). By analysing the linear amino acid sequence of both PcPCNA1 and PcPCNA-like1 proteins, all the above listed motifs characteristic for PCNA were identified in the PcPCNA1 protein (Fig. 1). In contrast to PcPCNA1, none of the motifs was found in PcPCNA-like1. The lack of these motifs in PcPCNA-like1 may be responsible for different biochemical properties of this protein compared with PcPCNA1.

Interestingly, it could be shown that human PCNA and PcPCNA1 effectively stimulated the activity of human DNA polymerase delta, whereas PcPCNA-like1 did not exhibit any stimulatory effect on this enzyme. Although human PCNA used in the same dose as PcPCNA1 had a greater stimulating impact on the activity of human DNA polymerase delta as compared to PcPCNA1, 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 PcPCNA1 and PcPCNA-like1 proteins performed under native conditions clearly showed that PcPCNA1 formed a homotrimer, and this feature is known to be necessary for its biological activity. Contrary to PcPCNA1, PcPCNA-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 PcPCNA-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

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**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.
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 DmPCNA2 showing 51.7% identity to DmPCNA1 (Ruike et al., 2006); and such a low similarity was also observed for PcPCNA1 and PcPCNA-like1. DmPCNA1 showed all features typical of PCNA, similarly to PcPCNA1 (Henderson et al., 1994). DmPCNA2 contains D41 and motif III, but its motifs I and II are incomplete. However, DmPCNA2, in contrast to PcPCNA-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 DmPCNA2 may function as an independent sliding clamp of DmPCNA1 during DNA repair (Ruike et al., 2006). In another organism containing two PCNA genes, Toxoplasma gondii, both gene products also contain D41 and motif III and are able to form homotrimers (Guerini et al., 2000). However, only TgPCNA1 probably serves as the major replisomal PCNA, whereas TgPCNA2 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, 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 PcPCNA proteins and showed that PcPCNA1 was co-precipitated with the p21 peptide, thus confirming their interaction, whereas the PcPCNA-like1 protein did not interact with the peptide most likely due to the lack of motif I in PcPCNA-like1. Another piece of evidence confirming differences between PcPCNA1 and PcPCNA-like1 in the structure and function was provided by Western blot analysis with the anti-human PCNA monoclonal antibody (PC10). This antibody cannot 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 (PcPCNA1; 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 PcPCNA-like1 epitope is only similar but not identical to sequences of other PCNA epitopes, and a degree of differentiation is obviously too high for PcPCNA-like1 to be recognized by the PC10 antibody. Since PcPCNA-like1 does not exhibit any features required for its function as the typical PCNA, it is likely that the PcPCNA-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 PcPCNA1 and PcPCNA-like1 by analysing their localization in the plant cell. These proteins (or at least PcPCNA1) 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 proteinsa |
|--------------------------|----------------|
| SDYEMKLMDL               | Homo sapiens PCNA |
| SDYEMKLMDL               | Mus musculus PCNA |
| SDYEMKLMDL               | Rattus norvegicus PCNA |
| SDFMKLMDI                | Pisum sativum PCNA |
| SDFMKLMDI                | Glycine max PCNA |
| ADFMKLMDI                | Arabidopsis thaliana PCNA1 and PCNA2 |
| SDFMKLMDI                | Phaseolus coccineus PCNA1 |
| SNFMELVDI                | 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 NP186517), 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, *PcPCNA1* and *PcPCNA-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 *PcPCNA1* 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, *PcPCNA1* 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, 1998a, 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 *PcPCNA1* as well as to *PcPCNA-like1* cDNAs. However, high similarity between nucleotide fragments coding for the ORF of *PcPCNA1* and *PcPCNA-like1* could cause recognition of the *PcPCNA-like1* sequence by the *PcPCNA1* 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 *PcPCNA-like1* protein might have lost some functions of the ancestral *PcPCNA* but retained and even gained other functions that are still unknown. It is theoretically possible that, after duplication, some functions of the ancestral *PcPCNA* were split into presently existing *PcPCNA1* and *PcPCNA-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 *DcPCNA2*; the identity level between the first 264 aa of *DcPCNA1* and *DcPCNA2* 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 Makorn1-p1 pseudogene that is expressed in mouse cells and regulates the expression of the functional Makorn1 gene by increasing the stability of a gene transcript (Hirotune 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 Makorn1-p1 that has premature stop codons (Hirotune 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 PcPCNAs 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 PcPCNA1 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 PcPCNA-like1. Since the PcPCNA-like1 gene most probably encodes a functional protein, the PcPCNA-like1 protein must exert as yet unknown functions, different from those of PcPCNA1.

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