

Research article

Cloning and characterization of a cowpea seed lipid transfer protein cDNA: expression analysis during seed development and under fungal and cold stresses in seedlings' tissues

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Received 10 April 2006; accepted 20 September 2006

Available online 09 October 2006

Abstract

Lipid transfer proteins (LTPs) are antimicrobial peptides (AMPs) involved in the defense of plants against pathogens. Our group has previously characterized and purified a LTP from cowpea (*Vigna unguiculata* (L.) Walp.) seeds which caused the inhibition of growth of fungal pathogens in vitro. The aim of this work was to obtain the cDNA encoding the cowpea LTP and after cloning, to use the cDNA as a probe for studying its expression profile during the development of cowpea seeds. In this work, the N-terminal sequence of the mature LTP peptide from cowpea was used to produce a degenerated oligonucleotide. This primer allowed the amplification of the LTP cDNA by RT-PCR from mRNA of cowpea seeds. The sequence analysis of the cloned cDNA, named *VULTP*, showed 494 bp which encoded a polypeptide of 91 amino acids. The deduced peptide presented high homology of similarity to plant LTPs of *Vigna radiata* var. *radiata* (94%), *Prunus domestica* (82%) and *Zea mays* (72%). The expression profile of the *VULTP* gene in cowpea was analyzed by Northern blot and revealed that the transcript is not accumulated in adult tissues. Conversely, *VULTP* mRNA is early and strongly accumulated during seed development. The results obtained to seedling of cowpea demonstrate that the *VULTP* gene presents differential expression in response to different stress. Further studies will be conducted to try to gain better understanding about the physiological role of this gene in cowpea.

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Keywords: *Fusarium oxysporum*; Northern analysis; RT-PCR; LTP; Seed germination; *Vigna unguiculata*

Abbreviations: AMP, antimicrobial peptide; DAP, days after pollination; Fop, *Fusarium oxysporum* f. sp. *phaseolus*; IEF, isoelectric focusing; LTP, lipid transfer protein.

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1. Introduction

Among the set of strategies evolved by plants to defend themselves against pathogens, an important aspect of the defense response is the production of antimicrobial peptides (AMPs). This common strategy is shared with a wide range of eukaryotic organisms like mammals and insects [1–3].

Lipid transfer proteins (LTPs) are among the AMPs produced by plants with two families characterized in plants. The LTP₁ family composes small, basic peptides of 90 to 94 amino acids, arranged in a three-dimensional structure formed by four α -helices with a long carboxy-terminal tail, generally devoid of defined secondary structure, except by the presence of one turn of 3₁₀ helix [4,5]. The most striking structural characteristic is an internal hydrophobic cavity in the peptide structure, whose volume is variable depending on the loaded molecule. Through this flexible cavity, LTP₁ are capable of reversibly binding and transporting a sort of hydrophobic molecules such as phospholipids, acyl CoA and fatty acids of different sizes [5–8].

The biological role fulfilled by LTP₁ in plants is still an unsolved question, but from previously reported data it might be suggested their involvement in cutin synthesis [8,9], β -oxidation [10], plant defense signaling [11–13] and plant defense against phytopathogens [14–18] which seems to have the stronger body of evidence. LTP₁ extracted from leaves and seeds of several plants have been demonstrated to inhibit fungal and bacterial phytopathogens [14–18]. The mechanism of fungal growth inhibition remains unclear but LTP₁ are able to permeabilize fungal plasma membrane to fluorescent dye [19], which possibly indicates that the permeabilization may be involved in the process of fungal growth arrest. Besides the inhibitory activity in vitro, the transgenic expression of LTP genes has been reported to confer enhanced tolerance to bacterial and fungal infection [20,21].

LTP₁ are in general codified by several genes which belong to a multigenic family, as demonstrated to *Arabidopsis* and rice [22,23]. Differential transcription levels of LTP genes have been shown in a variety of plants and plant tissues during diverse developmental stages and physiological conditions [22–24]. LTP₁ genes are also responsive to environmental changes such as drought, cold, salt stress and also infection with bacterial and fungal pathogens [25,26]. Signal molecules such as abscisic acid, salicylic acid, ethylene and methyl jasmonate are involved in the signaling pathway responsible for the expression of LTP genes [21,25,27,28].

The cowpea (*Vigna unguiculata* L. Walp.) is a tropical legume of West Africa origin and is an important cultivated bean in most tropical regions of the world. Their seeds represent the principal protein source on the daily diets for the most part of poor populations of these regions. Diseases caused by viruses, bacteria, fungi and nematodes constitute important constraints to production in all cultivated areas [29]. Thus, studies to improve agronomic and nutritional traits of cowpea are of main relevance [29,30]. Our group has previously characterized and purified a LTP from cowpea seeds which presents the ability to inhibit the growth of phytopathogens in vitro [17].

The aim of this work was to obtain the cDNA encoding the cowpea LTP and after cloning, to use the cDNA as a probe for studying its expression profile during the development of cowpea seeds. Its expression was also analyzed in different tissues of adult cowpea plants under normal physiological conditions. In addition, the expression of this gene was investigated under stress conditions imposed by fungal infection and cold in tissues of cowpea seedlings during the first 96 h after germination.

2. Results

2.1. Cloning and sequence analysis of the VULTP cDNA

A primer was designed based upon the amino acid sequence of the previously isolated mature LTP from cowpea seeds [17]. The RT-PCR amplification of mRNA of cowpea seeds resulted in a fragment which was cloned into the pCR2.1-TOPO vector and sequenced. The cloned fragment presents 494 bp and encodes a predicted peptide of 91 amino acids (Fig. 1). The VULTP cDNA sequence lacks AUG initiation codon which is consistent with the fact that this cDNA was amplified using a primer specific for the mature peptide, i.e. without signal peptide. By using the Computer pI/Mw Tool (<http://www.expasy.org>), the calculated isoelectric point (pI) and molecular weight of the deduced LTP were inferred to be 9.3 and 9244.68 Da, respectively. Both characteristics are shared by other plant LTP₁ family [23,31]. The sequence of the deduced peptide was compared at a data bank with known proteins and showed homology of 94%, 82% and 72% with LTP₁ from *Vigna radiata* var. *radiata*, *Prunus domestica* and *Zea mays*, respectively (Fig. 2). In the sequence comparison, the conserved cysteine residues which play an important role in stabilization of the peptide global fold are clearly observed [31].

The phylogenetic relationship of the cowpea LTP to other members of the LTP family from diverse plant genomes is displayed in Fig. 3 and shows that cowpea LTP was grouped with *Phaseolus vulgaris* and *Cicer arietinum* (chickpea) LTPs, following the botanical systematic (Fig. 3, LTP underlined) and the same was observed to the other LTPs. This result is confirmed when other programs are used (data not shown).

2.2. Expression analysis of the cowpea LTP during seed development and in different tissues of seedlings under biotical and abiotical stresses

The temporal expression pattern of the cowpea VULTP was examined by Northern blotting of total RNA isolated from developing seeds and from tissues of adult plants. The densitometric analysis showed that at the 18th days after pollination (DAP) the VULTP expression is about 10% of the maximum level reached at 39 DAP, dropping approximately to 5.5%, 3.7%, 3.4% and 4.7% between the 21st and 30th DAP, and increasing to 14%, 33%, 100% at the 33, 36 and 39 DAP, respectively. The levels of accumulation dropped at the dry seed stage at 42 DAP (Fig. 4A, B).

ATGACGTGTGGGCAAGTTACAGGGTAACCTGGCACAGTGCATAGGGTTCCTTCAAAATGGT
 M T C G Q V Q G N L A Q C I G F L Q N G
 GGGATTGTTCCGCCAGCATGCTGCAACGGGGTGAAGAATATCCTTAACGGTGCCAGAACC
 G I V P P A C C N G V K N I L N G A R T
 ACCGCCGATCGCCGTGGCATTGTAGCTGCCTGAAGTCTGCTGCTGGGGCCGTTCTGTGGT
 T A D R R G I C S C L K S A A G A V R G
 CTCAATCCCGCTAATGCTCAGGCTCTCCCTGGGAAGTGTGGGGTCAACATACCCTGGAAG
 L N P A N A Q A L P G K C G V N I P W K
 ATCAGCAGCTCCACCAACTGTAACACCATCAATTGAAAGGGAGAAAGATTTGGATGATA
 I S S S T N C N T I N -
 ATGGCTAAGAGAAGCCATGCGTATACTACATCTTAGTAAAGAGAATAAAAGGAGGATAG
 ATTCTCCAAAGCTTCTTGTGTTTCTTTATCCCAGTGTGAAGTTTATACACTTCCTATG
 GTTTTTATGTGAAGCTTTATAAATTGAATAAAAGAAAAGTTTGATGTTGGAAAAAACTCAAA
 AAAAAAAAAAAAAA

Fig. 1. Nucleotide sequence of the 494 bp fragment amplified by RT-PCR using the primer designed from the mature cowpea seeds LTP sequence and cloned into the pCR2.1-LTP. The deduced amino acid sequence of the *VULTP* is shown below the nucleotide sequence. The stop codon (TGA) is in bold. The amino terminal sequence used for the primer design is shown in light gray.

	1	10	20	30	40	50		
<i>VULTP</i>	1 M T C G Q V Q G N L A Q C I G F L Q N -	- G G I V P P A - C C N G V K N I L N G A R T T A D R R G I C S C L						
<i>V. radiata</i>	1 M T C G Q V Q G N L A Q C I G F L Q K -	- G G V V P P S - C C T G V K N I L N S S R T T A D R R A V C S C L						
<i>P. domestica</i>	1 I T C G Q V S S N L A P C I N Y V K G -	- G G A V P P A - C C N G I R N V N N L A R T T A D R R A A C N C L						
<i>D. carota</i>	28 L T C G Q V T G A L A P C L G Y L R S Q V N V P V P L T -	C C N V V R G L N N A A R T T L D K R T A C G C L						
<i>O. sativa</i>	28 I S C G Q V N S A V S P C L S Y A R -	- - - G L R P S A A C C S G V R S L N S A A S T T A D R R T A C N C L						
<i>Z. mays</i>	29 I S C G Q V A S A I A P C I S Y A R G -	- Q G S G P S A G C C S G V R S L N N A A R T T A D R R A A C N C L						
<i>Ace-AMP1</i>	29 N I C P R V N R I V T P C V A Y G L -	- - - G R A P I A P C C R A L N D L R - F V N T R N L R R A A C R C L						

	60	70	80	90	I%	P%
<i>VULTP</i>	G L N P A - N A Q A L P G K C G - - V N I P W K I S S S T N C N T I N 91				83	94
<i>V. radiata</i>	G I N P N - N A E A L P G K C G - - V N I P Y K I S T S T N C N S I N 91				83	94
<i>P. domestica</i>	G V N P N - N A A A L P G K C G - - V N V P Y K I S A S T N C A T V 90				64	82
<i>D. carota</i>	G L N L N - A A A G L P A R C G - - V N I P Y K I S P T T D C N R V 119				53	67
<i>O. sativa</i>	G L N A G - N A A S I P S K C G - - V S I P Y T I S P S I D C S R V N 117				45	71
<i>Z. mays</i>	G N L A G - N A A S I P S K C G - - V S I P Y T I S T S T D C S R V N 120				52	72
<i>Ace-AMP1</i>	G L R R N P R F Q N I P R D C R N T F V R P F W W R P R I Q C G R I N 121				24	31

Fig. 2. Comparison of the complete amino acid (in single letter code) sequences of various plant LTP₁ with the predicted peptide of 91 amino acids deduced from the clone pCR2.1-LTP. I% indicates the percentage of identical residues and P%, the percentage of positive residues (with similar physical-chemical characteristics); both indicate the degree of homology of the LTP₁ with the deduced peptide from *V. unguiculata*; (-) gaps are included to optimize alignment; numbers above the sequence indicate the extension of the peptides in amino acid numbers and based on the sequence of *VULTP*; numbers flanking the sequences indicate the position of the amino acids in the sequence of the mature peptides and the following sequences (*Daucus carota*, *Oryza sativa*, *Z. mays* and *Ace-AMP1*) were deduced from cDNA clones with omission of the putative signal peptides; the lines underneath the sequences represent α -helices of secondary structure elements according to Lee et al. [5]; The name of the species, abbreviations and the accession number on data bank are shown as follows: *VULTP*—deduced peptide from the clone pCR2.1-LTP; *V. radiata* var. *radiata* LTP (gi25090821); *P. domestica* (gi2927015); *D. carota* LTP (gi128384); *O. sativa* LTP (gi2497747); *Z. mays* LTP (gi128388); *Ace-AMP1* (gi2497758)—*Allium cepa* antimicrobial protein 1.

In sharp contrast to the expression of the LTP gene in cowpea seeds, the cowpea LTP transcript was not observed to be expressed in roots, leaves and flowers of adult plants under normal physiological conditions (data not shown).

In cowpea seedling tissues, the expression of *VULTP* was detected in leaves and gradually increased from 48 to 96 h after germination. In leaves of *Fusarium oxysporum* f. sp. *phaseolus* (*Fop*) inoculated seedlings, transcript level peaked at 72 h fol-

lowed by repression of *VULTP* expression by this fungus. When leaves of seedlings were exposed to the cold stress, the *VULTP* expression was maintained constant but at lower levels than under the control temperature condition (Fig. 5A). The densitometric analysis reinforced this visual information and demonstrated the repression of 63%, 60% and 86% occurred in this tissue after fungal stress and 72.4%, 70% and 85% after cold stress, in respect to the control samples.

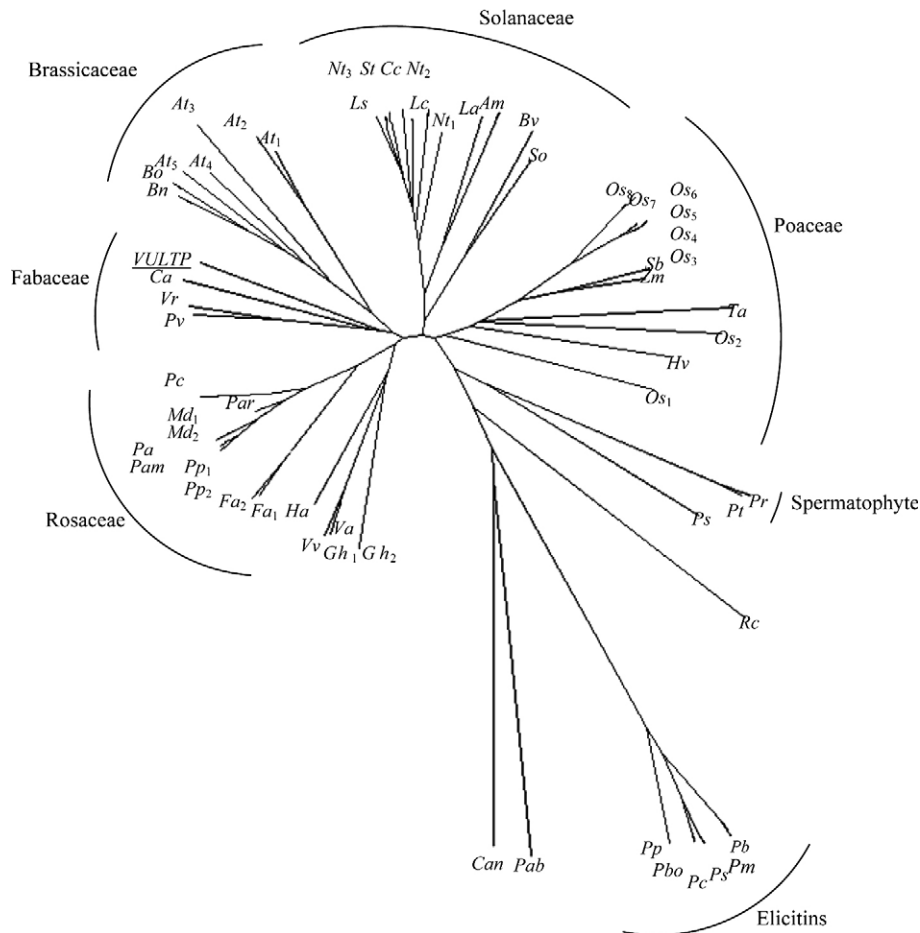


Fig. 3. Unrooted phylogenetic tree for known and putative LTPs of plants and elicitors from Oomycetes. Protein accession numbers are preceded by italic name of the species in which the peptide was identified. From plants: *At*₁ (Q9LLR6), *At*₂ (9LLR7), *At*₃ (Q9XFS7), *At*₄ (Q42589) and *At*₅ (Q9S7I3), *Arabidopsis thaliana*; *Am* (Q9AXF3), *Avicennia marina*; *Bv* (Q43748), *Beta vulgaris*; *Bn* (Q42614), *Brassica napus*; *Bo* (Q43304), *Brassica oleracea* var. *italica*; *Can* (Q6RK00), *Capsicum annuum*; *Cc* (Q5MJA3), *Capsicum chinense*; *Ca* (O23758), *Cicer arietinum*; *Fa*₁ (Q4PLT9) and *Fa*₂ (Q4PLU0), *Fragaria ananassa*; *Ha* (Q39950), *Gh*₁ (Q43129) and *Gh*₂ (Q42762), *Gossypium hirsutum*; *Ha* (Q39950), *Helianthus annuus*; *Rc* (P10975), *Ricinus communis*; *Hv* (Q42842), *Hordeum vulgare*; *La* (Q52RN7), *Leonurus artemisia*; *Ls* (P93224), *Lycopersicon esculentum*; *Lc* (Q3YMR2), *Lycopersicon chilense*; *Md*₁ (Q5GLH0) and *Md*₂ (Q5J026), *Malus domestica*; *Nt*₁ (Q42952), *Nt*₂ (Q8LK72) and *Nt*₃ (Q03461), *Nicotiana tabacum*; *Os*₁ (Q6ZX06), *Os*₂ (Q42976), *Os*₃ (Q42999), *Os*₄ (Q7XJ39), *Os*₅ (O22483), *Os*₆ (Q42978), *Os*₇ (O22485) and *Os*₈ (P23096), *Oryza sativa*; *Pab* (O22110), *Picea abies*; *Par* (P81651), *Prunus armeniaca*; *Pa* (Q9M5X8), *Prunus avium*; *Pam* (Q43017), *Prunus amygdalus*; *Pv* (O24440), *Phaseolus vulgaris*; *Pr* (Q9AXZ6), *Pinus resinosa*; *Pt* (Q41073), *Pinus taeda*; *Ps* (O24309), *Pisum sativum*; *Pp*₁ (Q5RZZ3) and *Pp*₂ (Q4VUZ0), *Prunus persica*; *Pc* (Q9M5X6), *Pyrus communis*; *So* (P10976), *Spinacia oleracea*; *St* (Q8H6L4), *Solanum tuberosum*; *Sb* (Q43194), *Sorghum bicolor*; *Ta* (P24296), *Triticum aestivum*; *Vr* (Q6WAT9), *Vigna radiata*; *VULTP*, LTP from *Vigna unguiculata*; *Vv* (Q850K5), *Vitis vinifera*; *Va* (Q6TKQ7), *Vitis aestivalis*; *Zm* (P19656), *Zea mays*; from elicitors, *Pbo* (Q5JBS4), *Phytophthora boehmeriae*; *Pb* (Q84JB1), *Phytophthora brassicae*; *Pc* (Q9T0M7), *Phytophthora cinnamomi*; *Pm* (Q8L690), *Phytophthora megasperma*; *Pp* (P41801), *Phytophthora parasitica* and *Ps* (Q84L73), *Phytophthora sojae*.

In the cotyledons of control seedlings a relatively strong expression of *VULTP* was detected, while in *Fop* infected material the transcript levels seemed to be repressed. In the cotyledons of seedlings stressed with cold, repression was observed at 48 h followed by induction of gene expression noticeable at 72 and 96 h (Fig. 5B).

In control roots, there was expression only at 96 h and, under cold stress, at 72 h. However both signals were relatively weak (Fig. 5C). Expression in the cold was observed 24 h ahead of control condition, indicating induction followed by repression at 96 h. It is important to mention that the control seedling tissues demonstrate the expression of the cowpea LTP gene during germination.

2.3. Expression of LTP in leaves of cowpea

Western blot demonstrated the presence of the LTP in leaves of seedlings under normal physiological conditions in all studied developmental stages and LTP was also detected in the adult leaves of cowpea (Fig. 6). The densitometric analysis demonstrated protein levels of 99.2%, 100% and 35% for the 48, 72 and 96 h studied times, respectively.

3. Discussion

Plant LTPs have considerable divergences in regard to the amino acid sequence, but some features are relatively con-

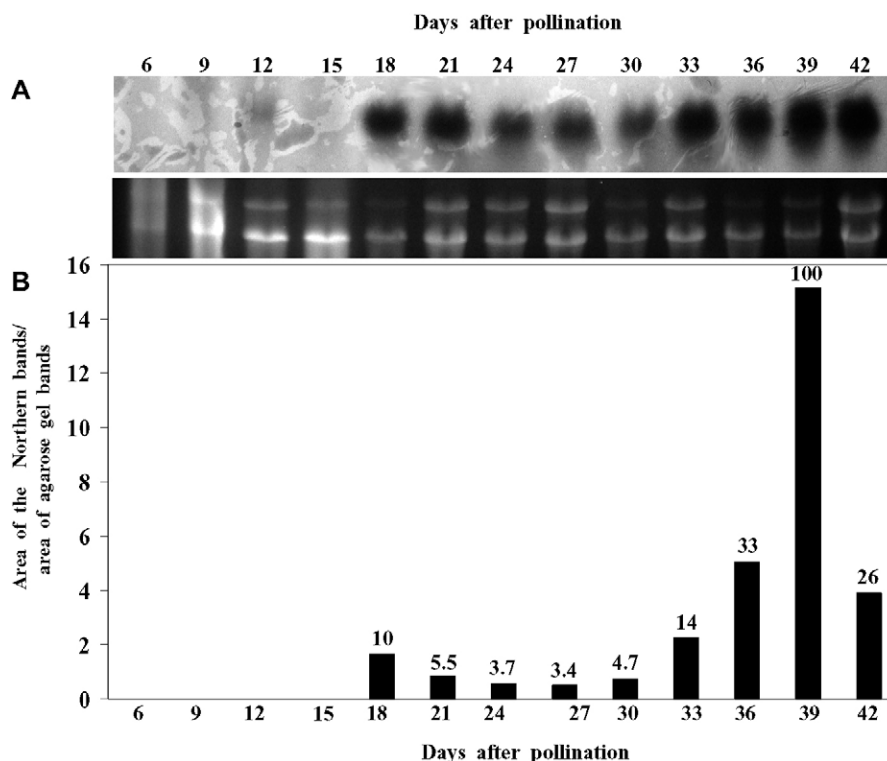


Fig. 4. (A) Northern analysis (upper panel) of the *VULTP* expression during the development of cowpea seeds from 6 to 42 (dry seeds) DAP. Total RNA (10 µg) was loaded onto the gel. Ethidium bromide stained rRNA bands are shown at the lower panel as a loading control. (B) Densitometric analyses of the Northern blotting. The numbers above the bars indicate the percentage of expression areas in regard to the expression area of the strongest band, in this case, 39 DAP, which was considered 100%.

served. Along the primary structure of LTP₁ family, eight cysteine residues are conserved in all sequences. They are linked to each other forming four disulfide bridges which are important to the stabilization of the peptide global fold and to the ability of binding and transporting hydrophobic molecules. The reduction of the disulfide bridges abolishes this ability [7]. The predicted peptide of 91 amino acids presents all of them at similar positions. In addition to the eight conserved cysteine, few residues are also conserved along the primary structure. They are Val₆, Pro₂₄, Asp₄₄, Pro₇₂, Arg₄₅, Arg₄₆, Leu₅₂, Ala₆₈ and aromatic residue at position 79. The predicted peptide presents all these conserved residues (Fig. 2). The alanine at position 68 has been shown to be involved in a hydrogen bond with a hydroxyl group of the ricinoleate (18:1, 9, 12-OH), with a possible role in cutin synthesis by transport of hydroxyl fatty acids such as cutin monomers [8,32]. The aromatic residue at the carboxyl terminal region, approximately at position 79, is positioned at the larger entrance of the hydrophobic cavity, in the three-dimensional structure [8]. In the case of maize LTP it was showed that a Tyr interacts with fatty acids and stabilizes the binding between the peptide and the hydrophobic molecule through a hydrogen bond, formed between the hydroxyl of the Tyr and the carboxyl group of the lipid polar head [8]. Future studies will analyze the relevance of the Trp₇₉ in the cowpea LTP.

The phylogenetic relationship of the cowpea LTP to other members of the LTP family from diverse plant genomes is displayed in Fig. 3. The phylogenetic tree shows that cowpea LTP

shares a common evolutionary origin based on conserved sequence and structural characteristics such as amino acid homology and conserved motifs with LTPs from Fabaceae species and thus was grouped with *P. vulgaris* and *C. arietinum* (chickpea), following the botanical systematic (Fig. 3, LTP underlined). This result is confirmed when other programs are used (data not shown). The other LTPs appear to be also grouped according to the botanical systematic, forming the clusters of the Solanaceae (*Nicotiana*, *Lycopersicon* and *Cap-sicum*), Brassicaceae (*Arabidopsis* and *Brassica*) and Rosaceae (*Prunus*, *Malus* and *Fragaria*). Some other LTPs seem to be more divergent and cannot be clustered consistently, such as the one from *Pisum sativum* (Fabaceae), grouped with LTPs from *Pinus taeda* and *Pinus resinosa*, both Spermatophytaceae. The other LTP from Spermatophytaceae, *Picea abies*, was grouped with the LTP from *Capsicum chinense*. These two were the most divergent peptides among the plant LTPs and seem to be more related with another class of lipid carrier protein, the elicitors, whose family forms an independent cluster.

Elicitors are peptides produced by species of phytopathogenic Oomycetes such as *Pythium* and *Phytophthora*. They resemble plant LTPs in some biochemical characteristics such as their small size (98 amino acids and 10 kDa), their basic pIs, the presence of three disulfide bonds with α -helix secondary structure and a hydrophobic pocket which endows them with the capacity to bind hydrophobic molecules, mainly sterols [33, 34]. Despite of these similarities the sequence identity is low between the two peptides, but at the tertiary level there are

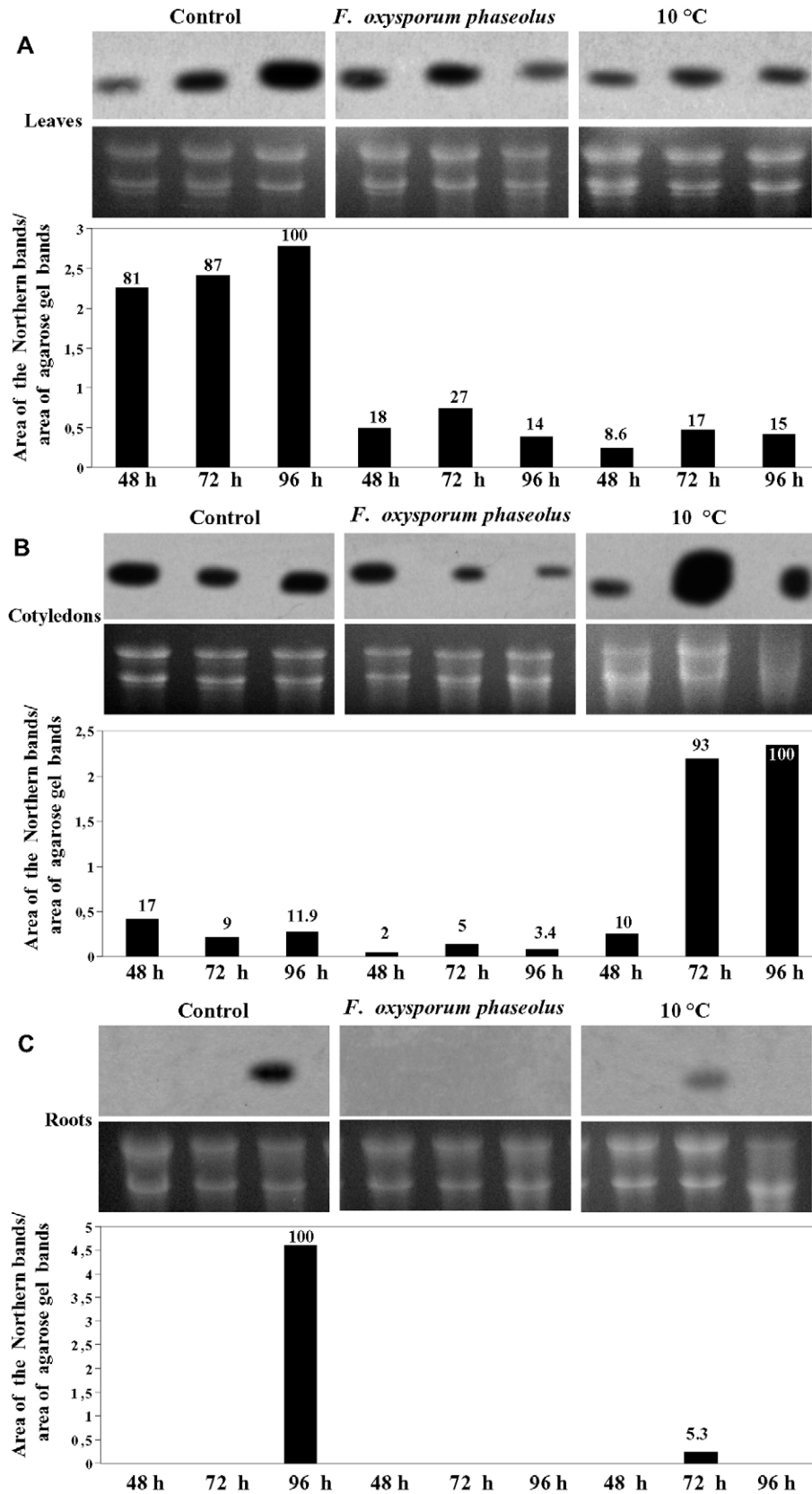


Fig. 5. Northern analysis (upper panel) of the *VULTP* expression in leaves (A), cotyledons (B) and roots (C) of cowpea seedlings at 48, 72 and 96 h after the infection with *Fop* and 8 h after the cold stress. Total RNA (10 µg) was loaded onto the gel. Ethidium bromide stained rRNA bands are shown at the lower panel as a loading control. The control conditions also indicate the expression of LTP gene during the seedling development. Densitometric analyses are shown below each Northern blot. The numbers above the bars indicate the percentage of expression areas in regard to the expression area of the strongest band which was considered 100%.

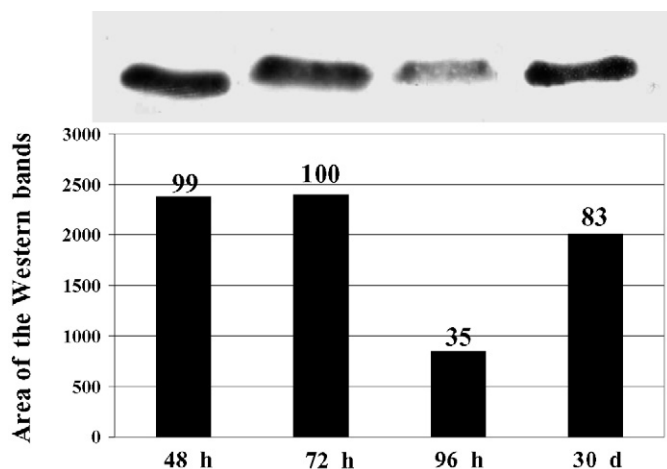


Fig. 6. Western blotting of crude protein extraction from leaves of cowpea seedlings (48, 72 and 96 h after germination) and adult leaf (30 days after emergence) showing the presence of LTP. Densitometric analysis are shown below the Western blotting. The numbers above the bars indicate the percentage of expression areas in regard to the quantity of the protein loaded on the gel and the highest value was considered 100%.

superimpositions of some helixes [34]. They were included on the phylogenetic tree because few years ago has emerged the hypothesis that plant LTPs could have a role or at least be involved in *Arabidopsis* defense signaling [13]. The authors suggested that this putative LTP interacts with a lipid derived molecule to function as a long distance signal complex. Giving support to the involvement of LTPs in plant signaling it has been shown that a LTP from wheat is able to bind to high affinity rates to the tobacco plasma membrane [11]. The binding site was the same used by elicitors as demonstrated by binding and *in vivo* competition experiments. Moreover, Buhot et al. [35] revealed that a recombinant LTP₁ from tobacco is able to bind jasmonic acid (JA) and the complex between LTP₁ and JA is able to bind to the elicitor receptor.

The temporal expression pattern of the cowpea *VULTP* examined by Northern blotting in developing seeds (Fig. 4A) showed that *VULTP* presents a similar pattern of expression with another AMP, the defensin, from radish seeds. The radish defensin transcript accumulated during seed development at the beginning of the desiccation stage (45 days postanthesis) and they are also found in dry seeds [36]. The radish defensins are released during seed germination and create a microenvironment around the seed where fungal growth is inhibited. This observation suggests an important protective role for these seed peptides against an eventual pathogen attack during germination [36]. The cowpea LTP was previously immunolocalized, by our group, at the cell wall and extracellular spaces of cotyledonary cells [17,37]. The extracellular location is indicative of a protective role, since this is the first contact area with an invader pathogen. It was previously demonstrated that the cowpea LTP is exuded during imbibition of cowpea seeds [38]. Such exudation is also an indication of the potential defensive role played by this peptide during seed germination and is in accordance with the work of Terras et al. [36].

The cowpea LTP transcript was not observed to be expressed in roots, leaves and flowers of adult plants under normal physiological conditions (data not shown). The *VULTP* expression seemed to be restricted to the developing seeds and seedling tissues.

Soufleri et al. [24] reported that three genes encoding LTP₁ in *Brassica napus* have their expression also restricted to seedling tissues similarly to the expression profile of the *VULTP* found in cowpea seedlings. The cowpea LTP gene was barely detected in seedling roots and was not detected in roots of mature plants and the three LTP genes of *B. napus* were not expressed in roots of seedlings. Another LTP gene, *CALTPIII*, was not expressed in leaves, roots and flowers of mature *Cap-sicum annuum* plants [25], also resembling the expression pattern found for *VULTP*.

Other reports demonstrate that the genes encoding LTPs are differentially regulated under normal physiological conditions and that some genes are not expressed in these conditions. In cowpea seedling tissues, the expression of *VULTP* gene presents differential expression in response to different stress. The expression of the *VULTP* in leaves of cowpea seedlings underwent small variation in the studied times under the stress condition. It was demonstrated for *C. annuum* plants inoculated with the tobacco mosaic virus a transient expression of the *caLTP1* gene. This gene expression was detected from 24 h after inoculation and increased until 48 h. From this time on the expression diminished between 60 and 72 h and slightly increased at 96 h [39]. The same profile was demonstrated for the compatible interaction with *Xanthomonas campestris* pv. *vesicatoria*, where the expression increased from 12 to 24 h and diminished at 36 h [39].

Results obtained by Jung et al. [25] showed that from the three genes that encode LTPs in plants of *C. annuum*, only the gene *CALTPII* did not have its expression changed by infection with pathogens and abiotic stresses. The two others, *CALTP1* and *CALTPIII*, were induced and the induction level was kept until the end of the studied times, on the contrary to the expression observed to *VULTP*.

Jung et al. [25] also demonstrated that only one of the three genes of LTPs in *C. annuum* was detected in leaves (*CALTPII*) and another in flowers (*CALTP1*) of this plant, neither of them being expressed in roots. Suelves and Puigdomènech [40] also reported that three LTP genes were undetectable in roots and leaves of *Prunus amygdalus* as demonstrate for *VULTP*. But in contrast to the absence of *VULTP* expression in cowpea flowers, in *P. amygdalus* flowers, the three genes were strongly expressed. In rice, three other LTP genes presented faint bands in root seedlings, and only the gene b21 was not detected in seedling leaves [22].

As exposed above, the LTP genes present a complex pattern of expression and thus additional studies will be necessary to gain better comprehension of the expression of *VULTP* gene, especially in tissues of cowpea infected with other pathogens.

By Western blot we demonstrated the presence of the LTP in leaves of seedlings and also in adult leaves under normal physiological conditions (Fig. 6). Some authors have demon-

strated the presence of LTPs in mature leaves of plants [9,15,16]. We had not been able to detect the expression of LTP in cowpea leaves, by Western blotting, previously. With the present results of Northern blots showing the expression of LTP in control leaves of cowpea seedlings, we were encouraged to search again. This time, we used an extraction method that is normally used for analysis of leafy proteins through isoelectric focusing (IEF). Although we did not find LTP transcript in adult leaves (data not shown), this may suggest that the cowpea LTP was deposited in this tissue, probably during leaves expansion, to fulfill some unknown function and is retained there. Similar results were demonstrated for a leafy broccoli LTP, whose transcript pattern of accumulation was similar to that found for the cowpea LTP. The broccoli LTP was associated with the wax layer and its level of transcript decreased as the leaves fully expanded and aged [9]. Another explanation is that the antibody, which was raised against LTPs isolated from cowpea seeds, is probably able to recognize LTPs from different cowpea tissues. In contrast, the nucleotide probe seemed to be more specific and due to its higher specificity did not recognize the LTP gene expressed in leaves of adult plants.

As previously demonstrated [37], there are at least two populations of LTP in cowpea seeds one extracellular and another intracellular, located in an organelle that was characterized as a protein storage vacuole [37]. In fact, some studies have revealed that LTPs are encoded by several genes [22,23,31] and each of these are spatially and temporally regulated. Thus, further studies will be performed in order to assess the full role of the cowpea LTP, as well as to understand the *VULTP* functions.

4. Materials and methods

4.1. Biological material

Cowpea (*V. unguiculata* L. Walp.) seeds, of the EPACE-10 cultivar, were supplied by Universidade Federal do Ceará, Fortaleza, Brazil.

The fungal isolate *Fop* was supplied by the Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Federal do Pernambuco, Brazil.

The chemically competent cells of *Escherichia coli* bacterial strain TOP10 (genotype: F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*) were supplied with the cloning pCR 2.1-TOPO Kit (Invitrogen).

4.2. Fungal growth condition and inoculum preparation

The fungus was maintained on agar Sabouraud (1.0% peptone, 2.0% glucose and 1.7% agar–agar). For the inoculum preparation, the fungal culture was transferred to Petri dishes containing agar Sabouraud for 12 days. After this period, 10 ml of sterile distilled water were added to the dish and this was gently rubbed with the help of a Drigalski loop for 1 min for

spore liberation. Spores were quantified in a Neubauer chamber for appropriate dilutions.

4.3. Vegetal samples

The cowpea seeds were sowed onto five-liter plastic pots filled with a sand/soil/organic material mixture (1:1:1, w/w/w). Six seeds were sowed per pot and 3 days after emergence, each pot was thinned out to two plants. Nitrogen was added to the plants 10 days after their emergence and they were kept in the greenhouse during the entire experimental period, February to May 2005. At the flowering stage, leaves, flowers and roots were collected from mature plants and seeds were collected after pollination on intervals of 3 days until completely dried. Samples were immediately frozen in liquid nitrogen. For biotical and abiotical stresses, cowpea seeds were surface-disinfected by immersing them on 30% hydrogen peroxide (Merck) for 10 min followed by 10 washes of five min in autoclaved distilled water. After disinfection, seeds were put on Petri dishes (six seeds per dish) containing 0.5% agar. After 20 h, when seeds were already germinated, the biotical stress was applied by inoculation of the seeds with 0.5×10^4 *Fop* spores ml⁻¹. After 40, 64 and 88 h, the abiotic stress was initiated by transferring the Petri dishes to cold (10 °C) and keeping them at this temperature for 8 hours before the collection. All seedlings, either stressed by the fungus or by cold, were collected at the times of 48, 72 and 96 h after initial placing of seeds on Petri dishes. From these materials, cotyledons, leaves and roots were separated and individually frozen in liquid nitrogen. All plant tissues were stored at -70 °C until use and total RNA for Northern blot analysis was extracted from them as described below and then used for hybridization.

4.4. RNA extraction from cowpea

Total RNA from cowpea tissues was extracted in 0.2 M Tris–HCl (pH 7.5), 0.1 M LiCl, 5 mM EDTA and 1% SDS followed by a conventional phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) extraction method and then freed from DNA by LiCl precipitation. Poly-A⁺ mRNAs were purified from total RNA by Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions.

4.5. RT-PCR amplification of the *VULTP* cDNA

Reverse transcription reaction mixture was prepared with 2 µg of mRNA, 1× first strand buffer (Invitrogen), 1 mM oligoT₁₈, 10 mM DTT, 1 mM dNTP, RNase^{OUT} 80 units, SuperScript II 250 Units (Invitrogen) and ultra-pure water to a final volume of 25 µl. The reaction was incubated for 90 min at 42 °C and then used for PCR amplifications.

The PCR primer, 5'-ATGACITG(CT)GGICA(AG)GT-3' (MWG-Biotech, USA), was designed based on the first six amino acids (MTCGQV) of the amino terminal sequence of previously purified cowpea LTP [17]. PCR was carried out in a Mastercycler gradient 22331 (Eppendorf) and the PCR reac-

tion mixture contained $1\times$ *Taq* buffer (Amersham Biosciences), dNTP 0.2 mM, 1 μ M poly-T₁₈, 1 μ M LTP primer, 3 Units *Taq* DNA polymerase I (Amersham Biosciences) in a final volume of 20 μ l reaction. This reaction was initially warmed at 95 °C for 5 min and subjected to a 40 cycles program as followed: 95 °C for 45 s; 50.1 °C for 45 s; 72 °C for 2 min; last PCR step included extension at 72 °C for 5 min. The PCR products were loaded onto a 1% agarose gel and purified using Wizard SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions.

4.6. Cloning and DNA sequencing of the LTP VULTP cDNA

The cDNA amplified by the RT-PCR was purified from the gel and cloned into the pCR2.1-TOPO vector (pCR2.1-TOPO vector Kit, Invitrogen), according to instruction manual. The resulting DNA construction was named pCR2.1-LTP. The nucleotide sequence of the cDNA insert was determined for both strands at an ABI PRISM 377 DNA Sequencer (Applied Biosystems) using a reaction mixture prepared with DYEnamic ET Terminator Cyclor Sequencing Kit according to instruction manual (Amersham Biosciences) and the M13 forward (–20) (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAG GAAACAGCTATGAC-3') sequencing primers. The two strands of four clones were separately sequenced, one set of four 5' → 3' strands was converted to the cDNA and aligned with the other set of four 3' → 5' strands. From this alignment the consensus sequence was deduced. The deduction of the amino acid sequence and protein alignment were carried out using BLAST (<http://www.ncbi.nlm.nih.org/Balst>) and Clustal algorithms (<http://www.expasy.org>) [41,42].

4.7. Phylogenetic analysis

The phylogenetic analysis was performed using LTPs sequences from plants obtained from GenBank which were aligned with ClustalW1.83 (<http://www.genebee.msu.su/clustal/basic.htm>) with default settings [42]. The graphic displayed was done with the Phylodendron Phylogenetic Tree Drawing (<http://www.iubio.bio.indiana.edu/treepp/>).

4.8. Northern analysis

The *VULTP* cDNA was used as a template for probe synthesis. Probe was labeled by random primer technique (T₇ Quick Primer Kit, Amersham) using [α -³²P]dCTP (30 Ci mmol⁻¹, Amersham Biosciences) according to instruction manual. Hybridization of ³²P-labeled probe, specific for LTP mRNA, was carried out after electrophoresis and transference of 10 μ g of total RNA to Hybond-N⁺ membranes (Amersham Biosciences) following standard procedures. Hybridizations were carried out at 42 °C. The membranes were washed two times at room temperature and one time at 65 °C with 0.2× SSC containing 0.1% SDS for 20 min and then developed by autoradiography.

4.9. Leaf protein extraction and protein content determination

Proteins of leaves were extracted according to a protocol used to IEF gel electrophoresis (<http://www.biotech.unl.edu/proteomics/mercaptoethanol.html>) with modifications. Leaves were ground to powder using mortar and liquid nitrogen. To each 0.1 g of fresh tissue, 300 μ l of the cold solution (10% TCA, 0.07% β -mercaptoethanol in acetone) were added. The extractions were kept at -20 °C for 2 h and then were centrifuged at 13,000 $\times$ g for 15 min at 4 °C. The pellets were washed with the solution containing 0.07% β -mercaptoethanol in acetone until the pellet has become white (approximately four times). The washed pellets were dried at 40 °C and re-suspended in the extraction buffer pH 5.4 (10 mM Na₂HPO₄, 15 mM NaH₂PO₄, 1.5% EDTA and 100 mM KCl). The extractions were heated at 60 °C and periodically agitated over 1 h. After this period the extractions were centrifuged at 13,000 $\times$ g for 7 min at 4 °C. The supernatants were collected in a new tube and protein content of all samples was determined as described by Bradford [43].

4.10. Gel electrophoresis and Western blotting

SDS-tricine-gel electrophoresis was performed according to the method of Schägger and Von Jagow [44], and after electrophoresis, proteins (5 μ g) were electrotransferred to nitrocellulose membranes, as described by Towbin et al. [45]. Nitrocellulose membrane was treated as follows: blocked with TTBS (10 mM Tris-HCl (pH 8.0), 150 mM NaCl containing 0.025% Tween 20) for 1 h, incubated for 18 h with anti-LTP/TTBS (1:2000, v/v) at 4 °C, washed 10 times for 5 min with TBS and incubated for 1 h with goat alkaline phosphatase-conjugated anti-rabbit IgG/TTBS (1:2000, v/v) and finally washed as afore mentioned. Immunoblots were developed using a NTB/BCIP Sigma fast Kit (Sigma) according to the manufacturer's instructions. Anti-LTP antibodies were raised as described by Carvalho et al. [17].

4.11. Densitometric analysis

Densitometric analysis were performed with the program Gel-Perfect [46,47] which divides the values of the areas of the Northern bands by the total area of the agarose gel bands. The highest value obtained from this division was considered 100% and from this, the percentage of expression of other bands was calculated. For Western blotting, calculations were done by dividing the areas of the positively immuno-probed bands by the total of protein loaded on the gel (5 μ g). The highest value obtained from this division was considered 100% and from this, the percentage of expression of other bands was calculated.

Acknowledgments

I would like to thank to Marcelo dos S. Ferreira, Verônica de S. Lima and Valéria C. L. Marques for help with the DNA

samples preparation for sequencing and to Marta T. Gobo and Luis C.D. Souza for technical assistance. This project was supported by the Brazilian agency CNPq, FAPERJ, FENORTE/TECNORTE and International Foundation for Science, Stockholm, Sweden, through a grant to C/2806–3F. This work is part of DS degree thesis of André O. Carvalho carried out at the Universidade Estadual do Norte Fluminense.

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