

Vegetative Storage Protein in *Litchi chinensis*, a Subtropical Evergreen Fruit Tree, Possesses Trypsin Inhibitor Activity

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- **Background and Aims** Vegetative storage proteins (VSPs) are commonly bioactive in herbaceous plants but few VSPs with bioactivity have been identified in trees. In addition, information on the characterization of VSPs in evergreen trees is limited. The objective of this study was to characterize the VSPs with bioactivity in evergreen trees.
- **Methods** The VSP in lychee (*Litchi chinensis*), an evergreen fruit tree, was characterized by a combination of cytological, biochemical and molecular biological techniques.
- **Key Results** The VSP in lychee was a 22-kDa protein. It accumulated in the large central vacuoles of protein-storing cells (PSCs) in two distinguishable forms, granular and floccular. The PSCs were of a novel type. The 22-kDa protein is distributed in mature leaves, bark tissues of branches, trunk and large roots, paralleling the distribution of PSCs. Its homologues were present in mature seed. During young shoot development and fruiting, the 22-kDa protein decreased apparently, suggesting a nitrogen-storage function. The 22-kDa protein had several isoforms encoded by a small multigene family. One gene member, *LcVSP1*, was cloned. The *LcVSP1* had no intron and contained a 675 bp open reading frame encoding a putative protein of 225 amino acids. *LcVSP1* was homologous to Kunitz trypsin inhibitors. The 22-kDa protein inhibited trypsin and chymotrypsin, but had no inhibitory effect on subtilisin.
- **Conclusions** Lychee is rich in a 22-kDa VSP with trypsin inhibitor activity. The VSP plays an important role in nitrogen storage while its possible defensive function remains to be elucidated.

Key words: Defence, evergreen tree, Kunitz trypsin inhibitor, *Litchi chinensis*, nitrogen storage, vegetative storage protein.

INTRODUCTION

Vegetative storage proteins (VSPs) occur in many herbaceous plants. The best characterized of these VSPs are the 27-, 29- and 94-kDa proteins in leaves of soybean where they specifically accumulate in large amounts in vacuoles of the paraveinal mesophyll cells (Franceschi *et al.*, 1983; Wittenbach, 1983; Tranbarger *et al.*, 1991) and act as temporary nitrogen reserves to support seed development (Staswick, 1994). By using transgenic soybean plants in which VSP synthesis is suppressed by a *vspA* antgene, however, Staswick *et al.* (2001) demonstrated that the VSPs in soybean play a very small, direct role in overall plant productivity. As the 27- and 29-kDa proteins are acid phosphatase active (De Wald *et al.*, 1992) and the 94-kDa protein is a lipoxygenase (Tranbarger *et al.*, 1991), VSPs could play some special roles under certain conditions (Tranbarger *et al.*, 1991; De Wald *et al.*, 1992). Proteins with biological activities are found in many other herbaceous plants, where they are present in flowers/flower buds (Berger *et al.*, 1995; Liu *et al.*, 2005), vegetative storage organs (Andrews *et al.*, 1988; Volenec *et al.*, 1996; Yeh *et al.*, 1997; Flores *et al.*, 2002; Meuriot *et al.*, 2004; Dhont *et al.*, 2006) and leaves (Graham *et al.*, 1986; Ryan, 1990; Moura and

Ryan, 2001; Horn *et al.*, 2005). These proteins are generally regarded as VSPs in herbaceous plants and have been suggested to play a dual role in nitrogen storage and defence (Liu *et al.*, 2005).

The best-characterized VSP in deciduous trees is the 32-kDa protein in poplar (for review, see Stepien *et al.*, 1994). The protein consists of several isoforms encoded by a small multigene family (Langheinrich and Tischner, 1991; Stepien and Martin, 1992; Stepien *et al.*, 1994). One gene member, *bspA*, has been cloned (Coleman and Chen, 1993). The 32-kDa protein acts as a seasonal nitrogen reserve on the basis of its seasonal fluctuation (for review, see Stepien *et al.*, 1994). Unlike the VSPs in herbaceous plants, the 32-kDa protein in poplar has no enzymatic or other biological activities (Stepien and Martin, 1992; Stepien *et al.*, 1994). Besides, WIN4 and PNI288 are also considered to be VSPs in poplar (Cooke and Weih, 2005). In addition to nitrogen storage, the two proteins as well as the 32-kDa protein are thought to play a role in defence against herbivores on the basis of the up-regulation of their genes by mechanical wounding (Davis *et al.*, 1993; Cooke and Weih, 2005). VSPs are also found in many other temperate deciduous trees (for review, see Stepien *et al.* 1994) and tropical deciduous trees (Wu and Hao, 1986, 1991; Hao and Wu, 1993; Tian *et al.*, 1998, 2003; Tian and Hu, 2004). The diversity in cytology and biochemistry of the VSPs suggests that VSPs with special

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bioactivities should be common in trees (Stepien *et al.*, 1994; Tian *et al.*, 1998). An open question is whether all of the VSPs in trees are generally biologically inactive.

Compared with deciduous trees, evergreen trees are poor in VSPs (Wetzel and Greenwood, 1989; Wetzel *et al.*, 1991; Roberts *et al.*, 1991; Arora *et al.*, 1992) and none of the VSPs have been well characterized. The absence of VSPs from evergreen trees is consistent with their role as seasonal nitrogen storage in deciduous trees. However, by studying proteins in lychee (*Litchi chinensis*), a subtropical evergreen fruit tree, using a combination of cytological, biochemical and molecular biological techniques, it was found that lychee is rich in a VSP, a 22-kDa protein with trypsin inhibitor activity.

MATERIALS AND METHODS

Plant materials and treatments

Five-year-old 'Feizixiao' lychee (*Litchi chinensis* Sonn.) trees were grown in the Lychee Plantation of Chinese Academy of Tropical Agricultural Sciences on Hainan Island, China. Ten comparable trees, in terms of size, growth vigour and phenological phase (phenophase) were chosen from the plantation and controlled to sprout twice a year in late June (the shoots produced were called the first autumn shoots) and late September (the shoots produced were called the second autumn shoots). Four plant parts were harvested during the second autumn sprouting, blossom and fruit development: (1) mature leaves; (2) stems of the first autumn shoots (i.e. twigs) and the second autumn shoots (i.e. terminal branches); (3) trunk samples that were taken 50 cm up from the ground level; (4) large root samples that were taken at a distance of 50 cm from the trunk base from the larger lateral roots in the surface soil. Samples from different trees at each time point were analysed, and representative data are given in the Results.

Light- and electron-microscopy

For histochemical staining, samples were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h. Paraffin blocks were prepared after dehydration through a graded series of ethanol. Microtome sections (10 µm thick) were stained with mercury-bromophenol blue, which is specific for protein (Wu and Hao, 1986).

For indirect fluorescence localization, samples were cut to size 5 × 5 mm and fixed in glutaraldehyde (0.5%) and paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 12 h. The samples were then dehydrated in ethanol and embedded in paraffin. Microtome sections (10 µm thick) were treated with the 22-kDa protein antiserum (1 : 1000) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgGs (SABC, Shanghai, China), and then examined with a Leica DMLN microscope (Leica, Wetzlar, Germany).

Electron microscopy was accomplished according to Tian *et al.* (2003).

TABLE 1. List of abbreviations used in the text

CTAB	Cetyl trimethyl ammonium bromide
FITC	Fluorescein isothiocyanate
LDS	<i>N</i> -Lauroylsarcosine sodium salt
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MS	Mass spectrometry
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PMF	Peptide mass fingerprinting
PSC	Protein-storing cell
PVPP	Polyvinylpyrrolidone
RACE	Rapid amplification of cDNA ends
SDS	Sodium dodecyl sulfate
UI	Inhibitor unit
UTR	Untranslated region
VSP	Vegetative storage protein

Protein extraction for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis

Samples (2 g fresh weight) were mixed with 0.5 g of polyvinylpyrrolidone (PVPP; see Table 1 for abbreviations used in the text) and ground to a homogenate in 5 mL of extraction buffer [0.1 M Tris base, 0.05 M sodium borate, 0.005 M ascorbic acid, 1% β-mercaptoethanol, 0.1% (v/v) Triton X-100, 1 mM PMSF (phenylmethylsulphonyl fluoride), pH 8.4]. The homogenates were centrifuged at 17500 g for 30 min at 4 °C and the supernatants were collected. Protein in the supernatants was determined according to the method of Bradford (1976) using bovine serum albumin as standard. The supernatants were used for SDS-PAGE after boiling for 5 min with an equal volume of 2 × Laemmli sample buffer (Laemmli, 1970). Protein samples for two-dimensional gel electrophoresis were purified by Sephacryl S-100HR and DEAE-Sepharose in turn.

SDS-PAGE

SDS-PAGE was performed using 14% SDS-polyacrylamide gels (or alternatives as shown in the figures), 25 mM Tris base, 192 mM glycine and 0.1% SDS running buffer, pH 8.3, at 25 mA gel⁻¹. Twenty micrograms of protein were loaded per lane (unless as shown in the figures). Gels were routinely stained with Coomassie brilliant blue R-250.

Two-dimensional gel electrophoresis

Thirty micrograms of the purified 22-kDa protein were mixed with 125 µL of rehydration buffer containing 8 M urea, 2% (v/v) Triton X-100, 65 mM DTT, 0.2% (v/v) Bio-lyte (pH 3.5–10.0) and a trace of bromophenol blue. Isoelectric focusing was carried out automatically in IPGphor (Bio-Rad Laboratories, Inc., USA) using 7-cm IPG strips (linear pH 3.0–10.0) (Bio-Rad Laboratories, Inc.). The equilibration and application of the IPG strips to SDS-polyacrylamide gels were according to the manufacturer's instructions. The gels were stained with Coomassie brilliant blue R-250.

Production of antiserum

The 22-kDa protein was extracted from the bark tissues of branches, purified by preparative SDS–PAGE, and emulsified with Freund's adjuvant (Sigma). This emulsion was used to immunize two New Zealand male rabbits by hypodermic injection. The antiserum was purified by ammonium sulfate precipitation.

Western-blotting analysis

Electrophoretic transfer of proteins from SDS–polyacrylamide gels to unmodified nitrocellulose was based on Towbin *et al.* (1979). The other steps were carried out according to Tian *et al.* (2003).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)

The visualized protein spots were excised from gels by an Ettain Spot Cutter (Amersham Bioscience, Sweden). After being destained and dehydrated, the protein was digested in-gel with sequencing grade-modified trypsin (Roche) (10 ng ml^{-1} in 25 mM ammonium bicarbonate) for 16 h at 37°C . The enzyme digest solution was spotted onto a sample plate with matrix (α -cyano-4-hydroxycinnamic acid, 8 mg mL^{-1} in 50 % trifluoroacetic acid) and allowed to air dry. Known trypsin autocleavable peptide masses (906.51 and 2273.16 Da) were used for a two-point internal calibration for each spectrum. Examples of peptide mass fingerprinting (PMF) were searched against NCBI protein databases with the search engine Matrix Science at <http://www.matrixscience.com>. All peptide masses were assumed to be monoisotopic and [M+H]⁺ (protonated molecular ions). Searches were conducted using a mass accuracy of 6100 ppm, and one missed cleavage site was allowed for each search.

Preparation of total RNA

Preparation of total RNA followed a routine protocol for RNA extraction (Sambrook *et al.*, 1989) with small modifications. Plant materials were ground to a fine powder in liquid nitrogen using a mortar and pestle. Two grams of the powder were mixed with 15 mL of extraction buffer [2 % cetyl trimethyl ammonium bromide (CTAB), 25 mM EDTA, 2 M NaCl, 100 mM Tris–HCl, 1 % *N*-lauroylsarcosine sodium salt (LDS), 2 % PVPP, 1.25 M NaBO₄, 2 % β -mercaptoethanol] in a 50-mL centrifuge tube for 3–5 min and incubated in water at 65°C for 20 min. The RNA samples were treated with RNase-free DNase I extraction buffer (2 % CTAB, 25 mM EDTA, 2 M NaCl, 100 mM Tris–HCl, 1 % LDS, 2 % PVPP, 1.25 M NaBO₄, 2 % mercaptoethanol) in a 50-mL centrifuge tube for 3–5 min and incubated in water at 65°C for 20 min. The RNA samples were treated with RNase-free DNase I (Fermentas, Canada) before they were used for reverse transcription.

Full-length cDNA cloning by rapid amplification of cDNA ends (RACE)

The N-terminal amino acid residues of the 22-kDa protein were sequenced by the Edman stepwise

degradation method using an Applied Biosystem Procise 491 (ABI, USA) at Peking University (Beijing, China). The degenerate primers were designed as 5'-TA(CT)GA(CT)AT(ACT)AA(CT)GGH GA(CT)GA(AG)GTH AT-3' (H = hypoxanthine). After reverse transcription (RT) with oligo dT-3site adaptor primer supplied by 3'-RACE kit (TaKaRa Biotech Co., Ltd, Dalian, China), the first-strand cDNA was used directly in a 3'-RACE PCR reaction. The amplified fragment was used to design one RT primer with its 5'-end phosphorylated (RTP: 5'-P-GCG GAC GAC AGC ACC TT-3') and two pairs of PCR primers (P1: 5'-CCA ACA CAG GTC GGA TTT ACA-3' and P2: 5'-GCA GCG TTC ATT TCT ACC AG-3'; P3: 5'-GTA GTC CAA TGA TGT TCT TTC CTG TCA A-3' and P4: 5'-CGG ATG GCT GAG ACG ATG TAA TAT TC-3') for the purpose of amplifying unknown 5'-end sequences of the cDNA by 5'-RACE according to the manufacturer's instructions (TaKaRa Biotech Co., Ltd, Dalian, China). To amplify the full-length cDNA, a pair of primers was designed (forward: 5'-ACC CAT CCC TCA AAA TAA GCG ACA TGA AGA G-3'; reverse: 5'-TTG GAT AAA CGA AAA GGA ACT CAT GTC CAT TAT TC-3') according to the sequence information from the 3'- and 5'-RACE analyses.

Southern-blotting analysis

Genomic DNA was isolated from young leaves of seedlings using the CTAB method. The DNA was digested completely with *Eco*RI, *Hind*III or *Xho*I overnight, and then separated by electrophoresis in 1.0 % agarose gels, blotted onto Hybond-N membrane and probed with the *LcVSP1* open reading frame (ORF) (³²P labelled). The hybridization was performed according to the standard method of Sambrook *et al.* (1989).

Construction of expression vector systems in *Escherichia coli* and purification of fusion protein

Expression vector systems for *LcVSP1* ORF were constructed using the P_{gex6p1} vector (Invitrogen Biotech Co., USA). Restriction sites for *Eco*RI and *Xho*I were introduced by PCR at the 5'- and 3'-end of the *LcVSP1* ORF, respectively. The amplified fragments were sequenced and digested with *Eco*RI and *Xho*I, and then ligated into P_{gex6p1} vector. This expression plasmid was used to transform the *E. coli* BL21(DE3) strain and the fusion proteins were purified by GSTrap FF column (Invitrogen Biotech Co., USA) according to the manufacturer's instruction. The inhibitory activity of the purified fusion protein on trypsin was determined according to the method described below.

Assays for proteinase inhibitor activity

Assays for the inhibitory activity of the 22-kDa protein on trypsin and chymotrypsin, as well as subtilisin, were performed according to Erlanger *et al.* (1961). Inhibitory activity was measured against 16.2 μM trypsin (TPCK treated; Sigma, St Louis, MO, USA) or 333 ng of chymotrypsin and subtilisin (TLCK treated; Sigma.). Synthetic

substrates *N*- \pm benzoyl-DL-arginine-*p*-nitroanilide hydrochloride for trypsin and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide for chymotrypsin and subtilisin were purchased from Sigma. Inhibitory activity was expressed as a percentage of the proteinase activity remaining after being incubated with inhibitor the 22 kDa protein for 30 min to the proteinase activity of control (without the 22 kDa protein). One trypsin inhibitor unit (UI) is defined as a decrease of 0.01 absorbance units at 410 nm 1 min^{-1} in 1 mL of reaction mixture under the assay conditions (Erlanger *et al.*, 1961).

RESULTS

Protein-storing cells in perennial tissues

Paraffin sections stained with mercury–bromophenol blue revealed protein-storing cells (PSCs) with dense vacuolar inclusions which appeared clear blue under a light microscope (Fig. 1). The vacuolar inclusions were proteinaceous as evidenced by their staining property, and therefore served as a mark by which the PSCs could be recognized and their distribution in perennial tissues could be traced.

In terminal branches (Fig. 1A–D) and twigs (Fig. 1E), PSCs were confined to the inner cortex and axial system of the secondary phloem and xylem. So, to a certain extent, the PSCs were not ordinary parenchyma cells. No PSCs were detected in the xylem of the trunk and large roots. In these organs, the PSCs were present in the secondary functional phloem or slightly beyond it and restricted to the axial system (Fig. 1F, G).

Electron microscopy revealed electron-dense masses in two different forms – granular and floccular – in the large central vacuoles of different phloem parenchyma cells (Fig. 2A). These masses should correspond to the proteinaceous inclusions in the PSCs observed under a light microscope (Fig. 1).

Isolation and identification of VSPs

A prominent protein band with molecular mass of about 22 kDa was detected in the SDS–PAGE profiles of soluble proteins from terminal branches (Fig. 3A, lane 2). By indirect immunofluorescence with polyclonal antibodies raised against the 22-kDa protein, materials labelled by FITC-specific blue-green fluorescence were observed in many of the axial parenchyma cells in the bark of terminal branches (Fig. 2B), corresponding to the proteinaceous inclusions in PSCs (Fig. 1B). The FITC-specific blue-green fluorescence could be easily distinguished from autofluorescence, such as the yellow autofluorescence emitted by tannin-like substances (Fig. 2B, C) and the weak autofluorescence by protein in the sections treated with pre-immune serum (Fig. 2C).

Distribution of the 22-kDa protein in the vegetative organs was analysed by SDS–PAGE and western blot. The protein was largely absent from mature leaves (Fig. 3A, B, lane 1), but abundant in the bark tissues of terminal branches (Fig. 3A, B, lane 2), twigs (Fig. 3A, B, lane 3), trunk (Fig. 3A, B, lane 4) and large roots (Fig. 3A, B, lane 5). The protein was also present in considerable amounts in the xylem of terminal branches

(Fig. 3A, B, lane 6) and twigs (Fig. 3A, B, lane 7), but could not be detected in the xylem of trunk (Fig. 3A, B, lane 8) and large roots (Fig. 3A, B, lane 9). Its homologues were present in the peel of immature (Fig. 3C, D, lane 2) and mature (Fig. 3C, D, lane 3) fruit as well as in the seed of mature fruit (Fig. 3C, D, lane 5), but absent from the pulp of mature fruit (Fig. 3C, D, lane 4).

The level of the 22-kDa protein in stems of the terminal branches was highest at the sprouting stage (Fig. 3E, lane 1) and then apparently decreased while the young shoot developed (Fig. 3E, lane 2) and during fruiting (Fig. 3E, lane 3).

Isolation and characterization of LcVSP1

The 22-kDa protein was transferred to a PVDF sheet and a partial amino acid sequence was determined by the automated Edman degradation. A sequence of the N-terminal 20 amino acid residues was: ASEPLYDINGDEVITGTEYY. Based on the genetic codes of the amino acid residues underlined, a degenerate primer was designed to amplify the 3'-end sequences of the protein cDNA by 3'-RACE. The nucleotide sequence of the amplified fragment was determined and used to design primers to amplify the unknown 5'-end sequences by 5'-RACE. In this way, a full-length cDNA from lychee was cloned. The cDNA, referred to as *LcVSP1* (GenBank accession number: DQ659678) was 855 bp long containing a 675-bp ORF encoding a putative protein of 225 amino acids, flanked by a 23-bp 5'-UTR (untranslated region) and a 157-bp 3'-UTR including a poly(A) tail of 16 bp. The predicted molecular mass of the protein is 25 kDa, with a pI of 6.47. The amino acid sequence deduced from the residue alanine-25 to the residue tyrosine-44 was identical to that obtained by the automated Edman degradation. By the SignalP 3.0 Server (<http://genome.cbs.dtu.dk/services/SignalP/>), the sequence from residue methionine-1 to residue glycine-24 was predicted to be a signal peptide and the most likely cleavage site was between residues glycine-24 and alanine-25. Thus, the amino acid at the N-terminus of the mature protein is expected to be alanine, which is consistent with the result from the automated Edman degradation.

To test whether *LcVSP1* contained intron(s), the primers used to amplify the full-length cDNA were used to amplify the gene from genomic DNA. A specific DNA fragment was obtained and its nucleotide sequence was identical to that of the full-length cDNA, suggesting that there was no intron in *LcVSP1*. Southern-blotting analysis confirmed that the *LcVSP1* identified belongs to a small multigene family (Fig. 4).

Blast analysis showed that *LcVSP1* was homologous to Kunitz trypsin inhibitors. The deduced amino acid sequence shares 42.4 %, 42.4 %, 39.0 % identity with the trypsin inhibitors from *Glycine max*, *Theobroma microcarpum* and *Populus balsamifera* subsp. *trichocarpa*, respectively. It also shares 44.3 % identity with the miraculin-like protein 2 from *Citrus jambhiri*, 41.5 % identity with the tumour-related protein from *Nicotiana tabacum*, 36.8 % identity with lem1r from *Lycopersicon esculentum*, and 27.2 % identity with the endopeptidase inhibitor from *Arabidopsis thaliana* (Fig. 5).

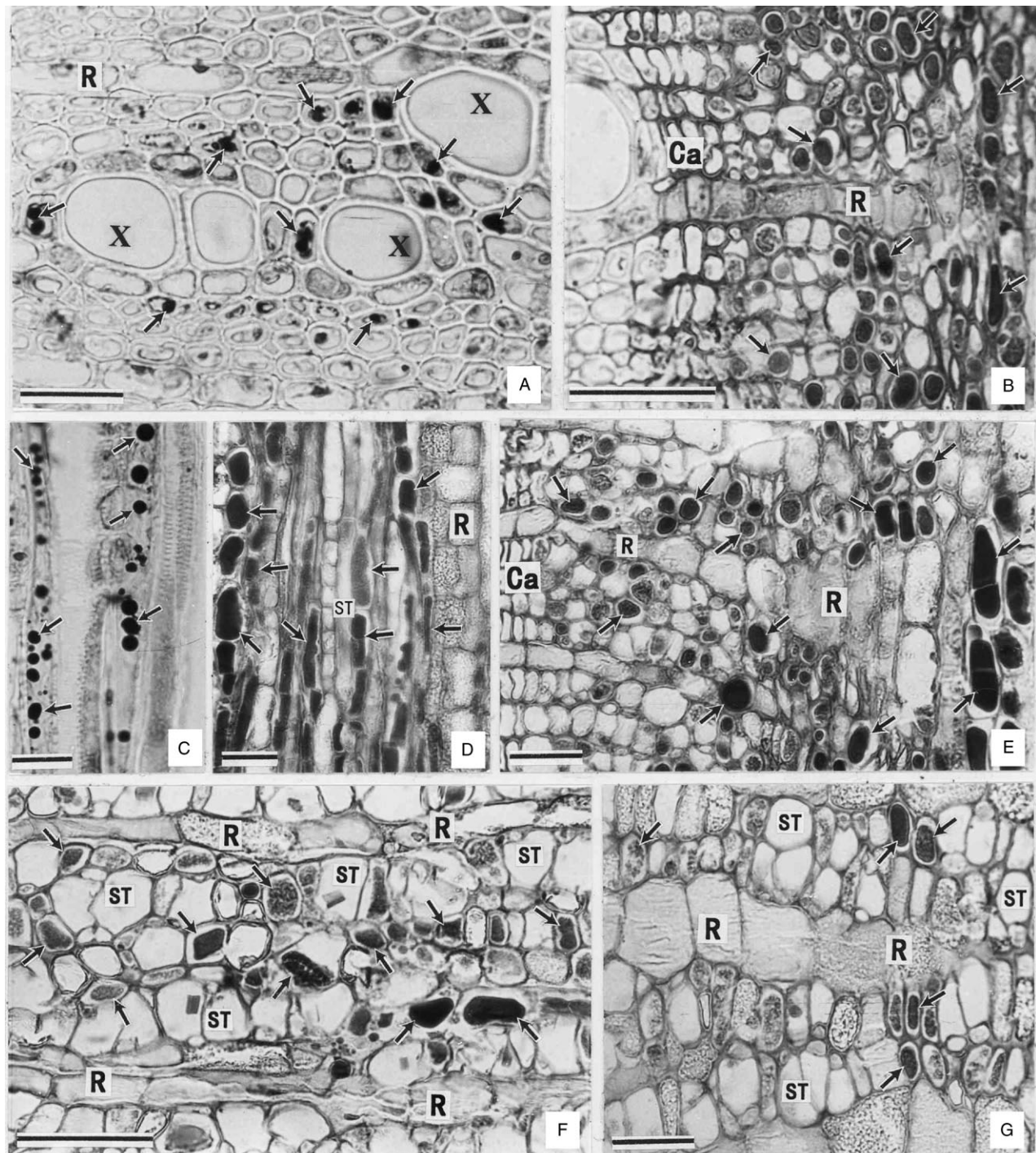


FIG. 1. Distribution of protein-storing cells (arrows) in the perennial tissues. Note: no PSCs are distributed in the radial system (ray). (A, B) Cross-sections of xylem (A) and bark (B) of terminal branches. (C, D) Radial sections corresponding to (A) and (B), respectively. (E–G) Cross-sections of twigs (E), trunk (F) and large roots (G). Ca, Cambia; X, xylem; R, ray; ST, sieve tube. Scale bars = 20 μm .

Biochemical properties of the 22-kDa protein

To demonstrate that the LcVSP1 possessed trypsin inhibitor activity, the ORF was expressed heterologously as a fusion protein with the glutathione *S*-transferase fusion vector in *E. coli* strain BL21(DE3). The fusion protein with the prospective size of about 50 kDa was

produced (Fig. 6A, lanes 2, 3). It was recognized by the polyclonal antibodies raised against the 22-kDa protein (Fig. 6B, lanes 2, 3) and had an inhibitory effect on trypsin. The average inhibitory activity of the fusion protein was 973 UI mg^{-1} protein at half maximum velocity of inhibition against 16.2 μg of trypsin.

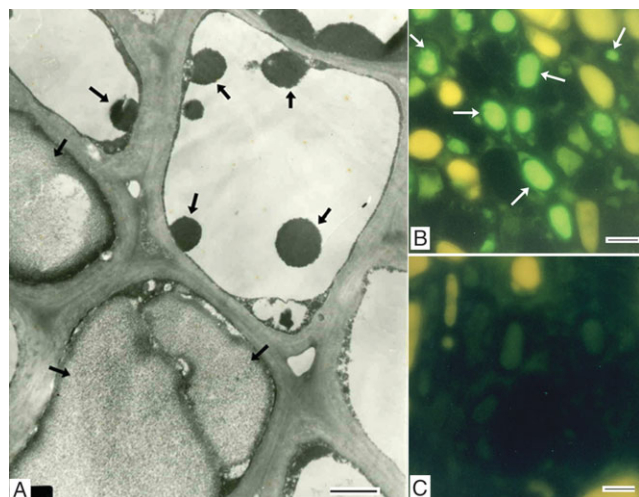


FIG. 2. Identification of vacuolar protein inclusions. (A) Electron micrograph of the secondary phloem parenchyma cells in the stem of terminal branches, showing different forms of vacuolar inclusions (arrows). (B, C) Indirect immunofluorescence localization of the 22-kDa protein in terminal branches. Sections were treated with the polyclonal antibodies (B) and pre-immune-serum (C). Arrows indicate the materials labelled by FITC-specific fluorescence. Scale bars: A = 2 μm ; B = 25 μm ; C = 12.5 μm .

The purified 22-kDa protein from lychee was separated by two-dimensional polyacrylamide gel into five detectable spots with a pI of about 6.13, 6.47, 6.75, 7.42 and 7.94, respectively (Fig. 6C). Spot 2 was the most prominent and its pI (6.47) was the same as the predicted pI of the putative protein LcVSP1.

MALDI-TOF MS showed that the PMF pattern of spot 2 (Fig. 6D) was very similar to that of spot 5 (Fig. 6E). Based on the criteria that the proteins identified must rank at the top hit with more than four matched peptides and a sequence coverage of more than 10% (Dai *et al.*, 2006), both of the PMF patterns of spot 2 and spot 5 were matched to the putative protein LcVSP1 (DQ659678). By searching against the NCBI protein database with the search engine Matrix science, five mass values matched among the six mass values searched with a sequence coverage of 20%. Thus, spots 2 and 5 and, probably, spots 1, 3 and 4 were isoforms of the 22-kDa protein in lychee.

The proteinase inhibitor activity of the purified 22-kDa protein was further tested *in vitro*. A typical purification is summarized in Table 2. The protein inhibited trypsin (Fig. 6F) and chymotrypsin (Fig. 6G, line 2) with a concentration of 2.5 $\mu\text{g mL}^{-1}$ and 0.45 $\mu\text{g mL}^{-1}$ at half maximum

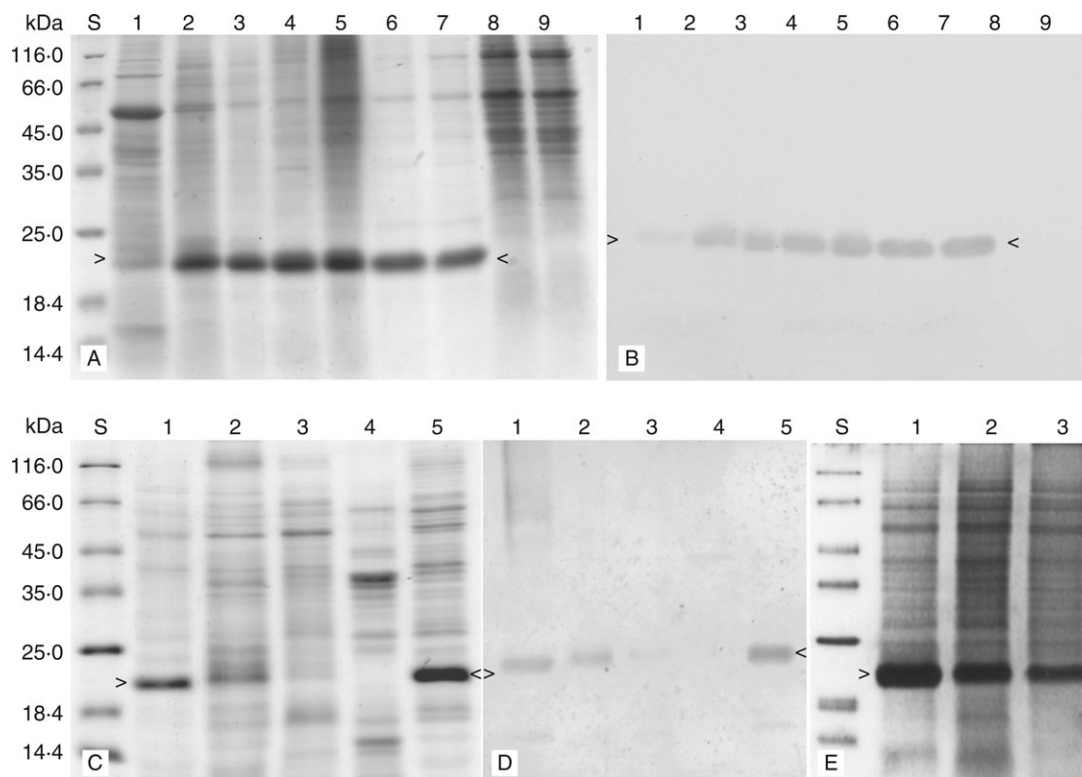


FIG. 3. Distribution and seasonal fluctuation of the 22-kDa protein in lychee. (A, B) Polypeptide profiles from SDS-polyacrylamide gel electrophoresis (A) and western-immunoblots of their duplications (B), showing the distribution of a 22-kDa protein in leaves (1), bark tissues of terminal branches (2), twigs (3), trunk (4) and large roots (5), and xylem of terminal branches (6), twigs (7), trunk (8) and large roots (9). (C, D) Polypeptide profiles from 12% SDS-polyacrylamide gel electrophoresis (C) and western immunoblots of their duplications (D), showing the distribution of the 22-kDa protein homologues in the peel of immature fruits (2) as well as the peel (3), pulp (4) and seeds (5) of mature fruits. Soluble proteins from the bark tissues of terminal branches (1) served as a positive control for the immuno-blot of the samples from fruits. (E) Polypeptide profiles from 12% SDS-polyacrylamide gel electrophoresis, showing the 22-kDa protein mobilization. Samples were harvested from the bark tissues of terminal branches at bud sprouting stage (1), new shoot development (2) and fruiting (3).

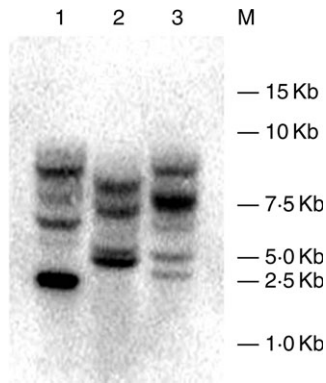


FIG. 4. DNA-blot profiles of the genomic DNA digested with *EcoRI* (1), *HindIII* (2) and *XhoI* (3), hybridized with the *LcVSP1* ORF probe. Five hybridizing regions could be detected in the three samples digested by different endonucleases, respectively, under highly stringent washing conditions.

velocity of inhibition against 16.2 µg of trypsin and 333 ng of chymotrypsin, respectively. The inhibitory effect of the 22-kDa protein on subtilisin could not be detected (Fig. 6G, line 1).

DISCUSSION

The 22-kDa protein in *Litchi chinensis* fits the criteria applied to the typical VSPs in plants (Clausen and Apel, 1991; Staswick, 1994), i.e. it is sequestered in the vacuoles of PSCs to a relatively high content and with an evident seasonal fluctuation.

The PSCs in the vegetative organs of trees have been divided into two types: *Hevea*-type and *Populus*-type (Tian *et al.*, 1998). PSCs in lychee (*Litchi chinensis*), however, are different from either the *Hevea*-type or the *Populus*-type of PSC. The PSCs in lychee are similar to the *Populus*-type of PSCs in that they occur in the xylem as well as in the bark tissues (phloem and cortex) of branches and the molecular mass of the VSP, 22 kDa, is within the range of 15- to 45-kDa, which is considered to be a feature of the VSPs in the *Populus*-type of PSC (Stepien *et al.*, 1994; Tian *et al.*, 2003). On the other hand, the PSCs in lychee differ from the *Populus*-type of PSC in that their distribution is confined to the axial system of the secondary phloem, which is the characteristic of the *Hevea*-type of PSC (Tian *et al.*, 1998). Thus, the PSCs in lychee can be considered as a novel type of PSC.

AAC63057	MKINQLFFPFLILAI SFNSLLSSAAESP...EVVDIDGKILRTGVVYIILPVVVRGGGLTMSIDKMC	68
AAC49969	MKTNQLFLPFLIFTISFNSFLSSAEAPP...AVVDIACKKLRITGIDYYILPVVVRGGGLTLDSTGNESC	68
NP_173228	MSSLLYIFLLAVFISHRGVTTEAAVEP...VKDINGKSLLTGVNYYIILPVIRGGGGLTMSNLKTETC	66
S16252	MKTATAVLLLLFAFTSKSYFFGVANAANS...PVLDTGDELQGTGVQYYVLSSTISGAGGGGLALGRATGQS	68
AAV41234	MKTATAVLLLLFALTSKSYFFRVANAANS...PVLDDIGDELRTGVQYYVVSALWGAGGGGLALGRATGQS	68
BAE79511	MKISLATTLSFLILALASNSLLVLTSSVPEPLLDVNCNKVESTLQYYIVSAIWAGGGGVSLHGGGRNGY	70
LcVSP1	MKSSLLT.LTFLVVFLSFLPNSLLGAS...EPLYDINGDEVITGTEYYIVSAIRGGGGGLNLFSGRNER	66
AAQ84217	MKSTLLVWLSFLLFVFLSVPSIEAYTEP...VLDIOGEEELKAGTEYIIGSIFFGAGGG...DVSATNKT	64
AAC63057	PLDAVVOEHNEDQGLPLTFTFPVDP...KKGVIRESIDLNIKFSANS...I...CVQITLWKLDDFDDETTG	130
AAC49969	PLDAVVOEQQEIKNGLPLTFTFPVNP...KKGVIRESIDLNIKFSAS...I...CVQITLWKLDDFDDETTG	130
NP_173228	PT.SVIQDQFEVQCGLPVKFSFYD...KSRITLPSVTDVNIKFS...TSIWELANFDETTK	120
S16252	CPEIVVQRSDLDNGTIPVIFSNADS...KDDVVRVSTDVNIKFSVPIR...DRLCSTSTVWRLDNYDNSAG	132
AAV41234	CPEIVVQRSDLDNGTIPVIFSNADS...KDGVVVRVSTDVNIKFSVPIR...DRLCSTSTVWRLDNYDNSAG	132
BAE79511	CPLDVIQLPSDTQNGIKLTLSPYNN...STIVRESADLNLRFVLLSGRDYCNQ.PLWKVDNYDAASG	135
LcVSP1	CPMDVFOHRSDLRQSPMMFFVNYNGEKGAVVRASIDMNIQFNVP...DACSQHSTVWVVDNYDEKGG	133
AAQ84217	CPDDVIQYSSDLLQGLPVTFSPASS...DDDVRVSTDLNIKFSIKK...ACDHSVWVKIQKSSNSEV	126
AAC63057	QYFITLGGDQGNPGVETISNWFKIEKYDRD...YKLLYCPITVCFCKVICRDIQIFIQD.GVRRRLALSDV	196
AAC49969	KYFILTIGGNEGNPGRETISNWFKIEKFERD...YKLLYCPITVCFCKVICRDIQIFIQD.GIRRLALSDV	196
NP_173228	KWFISTCGVEGNPGQRTVDNWFKIDKFEKD...YKIRFCPTVCFCKVICRDIQIFIQD.GKRRRLALSDV	186
S16252	QWVITTDGVKGPQPNLCSWFKIEKAGVLG...YKFRFCPSVCDSCITLCSDDIGRHSDDGQIRLALSDN	200
AAV41234	KWVITTDGVKGPQASTMSSWFKIEPAGGIG...YKFRFCPSVCDSCITLCSDDIGRHSDDGQIRLALSDS	200
BAE79511	KWFLITTGGLDGPAGETLLNWFKIEKIGNPGYKIVHCPSVCDSCVTKLNNVGRSFDGVRRLVLRDD	205
LcVSP1	AWFVITTVGVIQNPGRKTIHNFKFEKVGTDLNAKIVHCPSVCDSCVTLCSVQ...INVTQRRLALSSDS	202
AAQ84217	QWVITTGGEENPGIDTLTNWFKIEKAGILG...YKLVSCPEGICHGVLGRDICIYRENDGRRILSLSDK	194
AAC63057	P...FKVMFKKA	205
AAC49969	P...FKVMFKKAQVVKD	210
NP_173228	P...LKVMFKRAY	196
S16252	E...WAWMFKKA SKTIKQVVNAKH	221
AAV41234	G...WPWIFKKA SNTIKQVVNAKH	221
BAE79511	EPAFPVVLIPATERSTSV	223
LcVSP1	..QKPFMLMKVSDVQKYMGRFNAV	224
AAQ84217	LSPFLVLFKKVGLPLSSSI	212

FIG. 5. Amino acid alignment of LcVSP1 and other proteins. The lemir from *Lycopersicon esculentum* (AAC63057), tumour-related protein from *Nicotiana tabacum* (AAC49969), endopeptidase inhibitor from *Arabidopsis thaliana* (NP_173228), trypsin inhibitor homologue from *Glycine max* (S16252), putative 21-kDa trypsin inhibitor from *Theobroma microcarpum* (AAV41234), miraculin-like protein2 from *Citrus jambhiri* (BAE79511) and Kunitz trypsin inhibitor 4 from *Populus balsamifera* subsp. *trichocarpa* (AAQ84217). Residues that are identical throughout the proteins are shown in black. Residues conserved in >50 % of the proteins are shown in pink. Similar amino acid residues are shown in blue. Except for LcVSP1, all of the sequences were named conferring to their GenBank accession numbers.

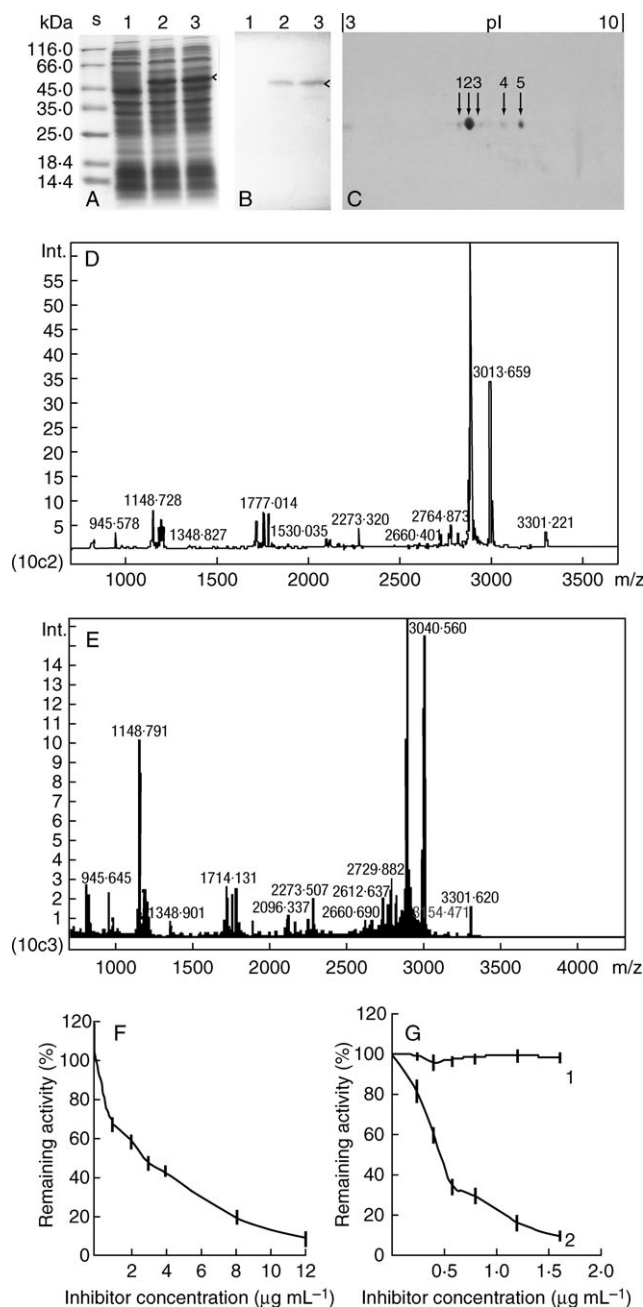


FIG. 6. Biochemical analysis of the VSP in lychee. (A, B) Polypeptide profiles from 10% SDS-polyacrylamide gel electrophoresis (A) and western immunoblots of their duplications (B), showing LcVSP1 heterologously expressed in *E. coli* strain BL21(DE3). Samples from the IPTG-untreated control (1) and those treated with 3 mM IPTG for 2 h (2) and 4 h (3). (C) Polypeptide profiles of the purified 22-kDa protein from two-dimensional gel electrophoresis. The pH range used for isoelectric focusing is 3–10 as indicated on top of the gel. Of the five spots detected, spots 2 and 5 were identified by MALDI-TOF MS. (D, E) PMF pattern of spot 2 (D) and spot 5 (E) generated by MALDI-TOF MS. (F) Inhibitory effect of the 22-kDa protein on trypsin. (G) Inhibitory effect of the 22-kDa protein on subtilisin (1) and chymotrypsin (2).

Previous data have shown that evergreen trees are poor in VSPs and the VSPs in the perennial tissues disappear completely in summer (Wetzel *et al.*, 1991; Arona *et al.*, 1992).

TABLE 2. Purification of the 22-kDa protein from the soluble proteins in bark

Fraction	Total activity (UI)	Protein (mg)	Specific activity (UI mg ⁻¹)	Yield (%)	Purification (-fold)
Bark protein	16575.30	78.93	210	100	1
supernant					
Sephacryl	6415.97	13.65	470	38.71	2.24
S-100HR					
DEAE-Sepharose	6057.8	6.06	1000	36.55	4.76

Here, it is demonstrated that lychee, an evergreen subtropical fruit tree, is rich in a 22-kDa VSP. The 22-kDa protein is mainly distributed in the bark tissues of branches, trunk and large roots, and occurs in large amounts in the stems of branches all the year round. The protein decreased during young shoot and fruit development, suggesting a role in nitrogen storage. The seasonal fluctuation of the 22-kDa protein in lychee (Fig. 3E) is similar to that of general stored nitrogen compounds in other evergreen trees (Kato, 1981, 1986), which is much less dramatic than that of the VSPs in deciduous trees where the abundant VSPs accumulated in the stem of branches are mobilized almost completely during young shoot development (Wetzel *et al.*, 1989; Tian *et al.*, 2003). Nevertheless, based on the fact that nitrogen application has little effect on fruit production of lychee (Menzel *et al.*, 1994), the stored nitrogen compounds in the vegetative organs, one of the main components of which should be the 22-kDa protein, may therefore play a pivotal role in supporting fruit growth and development.

Although the VSPs in many herbaceous plants are generally bioactive, none of the VSPs identified in trees, as far as is known, have been reported as having special bioactivities, except for the lectins, which behave as storage proteins (Nsimba-Lubaki *et al.*, 1986; Herman *et al.*, 1988; Baba *et al.*, 1991; Hankins *et al.*, 1998) and have a role in plant defence (Peumans and van Damme, 1995). The proteinase inhibitors that are considered to be VSPs in the leaves of some herbaceous plants belong to the Bowman–Birk trypsin inhibitor family. They accumulate in the large central vacuoles under biotic and/or abiotic stress conditions and their defence function has been well demonstrated (Graham *et al.*, 1986; Ryan *et al.*, 1990; Moura and Ryan 2001; Horn *et al.*, 2005). Here evidence is provided that a typical VSP, the 22-kDa protein in lychee, possesses trypsin inhibitor activity. The protein inhibits trypsin and chymotrypsin as is the case of the Kunitz trypsin inhibitor in *Peltophorum dubium* (Troncoso *et al.*, 2003), but has no inhibitory effect on subtilisin. Meanwhile, the putative LcVSP1 protein is homologous to Kunitz trypsin inhibitors. Taken together, the 22-kDa protein in lychee belongs to Kunitz trypsin inhibitor family. In poplar, a number of Kunitz trypsin inhibitor genes are up-regulated shortly after leaf damage by wounding (Christopher *et al.*, 2004). Considering the fact that all of the trypsin inhibitors that behave as VSPs in herbaceous plants are Bowman–Birk trypsin inhibitors, the possible defence function of the 22 kDa VSP, a Kunitz trypsin inhibitor in lychee, remains to be elucidated.

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