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Protein-storing Cells with a 67 kDa Protein in Regularly Tapped Hevea *Trees and in Trees Affected by Tapping Panel Dryness*

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Protein-storing cells (PSCs) are found in secondary phloem of Hevea *trees. In this study it was found that a 67 kDa protein based on SDS-PAGE might be the main component of the vacuole proteins in PSCs. SDS-PAGE analysis indicated that the 67 kDa protein varied in abundance at different levels of tree trunk in untapped and tapped trees, and that the protein also changed when tapped trees were affected by tapping panel disease (TPD). These changes were consistent with that of the amount of PSC vacuole proteins detectable by microscopy. The indirect immunocytochemistry in light microscopical level gave a more reliable evidence that the 67 kDa protein is quantitatively the more important component of the PSC vacuole inclusion. Latex exploitation caused a marked decrease in relative abundance of 67 kDa protein in the area under the tapping cut of the tree trunk. In comparison with healthy tapped trees, the trees which were affected by TPD and rested from tapping had much more 67 kDa protein in the bark tissues near the tapping cut. These facts indicate that the 67 kDa protein may be utilised as storage material for latex regeneration in the tapped trees and that the relatively abundant 67 kDa protein in TPD trees may be caused by resting the trees from latex exploitation.*

To understand the function of *Hevea* laticifers we should study not only the laticifers themselves but also the tissues around the laticifers as they function in co-operation with the surrounding tissues, especially bark tissues[1]. As to the biochemical characteristics of the bark tissues, Yip and Gomez[2] extracted proteins with isotonic sucrose from disintegrated bark tissues of the tapping cut and demonstrated that the proteins play a role in cessation of latex flow after tapping. Nataraja *et al.*[3] showed that in comparison with healthy tapped trees, the trees affected by tapping panel dryness (TPD) had a relatively abundant 70 kDa protein and some other new proteins in SDS-PAGE polypeptide profiles of bark tissues.

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Protein-storing cells (PSCs) were found in secondary phloem of *Hevea* tree[4,5] and available evidence indicate that a 67 kDa protein might be the major part of the vacuole protein inclusion of the PSCs[6]. The vacuole protein inclusion in terminal branchlets was shown to be used as reserve for growth flush of the branchlets[4-6] and the vacuole proteins in tree trunk were suggested to be related to both the girth growth and latex exploitation[4]. We show in this paper that tapping caused a marked decrease in the 67 kDa protein of PSCs in the area under the tapping cut of the tree trunk and that tapping rest of the tree affected by TPD might result in a relative increase in the 67 kDa protein of the PSCs.

MATERIALS AND METHODS

Plant Materials and Sample Collection

Hevea brasiliensis Mull. Arg. was grown on the experimental farm of the Chinese Academy of Tropical Agricultural Sciences on Hainan Island. Samples were collected in May 1998.

The *Hevea* clone RY7-33-97, eight years' old, was used for comparison between trees, tapped and untapped. Some of the trees were tapped for six months on 1/2S D/3 system and some untapped in the same field. Bark samples were collected with a 1.5 cm punch from the tapped and untapped trees. For each sample, a piece of it was used for microscopy and the remainder for protein analysis.

The *Hevea* clone RRIM 600 was used for comparison between healthy trees and TPD affected trees. In a field, 17-year-old trees were tapped on 1/2S D/3 system. For bark sample collection, five TPD affected trees which had been rested from tapping for 2~3 years were found. Samples were also collected from five healthy trees in the same field.

Light Microscopy

The samples were fixed in 80% alcohol. Paraffin blocks were prepared after dehydration through graded series of alcohol/n-butylalcohol. Microtome sections (thickness 20 μm) were stained with mercury-bromophenol blue to show general proteins[7]

Soluble Protein Extraction and Determination

Soluble protein was extracted as described previously[6]. Inner bark (1.5 mm from vascular cambium for *Hevea* clone RY7-33-97 and 3 mm for *Hevea* clone RRIM 600) of the samples were ground to a powder in liquid nitrogen and then homogenised in five volumes of extraction buffer (0.1*M* TRIS base, 0.05 *M* sodium borate, 0.05 *M* ascorbic acid, 1% β-mercaptoethanol, 0.1% *TritonX-100*, 1mM PMSF, and 2% PVPP, pH 9.0). The homogenates were centrifuged at 35 000 g at 4°C for 30 min and the supernatants were used for SDS-PAGE after boiling for 5 min in equal volume of 2x Laemmli sample buffer. Protein in the supernatants was determined according to the method of Bradford[8]

SDS-PAGE

Electrophoresis of the soluble protein extracted from inner bark was performed using 12% SDS-polyacrylamide gels, 0.025 *M* TRIS base, 0.192 *M* glycine, 0.1% SDS running buffer, pH 8.3, at 25 w/gel.

Production of 67 kDa Protein Antiserum

The antiserum production referred to Coleman *et al.*[9] and Mishkind *et al.*[10] The 67 kDa protein extracted from the terminal branchlet bark of *Hevea* tree clone RRIM 600

was purified by preparative SDS-PAGE. The purified protein was then used for the custom production of polyclonal 67 kDa protein antiserum in two male New Zealand rabbits. The antiserum was purified by ammonium sulphate precipitation.

Western Blotting

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose was based on Towbin *et al.*[11] and Burnette[12]. The electrode solution was composed of 20 mM TRIS base, 150 mM glycine and 20% methanol. Electrophoretic transfer was accomplished at 35 mA/gel for 20 h using a Peking DYY-III power supply. Localisation of bound alkaline phosphatase-conjugated antibodies was performed using the Sino-American Biotechnology Co. (SABC) alkaline phosphatase colour development kit, according to manufacturer's instructions. Controls were performed identically using pre-immune serum.

Light Microscopy Indirect Immunohistochemisty of 67 kDa Protein

Immunofluorescence localisation was based on Mishkind *et al.*[10]. Bark samples from the untapped trees were fixed in glutaraldehyde (0.5%) and paraformaldehyde (4%) in phosphate buffer (0.1 *M*, pH 7.4) at 4°C for 24 h. The samples were then dehydrated in ethanol and embedded in paraffin. The microtome sections (thickness 10 μm) were treated with the 67 kDa protein antiserum and FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgGs (SABC, Shanghai), and then examined in a AO fluorescence microscope with ultra-violet excitation filter.

RESULTS

Untapped Trees

In the tree trunk the PSCs were distributed in functional secondary phloem and slightly beyond it. A PSC in paraffin section stained with mercury-bromphenol blue had a mass of dense vacuole inclusion which appeared clear blue under a light microscope. In the cross-section of the secondary phloem the recognised mass of blue vacuole inclusion varied in number at different levels of the tree trunk as shown in *Figure 1* (A–D). More mass of inclusion were seen at higher levels (*Figure 1*, A–C) and much less mass were at 30 cm above graft union *(Figure 1, D)*. The reduced number of the inclusion mass in the phloem cross-section were caused by the decreased inclusion in a PSC, the long cell with a longitudinal axis parallel to the longitudinal axis of stem. The diminished inclusion was constricted to a small granule which was seen on rare occasions in the phloem cross-section prepared from ethanol-fixed sample[4].

The soluble protein content of inner bark varied at different levels of the untapped tree trunk *(Figure 2)* although no significant difference in statistics between the contents was present.

The SDS-PAGE profiles of the soluble proteins show that the 67 kDa protein was abundant at 130 cm, 150 cm and 190 cm *(Figure 3, lanes 2–4)* above the graft union of the trees and much less at 30 cm *(Figure 3, lane 1)*. This change in the relative abundance of the 67 kDa protein was consistent with the change in PSC vacuole inclusion detected in cross-sections of bark tissues *(Figure 1, A–D)*.

By indirect immunofluorescence technique, material labelled by specific FITC blue-green

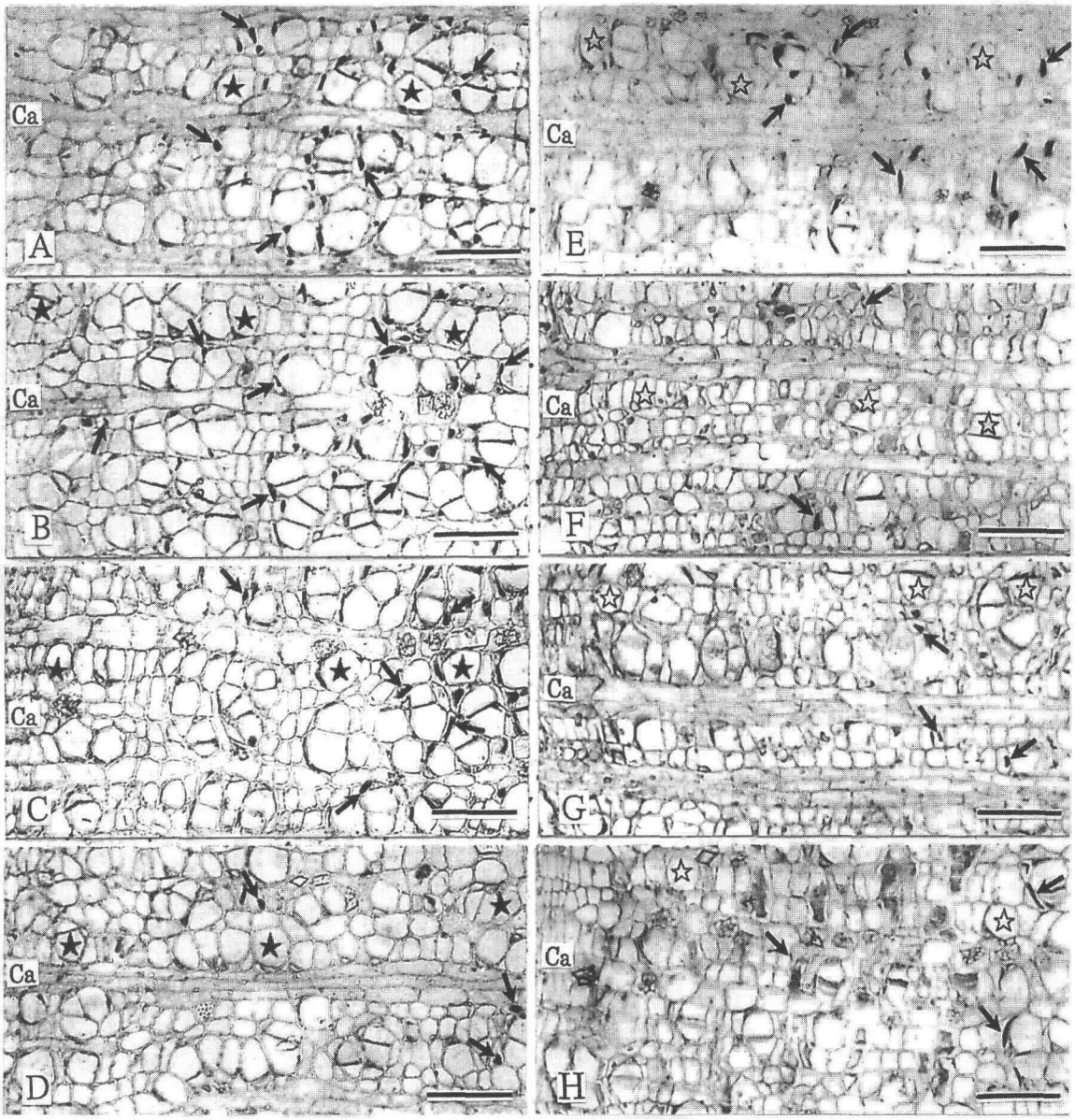

Figure 1. Protein-storing cells with a 67 kDa protein in Hevea *trees. Light micrographs of cross-sections of inner barks. E–H, tapped tree samples collected along the vertical line through the upper end of the tapping cut and in sequence at 190 cm, 150 cm, 130 cm above the graft union. A–D, untapped tree samples collected in the positions corresponding to that of the tapped tree. Note the mass of PSC vacuole protein inclusion (arrows). Asterisks:sieve element; Ca: cambium. Bar = 100 μm.*

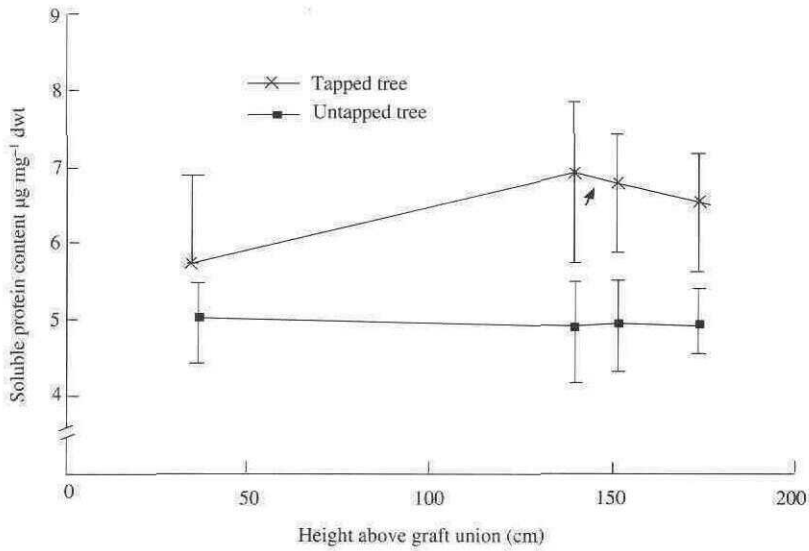

Figure 2. Protein-storing cells with a 67 kDa protein in Hevea *trees. Soluble protein content of inner bark. Values are the means of 5 trees, and error bars indicate ±SD. Arrow:tapping cut.*

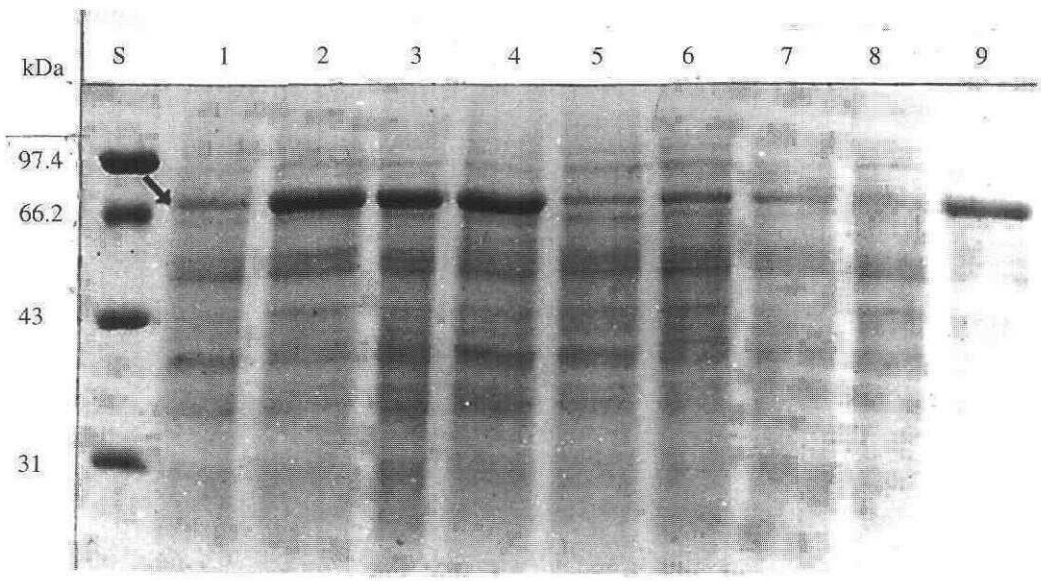

Figure 3. Protein-storing cells with a 67kDa protein in Hevea *trees. SDS-PAGE polypeptide profiles of soluble protein from inner bark. Lanes 1–4 from untapped trees in sequence at 30 cm, 130 cm, 150 cm and 190 cm above graft union. Lanes 5–9 from tapped trees in sequence at 30 cm, 130 cm, 140 cm (2 cm below tapping cut), 150 cm and 190 cm above graft union. 20 μg protein were loaded per lane. Lane S: standard proteins. Arrow: the 67 kDa protein.*

fluorescence was observed in some of the axial parenchyma sells in inner bark *(Figure 4, A)* This material with the fluorescence was then demonstrated to be protein inclusion of PSC vacuoles *(Figure 4, B)*.

Regularly Tapped Trees

In a cross-section of the secondary phloem of tapped tree trunk, more mass of PSC vacuole inclusion were recognised at 190 cm above graft union *(Figure 1, E)*, but much less of the inclusion mass at 150 cm (initial tapping cut height), 130 cm and 30 cm *(Figure 1, F–H)*. This showed that in comparison with the untapped tree, the vacuole protein inclusion of PSCs in the tapped tree had decreased dramatically under the tapping cut.

The content of the bark soluble proteins was higher in the tapped tree than in the untapped tree *(Figure 2)*. At different levels of the tree trunk, except 30 cm above the graft union, the protein contents between the tapped and untapped tree had a significant difference in statistics at the 95% confidence interval.

The 67 kDa protein band in SDS-PAGE profiles of the bark soluble proteins changed in parallel with that of the PSC vacuole inclusion. The protein band was much weaker at 130 cm and 150 cm above graft union and at 2 cm below the tapping cut *(Figure 3, lanes 6–8)*.

Trees Affected by TPD

In the cross-section of the secondary phloem at 2 cm below the tapping cut the detectable PSC vacuole inclusion was much more in the TPD tree than in the healthy tapped tree *(Figure 5, A–B)*.

The soluble protein content in the inner bark tissues of the TPD affected tree amounted to 10 $\mu g\ mg^{-1}$ dwt and the same level of the soluble protein content was found in the healthy tapped tree. The TPD affected tree had abundant 67 kDa protein in SDS-PAGE profiles of the soluble bark proteins and this formed a striking contrast with the healthy tapped tree which had a scanty 67 kDa protein *(Figure 6, A, lanes 1–3)*. By using the antiserum against the 67 kDa protein, the western blot analysis was done and a 67 kDa protein was detected in all the untapped, tapped and TPD trees, indicating that the protein in these trees was identical *(Figure 6, B, lanes 4–6)*.

DISCUSSION

The suggestion[6] that the 67 kDa protein based on SDS-PAGE analysis of bark soluble proteins might be the main component of the vacuole proteins in PSCs was confirmed in this study. The 67 kDa protein varied in abundance at different levels of the tree trunk in untapped and tapped trees, and the protein also changed in abundance when the tapped trees were affected by TPD. With microscopy, similar changes were found in the amount of the PSC vacuole inclusion mass detectable in bark cross-section of the trees. The immunocytochemistry in light microscopical level gave a more reliable evidence that the 67 kDa protein was quantitatively the more important component of the PSC vacuole inclusion. The investigation for more accurate localisation of the 67 kDa protein in PSCs by immunocytochemistry in electron microscopical level is in progress.

We found in the present study that latex exploitation caused a marked decrease in relative abundance of the 67 kDa protein in the area under the tapping cut of the tree trunk. The area is expected to be deeply affected by latex exploitation and it must have intensive latex regeneration. This indicates that the 67 kDa protein may be utilised as storage

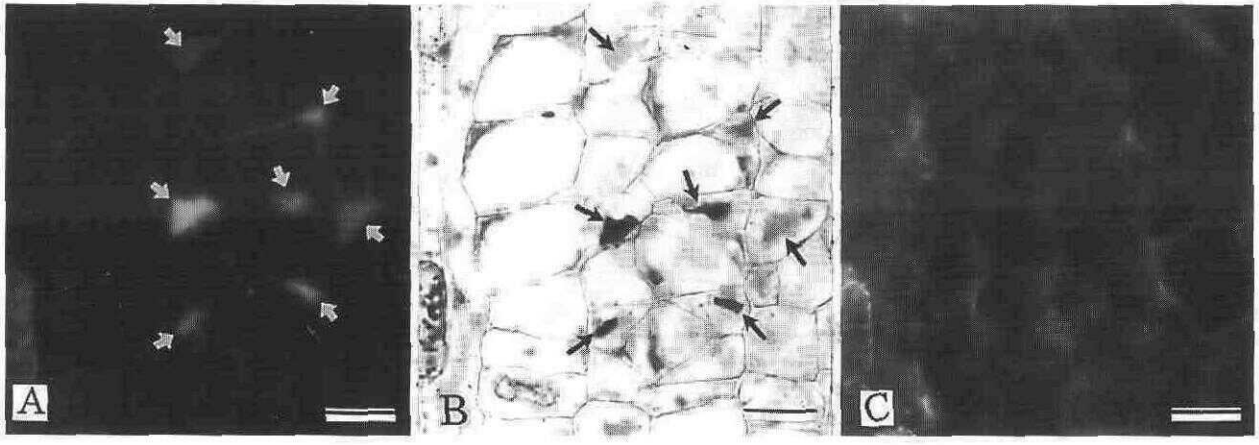

Figure 4. Protein-storing cells with a 67 kDa protein in Hevea *trees. Light micrographs of cross sections of inner bark from untapped tree showing immunolocalisation of PSC 67 kDa protein. Section A: treated with polyclonal 67 kDa antiserum (the first antibody) and FITC-conjugated goat anti-rabbit IgGs (the second antibody), under fluorescence. Section B: The section A stained with mecury bromophenol blue showing general protein, under bright field optics. Section C: treated with pre-immune serum as control. Note that the FITC-labelled mass in A (arrows) is identical to the mass of PSC vacuole protein inclusion in B (arrows), and no FITC-fluorescence is in Section C. Bar = 25 μm.*

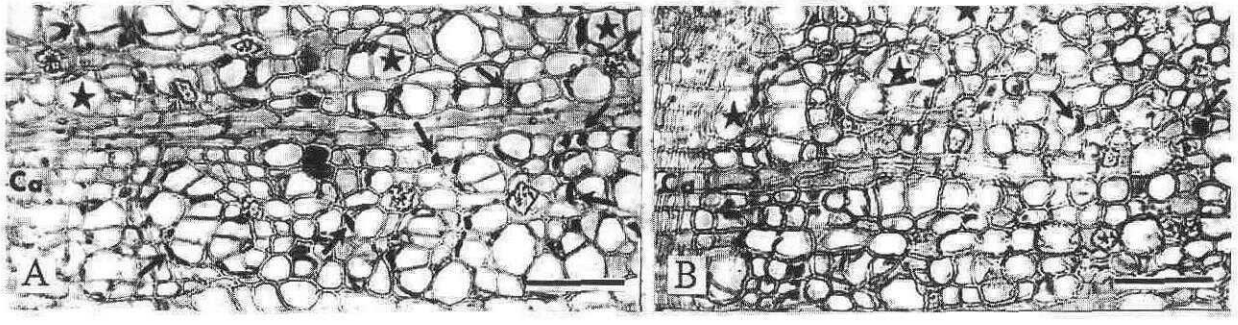

Figure 5. Protein-storing cells with a 67kDa protein in Hevea *trees. Light micrographs of cross-sections of inner barks at 2 cm below tapping cut, showing the distributions of vacuole protein inclusion mass (arrows) of PSCs in TPD affected (A) and healthy tapped (B) trees. Bar = 100 μm.*

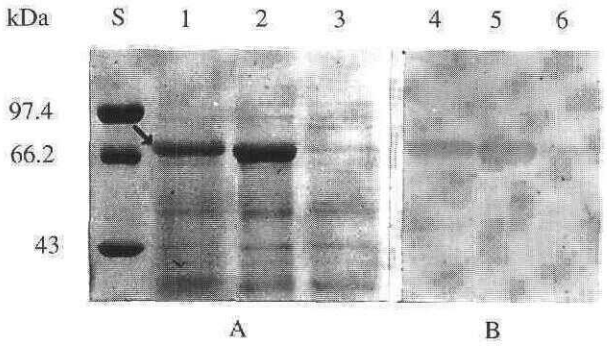

Figure 6. Protein-storing cells with a 67 kDa protein in Hevea *trees. Sodium dodecyl sulphate-gel (lanes 1–3) and western immunoblot of its duplication (lanes 4–6). Solube proteins from inner barks of untapped (lanes 1, 4), TPD affected (lanes 2, 5) and healthy tapped (lanes 3, 6) trees. 20 µg protein were loaded per lane. Lane S: standard proteins; Arrow: the 67 kDa protein.*

material for latex regeneration in trees in regular tapping.

Recently, efforts were made to find protein markers linked to TPD, a disease seriously concerning *Hevea* plantation. Our study showed that in comparison with the healthy tapped tree, the TPD affected tree had much more 67 kDa protein in the inner bark near the tapping cut and demonstrated that 67 kDa protein was the vacuole inclusion of PSCs. Since latex exploitation caused a marked decrease of the 67 kDa protein under tapping cut, the relative abundant 67 kDa protein in TPD tree may result from the tapping rest. In this regard, we suggest that the abundant 67 kDa protein in TPD trees might be identical with 70 kDa protein that Nataraja *et al.*[3] revealed by SDS-PAGE in TPD affected inner bark. Both of the proteins were extracted from the same tissues (inner barks 2mm~3mm from vascular cambium) by similar method and had similar molecular weight. In addition, by electrophoresis Lacrotte *et al.*[17] found some new and over-expressed proteins in the latex of TPD trees. In the future study it should be considered whether the changes in latex proteins in TPD trees are related to the changes in bark proteins.

The storage role of the vacuole proteins in PSCs was again emphasised in the present study. However, increasing evidence indicated that some vegetative storage proteins might have important defensive or other roles and in reverse, many proteins with other metabolic functions might function as reserves in some conditions[13–16]. Based on the characteristics of the *Hevea* PSCs and their 67 kDa protein we had assumed that the PSCs with their vacuole proteins might have other physiological functions[6]. To test this assumption, the biochemical properties of the 67 kDa protein are being investigated.

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REFERENCES

1 d'AUZAC, J , JACOB, J L AND CRESTIN, H *(eds)* (1989) *Physiology of Rubber Tree Latex* Boca Raton, Florida CRC Press

2 YIP, E AND GOMEZ, J B (1984) Characterization of Cell Sap of *Hevea* and Its Influence on Cessation of Latex Flow *J Rubb Res Inst Malaysia*, **32(1)**, 1–19

3 NATARAJA, K N , KRISHNAKUMAR, R , JACOB, J AND SETHURAJ, M R (1997) Analysis of the Protein Profiles of Healthy and TPD Affected *Hevea brasiliensis* Bark Tissues *IRRDB Workshop on TPD of* Hevea, *Hainan, China, 29–30 April 1997*

4 WU, J -L AND HAO, B-Z (1987) Protein-storing Gells in Secondary Phloem of *Hevea brasiliensis Kexue Tonbao (Peking)*, **32**, 118

5 WU, J -L AND HAO, B-Z (1987) Ultrastructure and Differentiation of Protein-storing Cells in Secondary Phloem of *Hevea brasiliensis* Stem *Ann Bot* , **60**, 505

6 TIAN, W -M , HAN, Y -Q , WU, J -L AND HAO, B -Z (1998) Characteristics of Protein-storing Cells Associated with a 67 kDa Protein in *Hevea brasiliensis Trees* **12**, 153

7 PEARSE, A G E (1960) *Histochemistry Theoretical and Applied* London J & A Churchill

8 BRADFORD, M M (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding *Anal Biochem* , **72**, 248

9 COLEMAN, G D , CHEN, T H H , ERNST, S G AND FUCHIGAMI, L (1991) Photoperiod Control of Poplar Bark Storage Protein Accumulation *Plant Physiol* , **96**, 686

10 MISHKIND, M L , PLUMLEY, FG AND RAIKHEL, N V (1987) Immunocheical Analysis of Plant Tissue *Handbook of Plant Cytochemistry Vol II Other Cytochemical Staining Procedures (Vaughn, K C ed)*, p 65 Boca Raton, Florida CRC Press

11 TOWBIN, H , STAEHELIN, T AND GORDON, J (1979) Electrophoretic Transfer of Protein from Polyacrylamide Gels to Nitrocellulose Sheets Procedure and Some Applications *Proc Natl Acad Sci USA*, **76(9)**, *4350*

12 BURNETTE, W N (1981) 'Western Blotting' Electrophoretic Transfer of Protein from Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radio-iodinated Protein A *Anal Biochem* **112**, 195

13 TRANBARGER, T J , FRANCESCHI,V R , HILDEBRAND, D F , AND GRIMES, H D (1991) The Soybean 94-Kilodalton Vegetative Storage Protein is a Lipoxygenase that is Localized in Paravenal Mesophyll Cell Vacuoles *Plant Cell* **3**, 973

14 STEPIEN, V , SAUTER, J J AND MARTIN, F (1994) Vegetative Storage Protein in Woody Plants *Plant Physiol Biochem* **32**, 185

15 STASWICK, P E (1994) Storage Protein of Vegetative Plant Tissues *Annu Rev Plant Physiol Plant Mol Bio* **145**, 303

16 BELL, E AND MULLET, J E (1991) Lipoxygenase Gene Expression is Modulated in Plant by Water Deficit, Wounding, and Methyl Jasmonate *Mol Gen Genet* **230**, 456

17 LACROTTE, R , GIDROL, X , VICHIT-CHOLCHAI, N , PUJADE-RENAUD, V , NARANGAJAVANA, J AND CHRESTIN, H (1995) *Hevea* Protein Markers of Tapping Panel Dryness *Plantations, recherche, developpement* **2(4)**, 40