Taiwania, **41**(3): 244-251, 1996

Immunofluorescent Localization of 24 kDa Protein in *Luffa cylindrica* Phloem Exudate

Yih-Yuan Chang[1] and Yung-Reui Chen[1,2]

(Manuscript received 24 August 1996; accepted 8 September 1996)

ABSTRACT : A 24 kDa phloem protein was isolated from phloem exudate of *Luffa cylindrica* fruits by the method of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal antibodies obtained from rabbit serum recognized both the native protein and denatured protein subunits. Immunofluorescent study was done by a FITC-conjugated goat-anti-rabbit IgG. Immunolabelled sections of pepo show that the antibodies strongly bound with 24 kDa protein in the bicollateral phloem tissues of vascular bundle. The intensity of green fluorescence is related to the degree of phloem differentiation. Similar results were also observed in stems and petioles of leaves.

KEY WORDS: Phloem protein, Immunofluorescence, Localization, *Luffa cylindrica*.

INTRODUCTION

Phloem proteins (p-proteins) are proteins that express specifically in phloem tissues of monocotyledons and dicotyledons (Arsanto, 1982). They are plentiful in the sieve elements and sometimes are also found in companion cells and phloem parenchymatous cells (Cronshaw and Sabnis, 1990). P-protein are filamentous, spherical, tubular, crystalline and amorphous in shape (Bouck and Cronshaw, 1965; Cronshaw and Esau, 1967). More than one kind of p-protein can be found in the same plants. Many organelles have been implicated in the formation of p-proteins due to their differences in structure, shape and ontogenesis (Cronshaw, 1975). The diversities in shape and the biogenesis of p-proteins related to plant species and developmental stages of phloem tissues are well documented (Behnke and Sjolund, 1990).

Early studies showed that the presence of slime plugs in mature sieve elements was cytochemically detected by mercuric bromophenol blue or Ponceaus S (Cronshaw and Esau, 1967; Esau and Cheadle, 1965). These slime plugs disappeared in sections treated with protease digestion *in situ* (Yapa and Spanner, 1972). Immunofluorescent studies on phloem proteins did reveal that sieve elements of whole plants or plant tissue cultures were recognized by polyclonal antibodies of phloem proteins (Sjolund, 1990). However, lectin genes of PP1 and PP2 were specifically expressed in companion cells of *Cucurbita maxima* (Bostwick *et al.*, 1992).

1. Department of Botany, National Taiwan University, Taipei 106, Taiwan, Republic of China.
2. Corresponding author.

A 24 kDa protein isolated from phloem exudate of *Luffa cylindrica* fruits is species-specific (Chang, 1995). In the present study, the immunocytochemical localization of the 24 kDa protein was examined with fluoresein isothiocyanate (FITC)-conjugate anti-rabbit IgG under fluorescence microscope.

MATERIALS AND METHODS

Fruits of *Luffa cylindrica* were harvested for the anatomical study. The outer parts of fruits containing exocarp and mesocarp were firstly cut into small cubes of 1 mm^3, prefixed in 0.1 M phosphate buffer containing 2.5 % glutaraldehyde for 4 hours, and after washing three times in buffer (15 min each time), postfixed in 0.1 M phosphate buffer containing 3 % osmium tetraoxide for 2-12 h. Fixed samples rinsed three times in buffer were dehydrated through a graded ethanol series and embeded in Spurr's resin (Spurr, 1969). Plastics obtained from polymerized resin at 70°C for 8 h were trimmed and cut with a glass knife. Semi-thin sections (1 μm in thickness) stained with Coomassie brilliant blue R250 were photographed by a Leitz Diaplan microscope.

The isolation, purification and immunolocalization of phloem 24 kDa protein followed the method of Chang (1995). For immunolabelling study, the method of Doonan and Clayton (1986) was described briefly as follows: sections 20-30 μm in thickness were firstly obtained from fruits, stems and petioles of *Luffa cylindrica* by means of free-hand sections; saturated with 1 % gelatin in phosphate buffered saline (PBS) for 15 min to reduce unspecific binding sites; incubated with rabbit antiserum in 1 % gelatin solution at 1 to 100 dilution after removal of unspecific bindings; washed three times with PBST (0.1 M phosphate buffer saline containing 0.05 % Tween-20); then, incubated with FITC-conjugated goat anti-rabbit IgG in the dark for 1 h. The indirectly immunolabelled sections were examined and photographed by a Leitz Diaplan microscope with a BP 350-460 excitation filter and a LP 520 barrier filter.

RESULTS

As shown in Figures 1A and 1B, the exocarp consists of uniseriate epidermal cells and the mesocarp consists of chlorenchyma, sclerenchyma, vascular bundles, and flesh parenchyma. The epidermal cells are brick-shaped, uniform in size, and their outer wall is covered with a cuticular layer (Fig. 1A). The subepidermal layers vary in width and mainly consist of parenchymatous cells that contain normal chloroplasts. The sclerenchymatous tissue is two to several-cell layers in thickness and in continuous layers or seated in mass between subepidermal parenchyma and flesh parenchyma (Fig. 1C). The cells are strongly lignified and most of them become scleroid fibers (Fig. 1D). Flesh parenchymatous cells occupy most of the mesocarp and extend to the center of the fruit; their sizes are correlated with their location in the parenchymatous tissue, generally increasing from the outer to inner layer of tissue. All of the flesh parenchymatous cells are juicy and have abundant intercellular spaces (Figs. 1A, 1C). The vascular system is bicollateral in arrangement. The outer and inner phloem tissues are present on both sides of xylem tissues (Fig. 1F). The

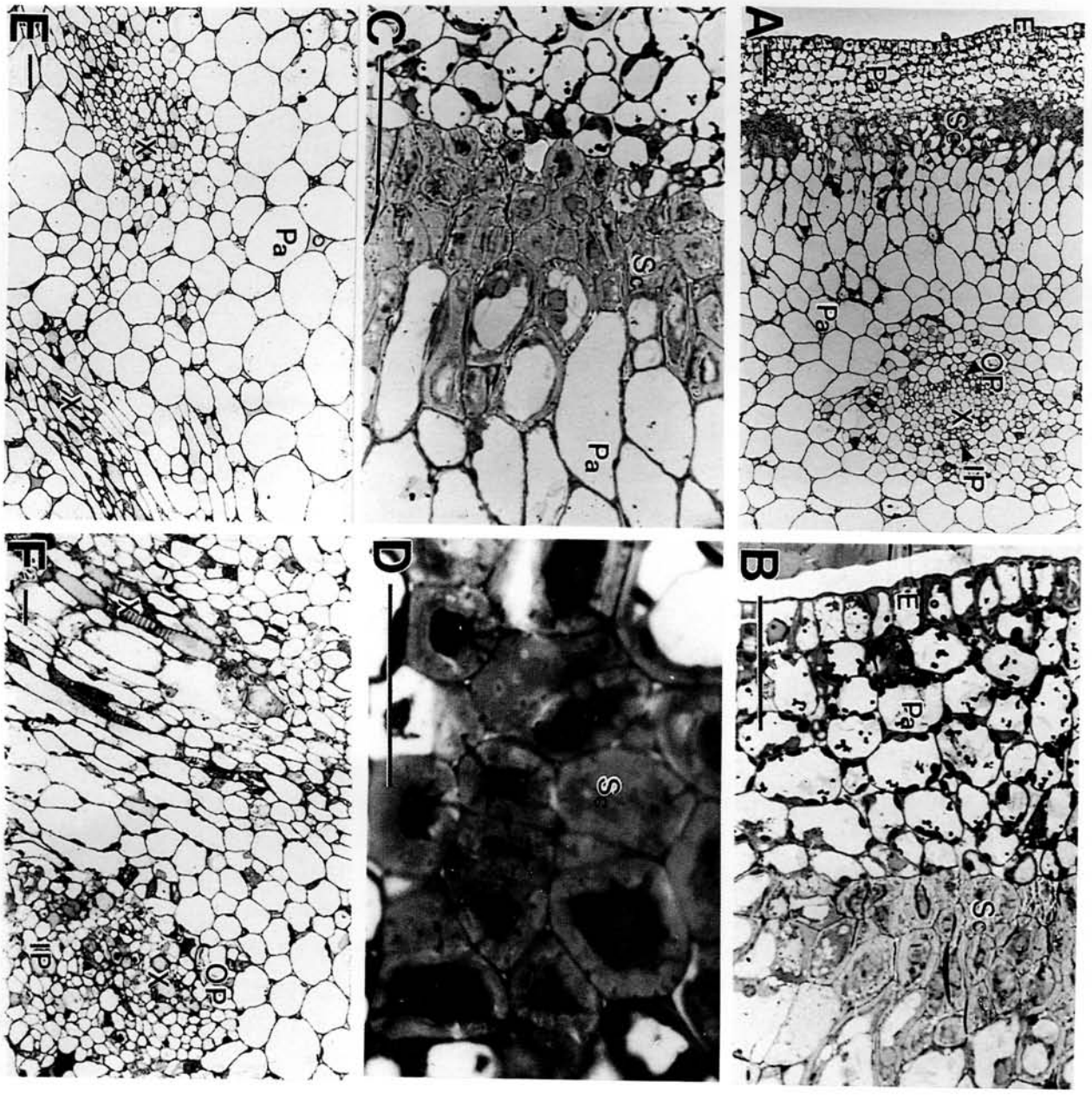

Fig. 1. Structure of exocarp and mesocarp of *Luffa cylindrica*. A, Cross section of exocarp and outer mesocarp showing the uniseriate epidermis covering the heterogenous tissues of mesocarp. B, Epidermis, subepidermal parenchymatous cells and sclerenchymatous tissue. C, Sclerenchymatous tissue between subepidermal and flesh parenchymatous tissues. D, Scleroid fibers in sclerenchymatous tissues. E, Parts of transverse interconnected vascular bundles. F, Cross section of vascular bundles in different orientation. All bars are 5 μm. (Ch, chlorenchyma; E, epidermis; IP, inner phloem; OP, outer phloem; Pa, parenchyma; Sc, sclerenchyma; X, xylem)

orientation of vascular bundles is multiple directional and forms an interconnected network. The small vascular strands are mostly contained in phloem strands and sparsely distributed in flesh parenchymatous tissues. Phloem exudate contained higher amounts of proteins than that reported for homogenates of other tissues of plants (Chang, 1995). Protein staining of pericarp with Coomassie brilliant blue R250 showed high stainability of protein dye in protein bodies of various tissues (Fig. 2A), especially in phloem tissues (Fig. 2B).

Autofluorescences of lignin and chlorophylls were found in unfixed sections without the incubation of FITC-labelled antibodies under fluorescence microscope (Figs. 2C). Te sites of lignin-autofluorescence are dependent upon the degree of lignification in cell wall thickening of different tissues or organs. In fruits, sclerenchymatous layers are the major site of yellow fluorescence of lignin and xylem is the minor one (Fig. 2C). In twigs, yellow fluorescence come from sclerenchymatous tissue, vascular xylem and the outer cuticular layer of outer epidermis; in young petioles, weak yellow fluorescence is emitted from xylem of vascular bundles (personal data). Autofluorescence of chlorophyll in red was observed in various tissues of fruits, twig and petiole (Figs. 2C-2E) even though the outer epidermal cells and inner flesh parenchymatous cells are all in red. This indicates that the observed materials have plastids containing chlorophyll-pigment.

Surveys of immunofluorescent section showed that the FITC-conjugated goat antirabbit-IgG of 24 kDa protein was limited to vascular bundles of the examined fruits, stems and petioles (Figs. 2D-F). As shown in Figure 2D, only inner and outer phloem tissues of the bicollateral vascular bundles in fruits were stained in green although, some xylem tissues were also seen in pale green. In young vascular bundles, green fluorescence was found in networked phloem tissues. Similar results were also found in twig and petioles (Figs. 2E, 2F). All of the above facts indicate that 24 kDa protein is found in the phloem of different plant organs.

DISCUSSION

The structure of the pepo includes exocarp, mesocarp and endocarp. The fruit wall mainly contains a massive, heterogeneous and pigment-containing mesocarp enclosed between exocarp and endocarp (Esau, 1965). Both the external and internal coloring of different cucurbitaceous fruits depend on the plastid interconversion of chromoplasts and chloroplasts. The outer coloring turning yellow in pumpkins is due to the conversion of chloroplasts to chromoplasts (Devide and Ljubesic, 1974). In watermelons, the yellow, pink, and red internal coloring is based upon formation of needle and plate crystallines in chromoplasts (Matienko, 1969 cited in Esau, 1977). However, in *Luffa cylindrica*, chlorophyll-containing plastids are indicated by their outer green compact layer and inner light-green juicy layers of pericarp.

The autofluorescence of chlorophylls in unfixed section could be overcome by the regular process of fixation and dehydration in section preparation (Chen *et al.*, 1995). However, the autofluorescence from lignin is always retained in sections. Careful interpretation of black and white photographs in comparison with control experiments is needed.

In fruits of *Cucurbita maxima*, PP1 (80 kDa) and PP2 (26 kDa) are immunologically cross-reacted phloem proteins (Read and Northcote, 1983). Immunoperoxidase localization with polyclonal antibody of PP2 shows that the strong binding occurred in sieve elements and companion cells (Smith *et al.*, 1987). With monoclonal antibody against phloem specific-protein, immunolabelled-FITC was observed in the phloem of whole plant and the redifferentiating phloem in calli of *Streptanthus tortuosus* (Toth *et al.*, 1994; Wang *et al.*, 1995). However, FITC-conjugated goat antirabbit IgG of phloem lectin was detected in axial and ray parenchyma cells (Yoshida *et al.*, 1994). In the present study, a 24 kDa

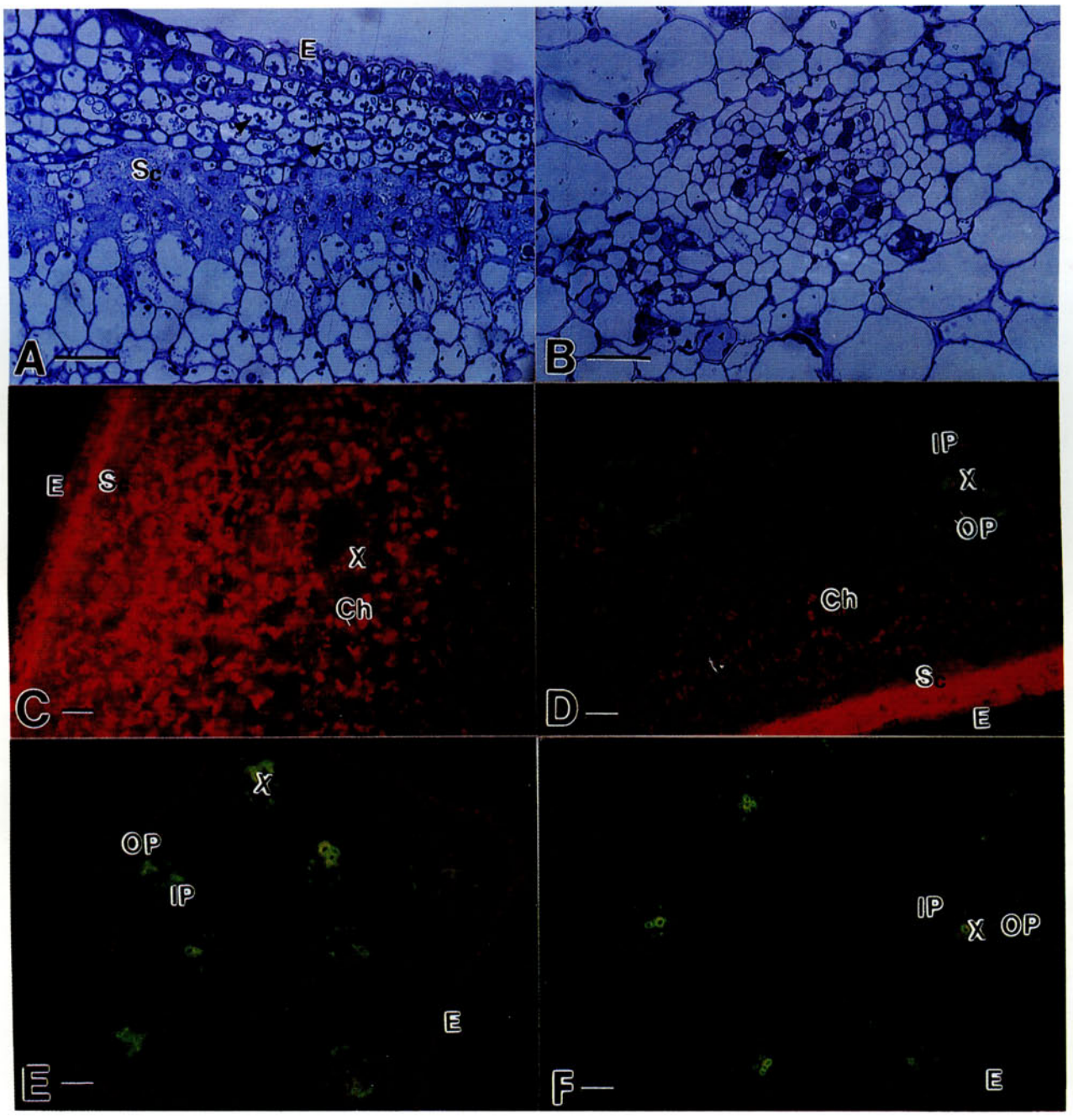

Fig. 2, Protein staining and immunofluorescent staining of 24 kDa p-protein in *Luffa cylindrica*. A and B, Exocarp and mesocarp stained with Coomassie blue showing protein bodies (arrowheads) in different tissues. C, Autofluorescences of lignin and chlorophylls in pericarp section of control experiment. D, E and F, Immunofluorescence micrographs of fruit, twig and petiole, respectively, and only inner and outer phloem tissues of the bicollateral vascular bundles stained in green. Autofluorescences of chlorophylls and lignin are red and yellow in color, respectively. All bars are 5 μm. (Ch, chlorenchyma; E, epidermis; IP, inner phloem; OP, outer phloem; Pa, parenchyma; Sc, sclerenchyma; X, xylem)

protein was immunocytochemically determined within the bicollateral vascular tissues. It is consistent with the results of immunoblotting in our previous study (Chang and Chen, 1995). However, the occurrence of pale green immunofluorescence in some vessels indicates that 24 kDa protein cross-reacted with some proteins in the xylem. This may suggest circulation between xylem and phloem.

ACKNOWLEDGEMENT

This study was financially supported by a grant from National Science Council. The generosity of Professor Ling-Long Kuo-Huang of our department for using Leitz Diaplan microscope and related facilities is also acknowledged.

LITERATURE CITED

Arsanto, J. P. 1982. Observations on p-protein in dicotyledons: substructural and developmental features. Am. J. Bot. **69**: 1200-1212.

Behnke, H. D. and R. D. Sjolund. 1990. Sieve Elements: Comparative Structure, Induction and Development. Springer-Verlag, Berlin.

Bostwick D. E., J. M. Dannenhoffer, M. I. Skaggs, R. M. Lister, B. A. Larkins and G. A. Thompson. 1992. Pumpkin phloem lectin genes are specifically expressed in companion cells. Plant Cell **4**: 1539-1548.

Bouck, G. B. and J. Cronshaw. 1965. The fine structure of differentiating sieve tube elements. J. Cell Biol. **25**: 79-96.

Chang, Y.-Y. 1995. Purification, identification and localization of *Luffa cylindrica* phloem proteins. Master thesis of Department of Botany, National Taiwan Univerity, Taipei.

Chang, Y.-Y. and Y.-R. Chen. 1995. Phloem proteins of *Luffa cylindrica*. 10th Joint Annu. Conf. of Biomed. Sci. p122.

Chen, Y.-R., C.-C. Chen, J.-C. Hsu and C.-M. Yang. 1995. Structure and function of mungbean testa. Elctr. Microsc. Symp. at National Chung Sun University, Kaoshiung, Taiwan, p7-9.

Cronshaw, J. 1975. P-proteins. In "Phloem Transport"(Aronoff, S., J. Dainty, P. R. Gorham, L. M. Srivastava, and C. A. Swanson eds.). Plenum, New York.

Cronshaw, J. and K. Esau. 1967. Tubular and fibrillar components of mature and differentiating sieve elements. J. Cell Biol. **34**: 801-816.

Cronshaw, J. and D. D. Sabnis. 1990. Phloem proteins. In: Behnke, H. D. and R. D. Sjolund (eds.). Sieve Elements: Comparative Structure, Induction and Development. Springer-Verlag, Berlin.

Devide, Z. and N. Ljubesic. 1974. The reversion of chromoplast to chloroplast in pumpkin fruits. Z. Pflanzenphysiol. **73**: 296-306.

Doonan, J. H. and L. Clayton. 1986. Immunofluorescent studies on the plant cytoskeleton. In: Wang, T.-L. (ed.) Immunology in Plant Science. Cambridge University Press, Cambridge.

Esau, K. 1965. Plant Anatomy (2nd ed.). John Wiley & Sons, New York.

Esau, K. 1977. Anatomy of Seed Plants (2nd ed.). John Wiley and Sons, New York

Esau, K. and V. I. Cheadle. 1965. Cytologic studies on phloem. Univ. Cal. Publ. Bot. **36**: 619-630.

Read, S. M. and D. H. Northcote. 1983. Chemical and immunological similarities between the phloem proteins of three genera of the Cucurbitaceae. Planta **158**: 119-127.

Smith, L. M., D. D. Sabnis and R. P. C. Johnson. 1987. Immunocytochemical localisation of phloem lectin from *Cucurbita maxima* using peroxidase and colloidal-gold labels. Planta **170**: 461-470.

Sjolund, R. D.1990. Sieve elements in plant tissue cultures: development, freeze-fracture and isolation. In: Behnke, H. D. and R. D. Sjolund (eds.). Sieve Elements: Comparative Structure, Induction and Development. Springer-Verlag, Berlin.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31-43.

Toth, K. F., Q. Wang and R. D. Sjolund. 1994. Monoclonal antibodies against phloem p-protein from plant tissue cultures. I. Microscopy and biochemical analysis. Amer. J. Bot. **81**: 1370-1377.

Wang, Q., J. Monroe and R. D. Sjolund. 1995. Identification and characterization of a phloem specific β–amylase. Plant Physiol. **109**: 743-750.

Yapa, P. A. J. and D. C. Spanner. 1972. The effect of protease digestion (*in situ*) on the slime substance of mature sieve tube. Planta **107**: 89-96.

Yoshida, K., K. Baba, K. Yamamoto and K. Tazali. 1994. Cloning of a lectin cDNA and seasonal changes in levels of the lectin and its mRNA in the inner bark of *Robinia pseudoacacia*. Plant Mol. Biol. **25**: 845-853.

絲瓜韌皮部蛋白質的免疫螢光定位

張奕元[1]、陳榮銳[1,2]

（收稿日期：1996年8月24日；接受日期：1996年 9月8日）

摘　　要

絲瓜韌皮部溢流物經電泳分析及高效能液態色層分析，取得 24 kDA 的韌皮部蛋白質。此蛋百質打入兔子產生多元的抗體，能認出原性蛋白質或其變性蛋白質次體。以 FITC 免疫螢光細胞定位法檢出該蛋白質存在位置時，觀察到絲瓜果實內的維管束是其主要存在位置，尤以其雙側型的內生及外生韌皮部最為顯著。類似結果也在枝條及葉柄的維管束表現。

關鍵詞：韌皮部蛋白質、免疫螢光、定位、絲瓜。

1. 國立臺灣大學植物學系，臺北市106，臺灣，中華民國。
2. 通信連絡員。