Taiwania, **53**(4): 394-400, 2008

Various Microscopic Methods for Investigating the Venuloid Idioblasts of *Pteris grevilleana* Wall.

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(Manuscript received 5 July 2008; accepted 12 October 2008)

ABSTRACT: Venuloid idioblasts are vein-like structures. In *Pteris*, they are long epidermal cells with very thick cell walls. In this study, venuloid idioblasts of *Pteris grevilleana* were investigated with various light microscopic (LM) and scanning electron microscopic (SEM) techniques and the main purposes of these microscopic techniques are summarized and discussed. To investigate the morphology and distribution of venuloid idioblasts, partial polarization LM technique and cryo-tabletop-SEM technique were used. The idioblasts had lobed margins, acute or round ends, and could be found on both upper and lower epidermis of fertile fronds and sterile fronds. They are distributed on veins, interveinal regions, and leaf margins, but not on costules, costae, and false indusia. By using histochemical staining and SEM with energy dispersive X-ray spectrometer (EDS), it was confirmed that the idioblasts contained silicon. In addition, the venuloid-idioblast-like silica bodies were extracted by wet oxidation method. These indicate that the venuloid indioblast in *P. grevilleana* is a kind of spicular cell (long epidermal cells containing silica bodies, which are found in Adiantoids and Vittarioids).

KEY WORDS: EDS, false vein, partial polarization, Pteridaceae, *Pteris grevilleana*, silica body, spicular cell, tabletop SEM, venuloid idioblast, wet oxidation.

INTRODUCTION

Venuloid idioblasts are tiny veinlet-like structures scattered in interveinal, submarginal, or sinus regions of laminas, which never attach to true veins (Wagner, 1978). These structures are known as false veins, a taxonomical character in ferns. In *Pteris*, they are specialized long epidermal cells with thickened cell walls, which can be observed by hand lens. This structure was first reported in *Pteris grevilleana* Wall. (Holttum, 1954), a terrestrial fern distributed over tropical and eastern Asia. In Taiwan, they can be found in the understory of low altitude broad-leaved forest (Fig. 1A).

Spicular cells are defined as long epidermal cells containing spicules of silica (Williams, 1927). They have hyaline and pronouncedly thickened cell walls which almost occlude their cell lumens (Nayar, 1961). This kind of cell is found in Adiantoids and Vittarioids. The molecular evidence shows the two taxa are sister groups nested in Pteridaceae *sensu lato* (Smith et al., 2006; Schuettpelz et al., 2007). However, spicular cells in these two taxa may not be homologous structures because of the difference in their distribution patterns (Nayar, 1961). In Vittarioids, spicular cells are distributed on foliar epidermis, but in Adiantoids they are distributed over the veins and restricted to areas contiguous with sclerenchyma sheaths of the veins.

The morphology of venuloid idioblasts is similar to the spicular cells (Wagner, 1978), but whether the venuloid idioblasts of *Pteris* is a kind of spicular cell is not sure. The silica contained is needed for testing. The distribution of venuloid idioblasts in *Pteris* also needs to be further tested. By Wagner's observation, they are never attached to true veins (Wagner, 1978). However, in *P. grevilliana*, idioblasts are similar to true veins under Nomarski interference contrast illumination (DIC) and in another species, *Pteris multifida* Poir., they tend to cluster along the veins.

In this study, several light microscopic (LM) and scanning electron microscopic (SEM) techniques were used to investigating venuloid idioblasts of *Pteris grevilleana* with emphases on containing silica and their distribution. We also summarized major observation characteristics of each microscopic technique used in this study. This may contribute to the future works on venuloid idioblast investigation in Pteridaceae.

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MATERIALS AND METHODS

Plant materials

Pteris grevilleana were collected from the understory of a secondary forest in Fuyang Eco Park (25° 01′ N, 121° 33′ E, altitude 50 m), in southern Taipei, Taiwan in October, 2007 and June, 2008. Pinnules from the middle of mature fronds were sampled (Fig. 1B). Voucher specimen (*Kao, Tzu-Tong 08051*) was made and deposited in TAI herbarium.

Dissecting microscope observation

Samples were directly examined under a WILD M3Z dissecting microscope (Leica). Photographs were taken by a COOLPIX 4500 digital camera (Nikon).

LM observation

Samples for LM observation were pretreated with five different ways: (1) direct observation, (2) epidermis impression, (3) clearing, (4) paraffin section, and (5) wet oxidation. The direct observation: samples were directly observed without any treatment. The epidermis impression (Hilu and Randoll, 1984): a thin layer of clear nail polish was applied on the frond surface. The applied nail polish was air dried and then peeled off by forceps. The peeled nail polish films were observed. The clearing: samples were boiled in 95% ethanol with a hot water bath for 3-5 hours to remove the chlorophyll. The samples were transferred into 4% NaOH aquatic solution for 5 days until the samples were clear (without color). Then the samples were stored in 70% ethanol. The paraffin section: samples were subdivided into fragments about 5 x 5 mm^2 and fixed in FPGA (Formalin: Propionic acid: Glycerol: 95% Ethanol: distilled water = 1: 1: 3: 7: 8) at 4℃ overnight. The fixed samples then were dehydrated by Alcohol-TBA (Tert-Butyl Alcohol) series at room temperature for a day, infiltrated by paraffin at 65℃ for three days, and embedded in paraffin. Next step, the embedded samples were sectioned into 20 μm thick sections by an 820 rotary microtome, AO. The sections were attached to a slide by Stay-On (Surgipath) and extended on a hot plate at 40℃. Continuously, slides were emerged into xylene to remove the paraffin. Then the slides were rehydrated in ethanol series and stored in 70% ethanol. The wet oxidation (Piperno, 2006): 1 g fronds were fragmented and washed with 1% potassium hypochlorite aquatic solution. The washed materials were boiled in concentrated nitric acid with a hot water bath for 2 hours and solid potassium chlorate was added during the procedure. The solutions were centrifuged 10 minutes at 3000 rpm and the supernatant was removed. The precipitates were resuspended in distilled water. The centrifuged processes were repeated three times. The final suspended solution was filtered with a 125 μm sieve and a 10 μm sieve (the latter one was under ultrasonic condition). The silica bodies were washed out from the 10 μm sieve and stored in a 1.5 mL microtube with 1.0 mL distilled water.

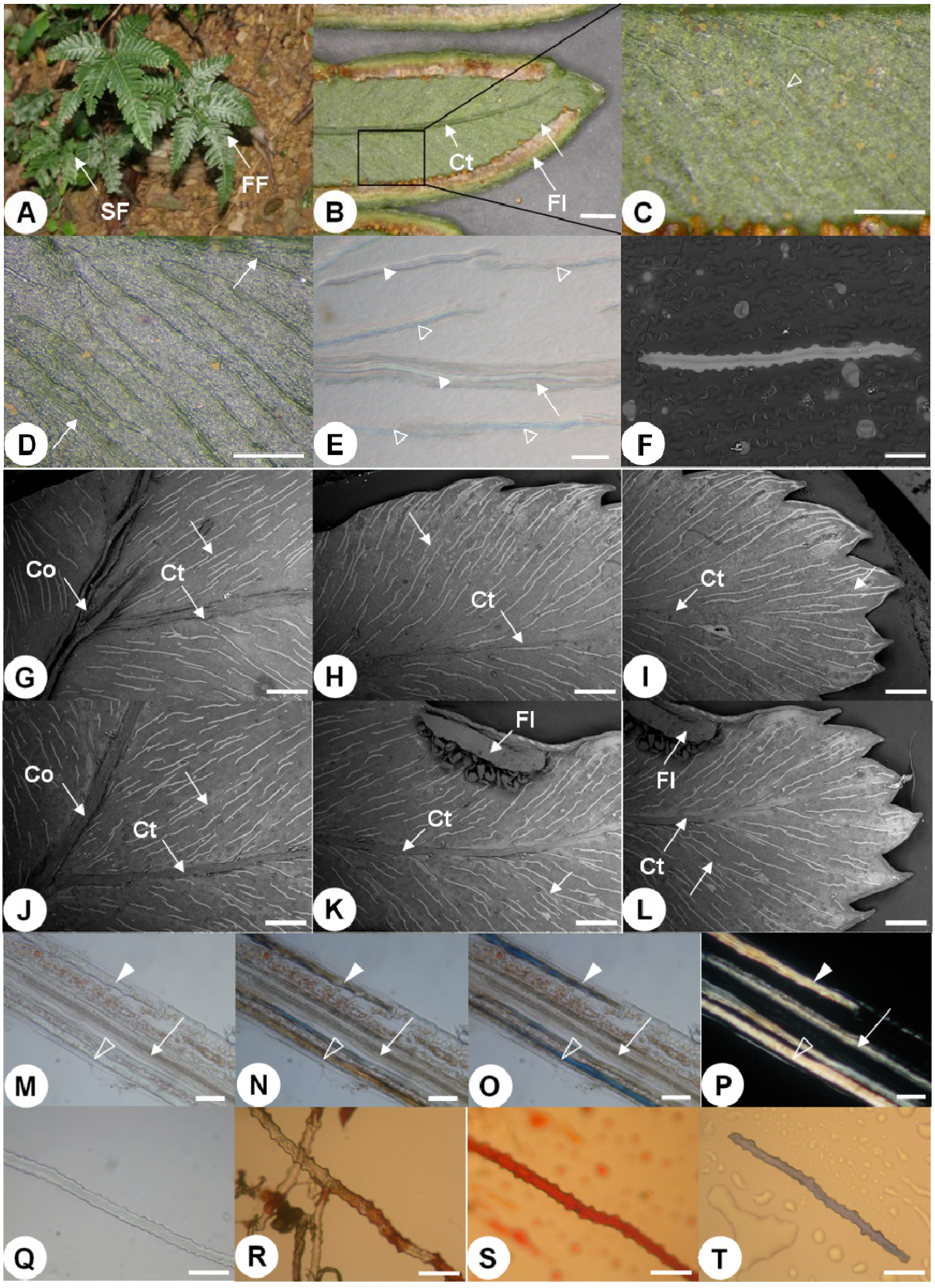
The pretreated samples were directly observed under LM or stained with silica body specific dyes, SAC (Silver-ammine chromate), CVL (Crystal Violet Lactone), and MR (Methyl Red) (Dayanandan et al., 1983). SAC was saturated Ag_2CrO_4 in 3% NH_4OH; MR was saturated methyl red solution in benzene; and CLV was 0.1% crystal violet lactone solution in benzene. For SAC staining, samples were transferred into distilled water and stained by SAC. For MR and CLV, samples were dehydrated by ethanol series (except silica bodies obtained from wet oxidation, which were direct air dried), transferred into benzene, and stained with MR and CLV.

The pretreated samples were examined under a LEITZ DMRB LM, Leica. Bright field (BF), dark field (DF), phase contrast (Phase), polarization contrast (Polarization) (including partial polarization), and DIC were used.

SEM observation

Two tabletop SEMs, TM-1000 (Hitachi) and PHENOM (FEI) were used. They were low pressure SEMs, which detected backscatter electrons and the accelerating voltages were 15 kV and 5 kV. Fresh pinnules and silica bodies obtained from wet oxidation were used. For fresh pinnule observation, samples were mounted on carbon stubs with carbon tape. The stubs were placed on a cold stage (Pre-cryogenic electron microscope specimen holder, patent pending), which was pre-frozen by liquid nitrogen, and observed as soon as possible. For silica bodies, 10 μL samples were dropped on carbon stubs (with carbon tape) and air dried overnight. The dried samples were directly taken for observation.

For ordinary SEM observation, fresh pinnules and samples pre-observed by tabletop SEMs were used. The samples were fixed in Karnovsky's fixative (Karnovsky et al., 1965) overnight at 4℃, washed by 0.1 M sodium phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, washed by 0.1M sodium phosphate buffer again, dehydrated in ethanol series, transferred into acetone, and dried with liquid CO_2 by HCP-2 CPD (Critical Point Dryer), Hitachi (Except for silica bodies which were directly taken for coating after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they were observed by tabletop SEM). The dried samples were mounted on carbon stages with carbon tape and coated with carbon by K950X, EMITECH. Samples were observed with S-2400 SEM, Hitachi. Besides, trace element analyses and Silicon dot mapping were done with KEVEX LEVEL4 Energy Dispersive X-Ray Spectrometer (EDS) (Lin et al., 2004).

RESULTS AND DISCUSSION

Venuloid idioblasts of *Pteris grevilleana* were found on both upper and lower surfaces (Figs. 1B-D, 1G-L) of fertile as well as sterile fronds. Under dissecting microscope, both venuloid idioblasts and veins are slightly protruded on frond surface and glistened when exposed to light (Figs. 1B-D). By LM and SEM, venuloid idioblasts are long epidermal cells parallel to the veins (free veins on fronds; rachids, costa, and costules are not included). They ranged from 100 to 1600 μm in length, which have lobed margins and acute or round ends (Figs. 1E & F). They could be found on veins, interveinal regions, and frond margin but not on costules, costae, and false indusia (Figs. 1G-L). Interestingly, by SEMs under backscatter electron detecting condition, the idioblasts were significantly lighter than ordinary epidermal cells (Fig. 1F). This indicates the difference in chemical composition between them.

Venuloid idioblasts had thick cell walls which almost occupied the cell lumens (Figs. 1M-P). They showed different colors under different polarization conditions, which could easily be distinguished from other cells. At 0°, they are hyaline (Fig. 1M); at around 30°, the inner parts (regions near the cell lumen) of cell walls are brown and the outer part is hyaline (Fig. 1N); at around 60°, they are blue (Fig. 1O); and at 90°, they are light yellow or white (the back ground was black) (Fig. 1P). This indicates something in the cell wall is arranged in a particular way. Other LM techniques, including dark field, phase contrast, and DIC were also used. Under dark field and phase contrast venuloid idioblasts were brighter than other cells. Under DIC, the result was similar to the result of polarization (data not shown).

SEM trace element analysis showed the idioblasts contain mass of silicon (Fig. 2A) and Si dot mapping showed the silicon composition was significantly higher than other epidermal cells (Figs. 2B & C). Venuloid-idioblasts-like silica bodies were obtained by wet oxidation, which can be stained with silanol group [-SiOH] specific dyes SAC, MR, and CLV (Fig. 1R-T). They have characteristically large silica bodies (Figs. 1Q and 3) (15~25 μm in width and the length could up to 1mm), which is specific to Polypods (Piperno, 2006). They have undulated lateral surfaces and flat upper and lower surfaces with wavy protrusions. The upper surface is coarser and smaller than the lower surface and often had a concave long axis. On the other hand, they could not easily react with SAC (in Fig. 1R, an hour was spent for staining and the red brown sliver chromate participates did not be formed homogeneously on silica body). This may be resulted from the tightly compact silica on outer boundaries of silica bodies (Dayanandan et al., 1983). However, the silica bodies could easily be stained with MR and CLV. This indicates the surfaces of silica bodies have entrances large enough for the MR and CLV molecules to pass through (The molecular area of MR and CLV are about 1.16 nm^2 and 1.64 nm^2 respectively) (Dayanandan et al., 1983).

Investigating methods which were used in this study were summarized in Table 1. Among them, two methods are most efficient on investigating the morphology and distribution of venuloid idioblasts; one is partial polarization LM technique; the other is cryo-tabletop-SEM technique (under backscatter

Fig. 1. Morphology, distribution and histochemistry of venuloid idioblasts. A: Habitat of *P. grevilleana*, an understory terrestrial fern. Venuloid idioblasts can be found on both fertile fronds (FF, erect and have longer stipes ones) and sterile fronds (SF). B: Lower surface of a mature fertile pinnule. The veins are oblique joined to costae (Ct) and the false indusia (FI) is formed by reflexed frond margins. (Bar = 1 mm). C: Partial enlargement of Fig. B., glisten lines parallel to veins are venuloid idioblasts. (Bar = 500 μm) D: Upper surface of a mature fertile pinnule. Two veins are showed in the micrograph. All the other vein-like raised lines are venuloid idioblasts. (Bar = 500 μm) E: Using partial polarization LM technique to observe the idioblasts, the sample was pre-treated with clearing technique. The idioblasts can be easily distinguished from other cells. Note that the idioblasts can be found on true veins. (Bar = 50 μm) F: Using cryo-tabletop-SEM (scanning electron microscope) technique to observe the idioblasts, the idioblast is significant lighter than ordinary epidermal cells. (Bar = 50 μm) G-L: Distribution of venuloid idioblasts. (Bar = 500 μm) Upper (G-I) and lower surface (J-L) of pinnules were observed with a tabletop SEM TM-1000, Hitachi. Venuloid idioblasts (white bright lines) are randomly dispersed on both upper and lower epidermis. They distribute on veins, interveinal regions, and leaf margins, but cannot be found on costae (Co), costules (Ct), and false indusia (FI). M-P: Paraveinal section of a frond, the idioblasts can be found on upper and lower epidermis of a vein. The thick hyaline cell walls almost occupy the cell lumens of the idioblasts, which show different color under different partial polarization conditions ((M) 0° (N) 30° (O) 60° and (P) 90° respectively). (Bars = 50 μm) Q: Morphology of the venuloid-idioblast-like silica body. (Bars = 100 μm) R-T: Histochemical staining of the silica body. (Bars = 100 μm) (R) Staining with silver ammonium chromate (SAC), red brown silver chromate is deposited on surface of silica bodies. (S) Silica bodies show red color when stain with methyl red (MR). (t) Silica bodies shows blue or purple color when stain with crystal violet lactone (CLV). Arrow: veins; Solid arrow heads: venuloid idioblasts on upper epidermis; Empty arrow head: venuloid idioblasts on lower epidermis.

Table 1. The major observation characters of investigating methods which were used in this paper.

Investigating methods	Major observation characters
Dissecting microscope	
direct observation	position of venuloid idioblasts on plant body and preliminary observations
Light microscope	
direct observation	preliminary observations
epidermis impression	contour of epidermal tissues
clearing	morphology and distribution of venuloid idioblasts on frond
paraffin section	anatomical structure of venuloid idioblasts
wet oxidation	extracting silica bodies for further observations
* histochemical staining	confirm the venuloid idioblast containing silica and distinguish them from other structures
*LM techniques (especially partial polarization)	optical characters of venuloid idioblast and distinguishing them from other structures
Scanning electron microscope	
** tabletop SEM	morphology and distribution of venuloid idioblast and distinguishing them from other structures
** EDS	trace element analysis and check the Silicon-containing

* Partial polarization can easily distinguish venuloid idioblasts from other cells, the containing of silica could be further checked by histochemical staining.

** Table top SEM can easily distinguish venuloid idioblasts from other cells. After observation, the samples can be recycled for trace element analysis under SEM to confirm the containing of silicon.

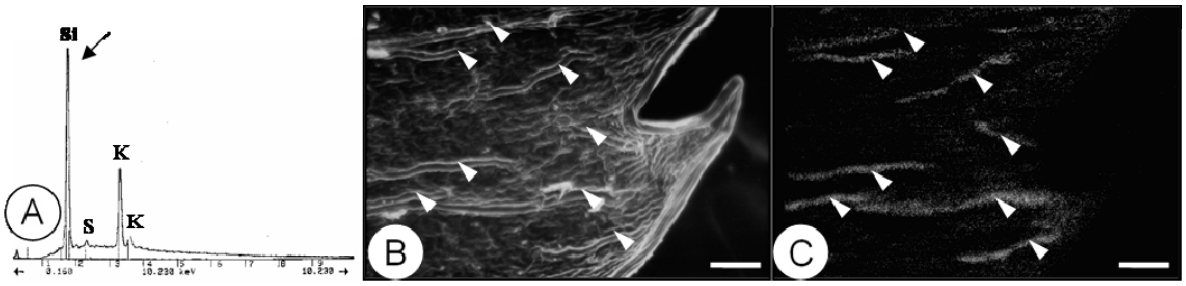

Fig. 2. SEM trace element analysis and silicon dot mapping show the venuloid idioblast containing silicon. A: Trace element analysis shows that a venuloid idioblast containing mass of silicon (arrow). B and C: Si dot mapping method shows the silicon depositions corresponds to the distribution of venuloid idioblasts (arrow head). (Bar = 100 μm)

electron detecting condition). Because of the difference in physical properties (contain mass of silicon), the idioblasts can be distinguished from other cells by these two techniques. For silica body investigation, wet oxidation is a reliable and widely used method for extracting silica bodies from plant materials. Using this method, further quantitative and qualitative studies on silica bodies can be done. Silica bodies have several functions in plant bodies. They take part in growth, development, and stress resistance of plants in several ways. Mechanical support, photosynthesis improvement, ion balancing, and pathogen resistance are four well known functions of silica bodies (summarized from Prychid et al., 2004; Ma and Yamaji, 2006). On the other hand, they are important in pharmacognosy (Komatsu et al., 1996), plant systematics (Rapp and Mulholland, 1992), and archaeoecology (Piperno, 2006). However, a systematically study on silica bodies in Polypods is still lacking (Wang and Lue, 1993).

The venuloid idioblast of *Pteris* is a kind of spicular cell (long epidermal cells containing spicules of silica), which is found in all species of Vittariaceae (or except *Monogramma*) (Willams, 1927; Ogura,

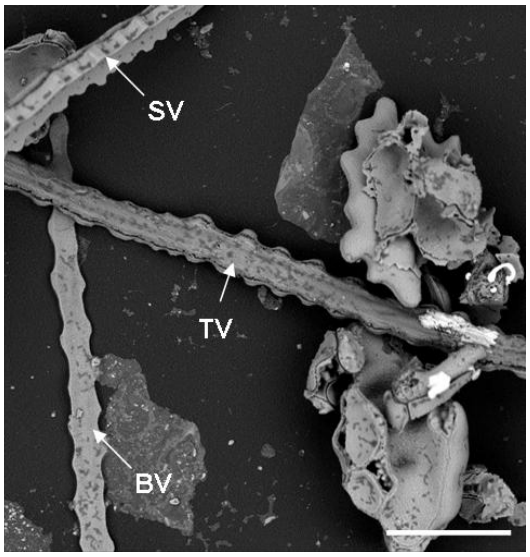

Fig. 3. Morphology of silica bodies. The bottom view (BV), top view (TV), and side view (SV) of the silica bodies are showed. A silica body has plate-like upper and lower surfaces and a pair of undulating ridges. The lower surface is smoother and larger than the upper surface. (Bar = 50 μm)

1972) and most *Adiantum* (Nayar, 1961). Similar cells can be found in *Lygodium*, *Onychium* (Nayar, 1961), and *Pityrogramma* (Gracano et al., 2001). Except for *Lygodium*, all of these taxa belong to Pteridaceae *s.l.* (Smith et al., 2006). Nevertheless, not all taxa in Pteridaceae *s.l.* contain spicular cells, at least they were not found in *Taenitis*, *Syngramma* (Holttum, 1974), some species in *Hemionitis*, *Pteris* (Gracano et al., 2001), and *Adiantum* (Sundue and Prado, 2005). The distribution of spicular cells on fronds is another issue. In Vittariaceae and some species in *Pteris* this structure is scattered on fronds, but in *Adiantum*, *Lygodium*, *Onychium*, *Pityrogramma* and *Pteris multifida* they are restricted to veins. The latter type is easy to be neglected because it is not easily distinguished from the long epidermal cells on veins. In this study, we found partial polarization LM technique, cryo-tabletop-SEM technique are useful methods on spicular cell identification.

Spicular cells (or venuloid idioblasts) are long and silica containing epidermal cells, which are found in Pteridaceae *s.l.* However, they are not present on all species in Pteridaceae *s.l.* and their distribution on frond is different between genuses. Thus, the evolution and function of such a characteristic cell in Pteridaceae *s.l.* is an interesting question. This study provides us some efficient investigation methods for further studies on spicular cell in Pteridaceae *s.l.*

ACKNOWLEDGMENTS

We would like to thank C.-Y. Tang and C.-Y. Lin for providing technical support and operating assistance in the SEM portion of this study. This study was supported by a project grant (NSC-92-2313-B-002-045) from the National Science Council of Taiwan.

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利用不同的顯微鏡技術研究翅柄鳳尾蕨 (*Pteris grevilleana* Wall.) 的脈狀異形細胞

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(收稿日期：2008 年 7 月 5 日；接受日期：2008 年 10 月 12 日)

摘　　要

脈狀異形細胞 (venuloid idioblast) (亦稱為假脈) 泛指外觀像是葉脈的構造。在鳳尾蕨屬 (*Pteris*) 中的脈狀異形細胞，是一種細胞壁特別厚的長形表皮細胞。在本研究中，我們利用各種光學顯微鏡及掃描式電子顯微鏡技術研究翅柄鳳尾蕨的脈狀異形細胞。利用半偏光的光學顯微鏡技巧以及冷凍式桌上型掃描式電子顯微鏡技術，可以有效的觀察該異形細胞的形態並且偵測其分布。該異形細胞的邊緣為波浪狀，兩端尖或圓，分布在營養葉及孢子葉的上下表皮，其可在脈上、脈間或葉緣被找到，但不會出現在羽軸、小羽軸或者假孢膜上。就能量分散 X 射線光譜儀及組織化學染色的結果，顯示該異形細胞具有二氧化矽，而運用濕式灰化法 (wet oxidation) 得知其形態即類似於該異形細胞的矽酸體 (silica body)。因此，翅柄鳳尾蕨的脈狀異形細胞可歸類為一種過去在鐵線蕨及書帶蕨類群中所被發現的矽異形細胞 (spicular cells)。

關鍵詞：能量分散 X 射線光譜儀、假脈、半偏光、鳳尾蕨科、翅柄鳳尾蕨、矽酸體、矽異形細胞、桌上型掃瞄式電子顯微鏡、脈狀異形細胞、濕式灰化法。

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