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Research article

Molecular cloning and biochemical characterization of a novel cystatin from *Hevea* rubber latex

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ABSTRACT

A novel cDNA encoding a cysteine proteinase inhibitor or phytocystatin was isolated from *Hevea brasiliensis* RRIM600 rubber latex cDNA library. The full-length *HbCPI* obtained from rapid amplification of cDNA ends contains 588 bp. An open reading frame of 306 bp encodes for a protein of 101 amino acids with the typical inhibitory motifs of phytocystatin superfamily, namely the central signature motif QXVXG, a GG doublet and LARFAV-like motifs in the N-terminal part, and conserved A/PW residues in the C-terminal region. Sequence comparison showed that the deduced amino acid sequence was similar to that of cysteine protease inhibitor from *Manihot esculenta* (84% identity). The *HbCPI* was subcloned into expression vector pQE-40 and then overexpressed in *Escherichia coli* M15 strain (pREP4) as a His-tagged recombinant protein with molecular mass approximately 13 kDa. The purified HbCPI showed thermal stable property and efficiently inhibited the protease activity of papain by non-competitive inhibition with K_i value of 15.4 nM. Beside latex, *HbCPI* also transcripted in leaf and young seed. The *HbCPI* message accumulation was induced by phytopathogenic fungi *Phytophthora palmivora* infection. These data suggest that HbCPI might play crucial roles in defense mechanism against biotic stimuli.

1. Introduction

Cysteine proteinase inhibitors or cystatins have been identified in vertebrates, invertebrates and plants. They contain three conserved sequence signatures which interact with the active site clefts of cysteine proteinases whereby an N-terminal region contains one or two glycine residues, a reactive site QXVXG that generally located in the central region of protein sequence and a tryptophan residue is near the C-terminal region [1,2].

Cystatins have been classified into four families on the basis of their primary structure characteristics, the presence and position of intrachain disulfide bonds, and the molecular mass of the protein. Family 1 or stefin family is a large group of small proteins and their molecular mass are approximately 11 kDa in size which lack disulfide bonds and carbohydrates. Family 2 or cystatins are small proteins of approximately 12–13 kDa containing two disulfide bonds towards their C-terminus. Family 3 or kininogens are large acidic proteins (45–65 kDa) containing one or several cystatin domains. Family 4 or phytocystatins have a molecular mass from 12 to 13 kDa with a consensus sequence [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N found within a predicted amino-terminal alpha-helix. Most phytocystatins do not contain free cysteine residues and lack disulfide bonds; however, some of them are considerably large and consist of several cystatin domains [2,3].

Plant cystatins or phytocystatins comprise more than 30 members from different monocotyledon and dicotyledon. They have been reported to be involved in the endogenous regulation of proteolysis during seed maturation and germination, and in programmed cell death [4]. Additionally, they also have a significant role in plant defensive processes against biotic and abiotic stresses [5].

In the present study, we cloned cDNA encoding a cysteine proteinase inhibitor from latex of rubber tree (*Hevea brasiliensis* RRIM600) and expressed as a His-tagged fusion protein in *Escherichia coli*. The inhibitory activity of the corresponding recombinant protein against papain and its thermal stability has been investigated. In addition, the expression pattern of *HbCPI* and intron–exon organization is also studied.

2. Results

2.1. Isolation and molecular characterization of Hevea cystatin

The sequence of *Hevea* cystatin was previously screened in latex cDNA library constructed by Chotigeat et al. [6]. The full-length

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cDNA obtained from rapid amplification of cDNA ends (RACE) contains 1004 bp with an open reading frame (ORF) of 306 bp beginning with a methionine codon (ATG) at nucleotide position 512 and ending with a TAG termination codon at nucleotide position 807. The theoretical 101 amino acid polypeptide has a calculated molecular mass of 11.2 kDa and predicted pI = 5.45. The possible glycosylation sites predicted by NetNGlyc software (ExPASy) were not found in this sequence. The *Hevea* cystatin cDNA sequence and deduced amino acid sequence has been submitted to the NCBI GenBank as accession number FJ850964. Sequence analysis with the BLAST algorithm of this deduced amino acid sequence has the highest similarity to cysteine protease inhibitor or phytocystatin from *Manihot esculenta* (GenBank accession no. AAF72202) at 84% (1^{e-42}).

Multiple alignments of HbCPI with other phytocystatins from other plants confirmed that phytocystatin from *M. esculenta* and HbCPI were most similar (Fig. 1). From the data, the polypeptide of phytocystatins and HbCPI contain conserve region which are responsible for cysteine protease inhibitory function. The typical inhibitory motifs include a GG doublet in the N-terminal region, the central signature motif QXVXG (where X is any amino acid), and W residue in the C-terminal part, about 30 amino acids distant from the central motif. In addition, a unique consensus motif, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N, was found at the N-terminal proximity of phytocystatins.

The comparison of cDNA sequence and PCR fragments amplified from genomic DNA shows one intron (317 bp) located between the sequences encoding the LARFAV-like motif and the reactive site QXVXG (Fig. 2). The phylogenetic dendogram (Fig. 3) constructed based on the deduced sequence of the mature cystatins retrieved from Genbank to investigate the evolutionary relationship among the cystatins of different species, clearly indicated that the HbCPI was closely related to *M. esculenta* cystatin (GenBank accession no. AAF72202).

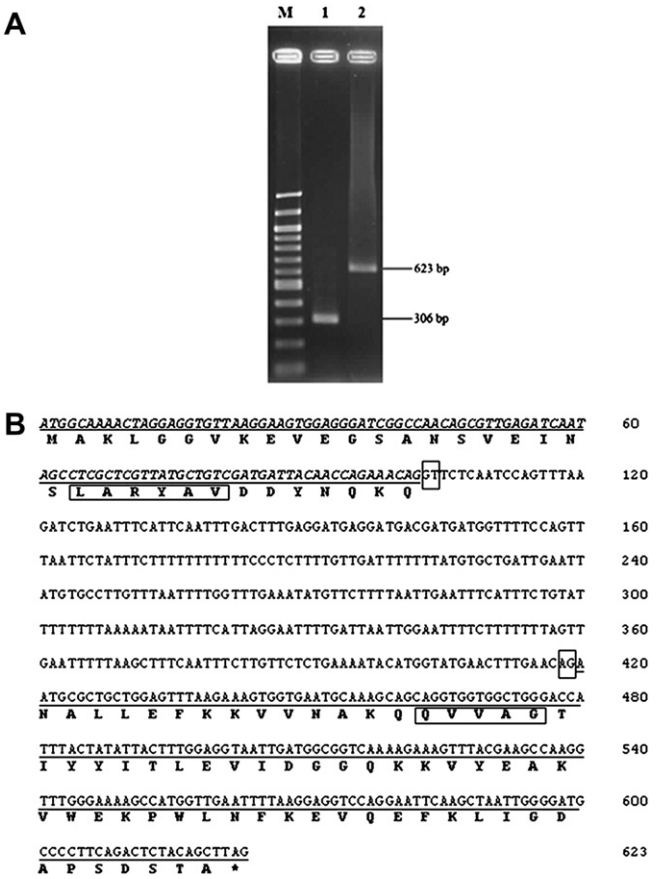

Fig. 2. Exon–intron organization of the cysteine proteinase inhibitor of *Hevea* rubber latex. The comparison of *Hevea* rubber latex (*HbCPI*) gene obtained from total RNA and genomic DNA (Fig. 2A, M: 100 bp ladder, 1: RT-PCR product amplified from total RNA, 2: PCR product amplified from genomic DNA). The *HbCPI* gene contains two exons (underlined letter) and one intron (Fig. 2B). The LARFAV-like motif and QXVXG, and the typical GT/AG sequences are boxed.

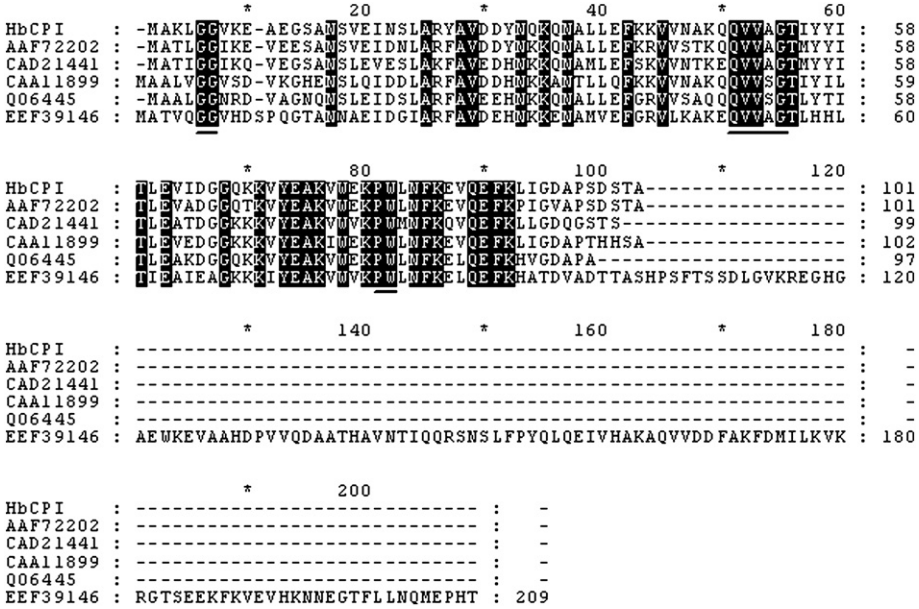

Fig. 1. Comparison of the amino acid sequence of *Hevea* rubber latex cysteine proteinase inhibitor with those of others phytocystatins. The cysteine proteinase inhibitor of *Hevea* rubber latex (HbCPI) was aligned to the phytocystatins from *Ricinus communis* (GenBank accession no. EEF39146), *Manihot esculenta* (GenBank accession no. AAF72202), *Rumex obtusifolius* (GenBank accession no. CAD21441), *Castanea sativa* (GenBank accession no. CAA11899) and *Vigna unguiculata* (GenBank accession no. Q06445). The sequences were aligned by Clustal algorithm. The dash represents a gap at the indicated proteins. The primary reactive site GG and the consensus sites of the secondary contact, QXVXG and PW are underlined.