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

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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* anti-ge, 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; Volenc 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 (Langeinrich and Tischner, 1991; Stepien and Martin, 1992; Stepien et al., 1994). One gene member, *vspA*, 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 Matin, 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 bio-chemistry of the VSPs suggests that VSPs with special...
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 x 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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CTAB</td>
<td>Cetyl trimethyl ammonium bromide</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>LDS</td>
<td>N-Lauroyl sarcosine sodium salt</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization–time of flight mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>Peptide mass fingerprinting</td>
</tr>
<tr>
<td>PSC</td>
<td>Protein-storing cell</td>
</tr>
<tr>
<td>PVPP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>UI</td>
<td>Inhibitor unit</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VSP</td>
<td>Vegetative storage protein</td>
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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 x 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 gell–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 antisem

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 antisem 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 mM NaCl, 100 mM Tris–HCl, 1 % N-lauroylsarcosine sodium salt (LDS), 2 % PVPP, 1.25 mM NaBO₄, 2 % β-mercaptoethanol] in a 50-mL centrifuge tube for 3–5 min and incubated at 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 mM NaCl, 100 mM Tris–HCl, 1 % LDS, 2 % PVPP, 1.25 mM 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(TG) GTA(ACT) AT(ACT) AA(CAT) GGH GA(CAT) GA(A) 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 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′ and reverse: 5′-TTG GAT AAA CGA AAA GGA GAT CAT GTC CAT 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′ and reverse: 5′-TTG GAT AAA CGA AAA GGA GAT CAT GTC CAT 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′ and reverse: 5′-TTG GAT AAA CGA AAA GGA GAT CAT GTC CAT 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). The other steps were carried out according to Tian et al. (2003).

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

Expression vector systems for LcVSP1 ORF were constructed using the Pgex6p1 vector (Invitrogen Biotech Co., USA). Restriction sites for EcoRI, HindIII or XhoI 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).

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 \( \text{LcVSP1} \)**

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: \( \text{ASEPLYDINGDEVITGTEYY} \). 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 \( \text{LcVSP1} \) (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 pl 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 \( \text{LcVSP1} \) 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 \( \text{LcVSP1} \). Southern-blotting analysis confirmed that the \( \text{LcVSP1} \) identified belongs to a small multigene family (Fig. 4).

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

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 \(\mu\)m.
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 μg mL⁻¹ and 0.45 μg mL⁻¹ at half maximum
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.

**Fig. 4.** DNA-blot profiles of the genomic DNA digested with *Eco*RI (1), *Hind*III (2) and *Xho*I (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.

**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 cacao* subsp. *cacao* (AAP41234), miracle-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 according to their GenBank accession numbers.
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).

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 bio-activities, 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.

![Biochemical analysis of the VSP in lychee.](image)

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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity (UI)</th>
<th>Protein (mg)</th>
<th>Specific activity (UI mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
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<tr>
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<td>16575.30</td>
<td>78.93</td>
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<td>100</td>
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<tr>
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</tbody>
</table>

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 bio-activities, 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.

![Biochemical analysis of the VSP in lychee.](image)

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

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

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