Plant Biotechnology 25, 407–411 (2008)

Technical Note

Investigation of metal exudates from tobacco glandular trichomes under heavy metal stresses using a variable pressure scanning electron microscopy system

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Received March 24, 2008; accepted May 19, 2008 (Edited by K. Hiratsuka)

Abstract Tobacco (*Nicotiana tabacum*) was recently shown to detoxify heavy metals by exudation of metals as a metal-substituted calcite (calcium carbonate) through leaf trichomes. In this paper, we describe the applications of the variable-pressure scanning electron microscopy (VP-SEM) system to investigate tobacco trichomes exudates after heavy metal treatment. An energy-dispersive X-ray analysis (EDX) system fitted to VP-SEM revealed that the exudates contain amounts of heavy metals. Overexpression of cysteine synthase confers cadmium (Cd) tolerance to tobacco, and the endogenous concentration of Cd was 20% less in transgenic plants than in wild-type plants. We evaluated the numbers of trichomes on the leaf surfaces of wild-type and transgenic plants using VP-SEM. The numbers of both long and short trichomes in the transgenic plants were 25% higher than in that of wild-type plants, indicating the active excretion of Cd from trichomes in transgenic plants. The VPSEM-EDX system is a powerful tool to investigate plant epidermal structures and functions.

Key words: Cadmium, cysteine synthase, trichome, tobacco, variable-pressure scanning electron microscopy.

Trichomes are specialized unicellular or multicellular structures derived from the epidermal cell layer, and may have various functions depending on the plant species and organ (Wagner et al. 2004). A major distinction is made between glandular and nonglandular forms. The glandular trichomes differ in morphology and in the spectrum of compounds, which are secreted or accumulated.

Tobacco (*Nicotiana tabacum*) has multicellular glandular trichomes and is known to excrete various alkaloids, including nicotine, whereas the short trichomes excrete terpenoids (Hallahan et al. 2000) and defensive proteins (Shepherd et al. 2005). Smoking tobacco leaves is one of the principal routes of exposure to heavy metals. Metals contained in tobacco leaves originate from root uptake and transfer to the shoots. These metals also originate from the deposition of aerosol particles on the leaves (Fleisher and Parungo, 1974). Tobacco is also a candidate for the phytoremediation of heavy metal contaminated soils, because it has several advantages, including a high biomass, moderate soil requirements, fast growth rate, and ease of harvesting, even though this plant species is not a hyperaccumulator. In spite of the interest in the metal homeostasis mechanism, little is known about the mechanisms of metals accumulation and detoxification in tobacco.

Recently, we showed that tobacco develops an original mechanism of metal detoxification by the exudation of metal/Ca-containing particles through leaf trichomes (Choi et al 2001; 2004; Choi and Harada, 2005; Sarret et al. 2006). Upon cadmium (Cd) or zinc (Zn) treatment, the number of trichomes was increased more than 2-fold (Choi et al. 2001; Sarret et al. 2006). Confocal laser scanning electron microscopy (CLSM) showed metal accumulation in the tip cells in trichomes (Sarret et al. 2006). The chemical forms of the exudated grains were identified as metal-substituted calcite (calcium carbonate) by using synchrotron-based X-ray microanalyses (Sarret et al. 2006; 2007). Other epidermal structures, such as the salt glands of *Armeria maritima* (Neumann et al. 1995), *Avicennia marina* (MacFarlane and Burchett, 1999), and *Silene vulgaris* (Bringezu et al. 1999), have been also shown to excrete Ca/metal-containing grains. Accumulation in nonglandular trichomes of several hyperaccumulating plants, including *Arabidopsis halleri* (Küpper et al. 2000; Sarret et al. 2000; Zhao et al. 2000), *Alyssum* sp. (Broadhurst et al. 2004), and non-hyperaccumulating plants, including *Brassica juncea* (Salt et al. 1995), *Arabidopsis thaliana* (Ager et al. 2003; Domínguez-Solís et al. 2004), pumpkin (*Cucurbita moschata*, Iwasaki and

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Abbreviation: EDX, energy-dispersive X-ray analysis; VP-SEM, variable-pressure scanning electron microscopy
This article can be found at http://www.jspcmb.jp/

Matsumura 1999), and waterlily (*Nymphaea* sp., Lavid et al. 2001), has been reported. However, the mechanism of metal accumulation and excretion of metals in these structures and their exudates are still under investigation.

Scanning electron microscopy (SEM) has been one of the most convenient and useful instruments for the observation of the surfaces of the samples in a variety of application fields. In particular, variable-pressure SEM (VP-SEM) allows the detailed observation of wet living materials without pre-treatment, so that epidermal structures as well as exudates can be realized (Kuboki and Wada, 1995; 1996; Mathieu 1999). Elemental analysis on the surface is also performed by an energy-dispersive X-ray analysis (EDX) system attached to VP-SEM. The application of VPSEM-EDX has so far been reported for *in situ* analysis of elemental distribution in the living pollen of *Brassica* (Iwano et al. 1999) and petunia (Iwano et al. 2004), and in Ca deposition in idioblasts of mulberry (*Morus* sp.) leaves (Sugimura et al. 1999). In this paper, we describe the applications of the VP-SEM system for the characterization of tobacco trichomes after heavy metal treatment.

The culture conditions of plants were described in Choi et al. (2001) and Sarret et al. (2006). Briefly, for the hydroponic cultures, seeds were germinated on solid medium-filled PCR tubes and transferred after 3 weeks to 1.5-L pots (three plants per pot) filled with one-tenth-strength Hoagland medium. For *in vitro* culture, seed-derived plants at about 5-cm height were transferred to one-third-strength Murashige and Skoog medium in 300-mL glass culture bottles containing 0.2 mM $CdCl_2$ or 1 mM $ZnSO_4$, and 1% sucrose solidified with 0.7% agar. For hydroponic plants, 0.25 mM $ZnSO_4$ was added to the medium. Transgenic tobacco lines expressing a cytosolic rice cysteine synthase (*rcs1*) were produced as described in Harada et al. (2001). *Arabidopsis thaliana* (Col-1) was grown in the hydroponic culture in one-tenth-strength Hoagland medium.

The grains were collected from leaves of metal-treated tobacco plants using toothpicks under a stereomicroscope (Olympus SZX11-ST). The isolated grains were then glued on an aluminum stub using carbon tape (Nisshin EM, Japan). Hydrated leaves were mounted using glue (aqueous emulsion type, Konishi, Japan). The samples were then set in a chamber stage after cooling to −20°C and observed by VPSEM-EDX using a Hitachi S-3500N fitted with a Horiba EMAX-7000 X-ray detector. The chamber pressure was 40 Pa and the accelerating voltage, 15 kV.

Generally, SEM analysis requires specimen preparation, which includes dehydration, and drying and coating by metal or carbon. However, the stereoscopic observation of various fresh living tissues can be directly realized under the VP-SEM system (Figure 1A). The operating principle of low vacuum analysis of the VP-SEM system is shown in Figure 1B. The VP-SEM system coupled with a cooling stage enabled us to observe the water-containing wet specimens simply by suppressing vaporization of water under a low vacuum condition. VP-SEM has a specimen chamber with pressure from 1 to 270 Pa. Under this condition, the specimens can be kept without freezing, and controlled not to bring ice damage or frost formation and also controlled to reduce the thermal damage caused by the irradiation of the electron beam (Kuboki and Wada 1995; 1996). As shown in Figure 2, the intact structures of Arabidopsis and tobacco trichomes are recognized by VP-SEM. To observe the living materials, the aqueous emulsion type glue may give better results than carbon tape because the humidity of the chamber can be maintained, thus enabling longer observation of living materials. Exudated grains (Figure 2B) were not recognized by an ordinary SEM system because they were washed out in pre-treatment steps.

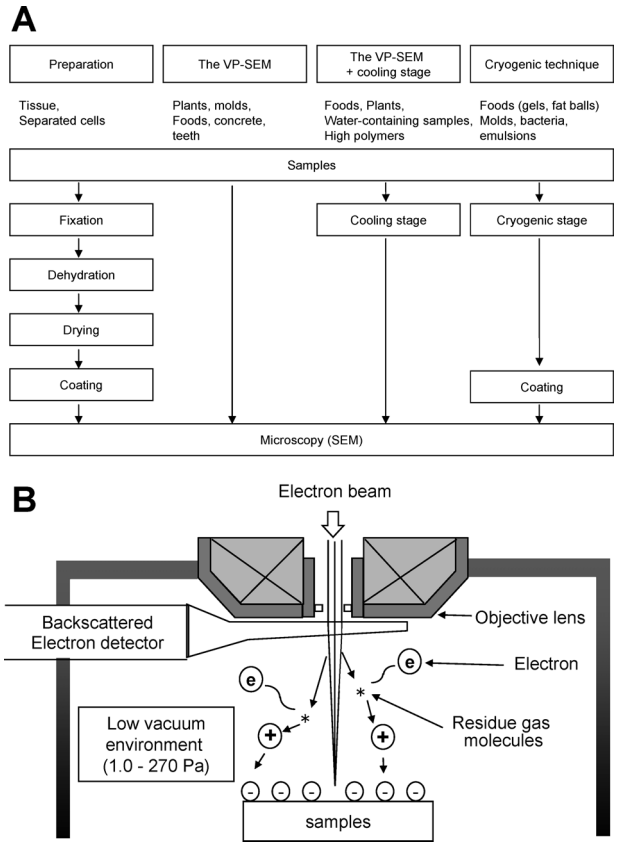

Figure 1. Application and operating principle of VP-SEM. (A) Various samples and preparation techniques for VP-SEM analysis (Figure modified from Kuboki and Wada, 1995). (B) The VP-SEM operates at a relatively high specimen chamber pressure (low vacuum) of 1 to 270 Pa. The residual gas molecules are efficiently ionized by the primary electron beam. The produced positive ions are neutralized by the minus-charged surface of the samples without taking an electrical charge (Figure modified from Kuboki and Wada, 1996).

So far we have shown that the trichomes of tobacco exposed to Cd or Zn produced metal-containing grains

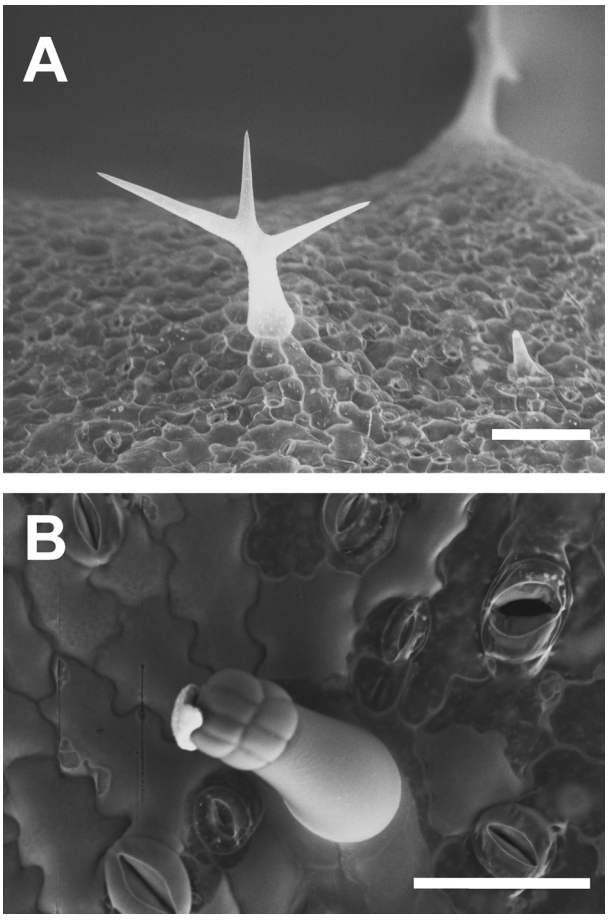

Figure 2. Trichomes on the leaf surface observed under VP-SEM. (A) A trichome on an Arabidopsis leaf. Bar=100 μm. (B) Exudate on a short trichome on a wild-type tobacco leaf treated with 0.2 mM Cd for 1-week. Bar=100 μm.

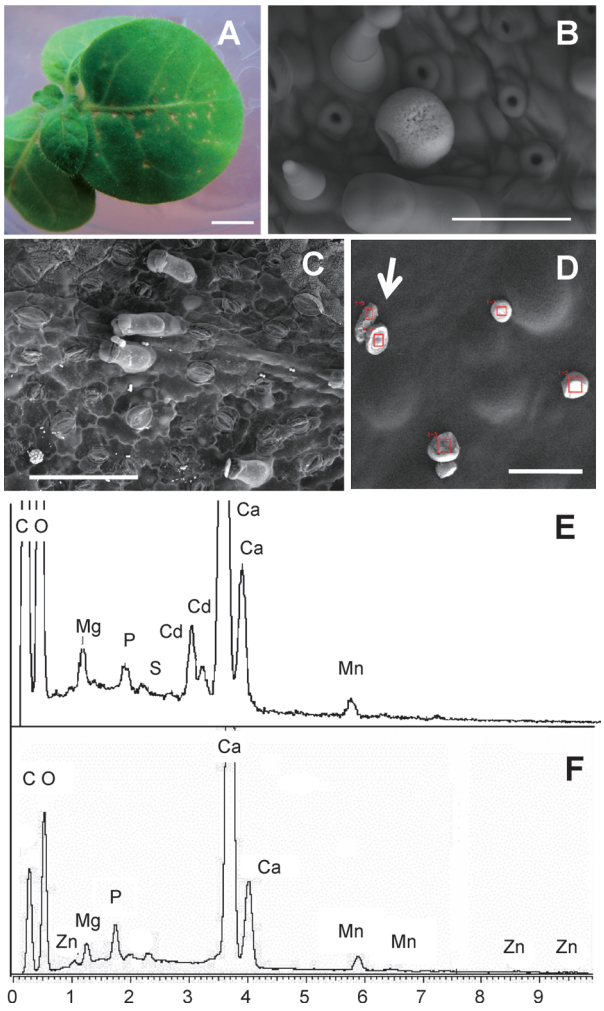

Figure 3. The symptoms and responses observed in tobacco leaves after heavy metal treatment. (A) A tobacco leaf treated with toxic levels of Zn for 3-week. Bar=1 cm. (B) Single exudated grain observed directly on the leaf surface of a Zn-treated tobacco plant under VPSEM-EDX. Bar=100 μm. (C) A tobacco leaf treated with toxic levels of Cd for 3 weeks. The grains were sometimes dropped on the leaf surface. Bar=250 μm. (D) Grains were isolated from Cd-treated tobacco and mounted on the carbon tape. EDX spectra were recorded on the surfaces of several grains (marked with red squares) Bar=250 μm. (E) VPSEM-EDX analysis of a grain for D (arrow) (F) VPSEM-EDX analysis of a grain for B.

(Choi et al. 2001; 2004; Choi and Harada 2005; Sarret et al. 2006). VPSEM-EDX was used to show the grains on the leaf surface (Figure 3). Elemental mapping demonstrated the distribution of isolated grains from Zn-treated plants (Figure 4). Exudated grains contain a certain amount of metals, indicating that they are involved in the metal detoxification mechanism of toxic heavy metals (Figure 3E, F, 4).

We previously reported that transgenic tobacco plants expressing *rcs1* are tolerant to toxic levels of Cd. The level of metal chelator phytochelatin was higher in transgenic than in wild-type plants, suggesting that Cd actively trapped thiol moieties in phytochelatin (Harada et al. 2001). However, the Cd concentration per g fresh weight of whole transgenic plants was 20% lower than that of wild-type plants. The reason for the reduced endogenous concentration of Cd might be caused by the active excretion of the toxic metals in transgenic plants.

In this report, we evaluated the numbers of trichomes on the leaf surfaces of wild-type and the *rcs1* transgenic plant line 2 (TRCS2), because excretion of Cd from trichomes may decrease endogenous concentration of Cd in transgenic plants. Leaf or stem segments were put on an aluminum stab using glue and observed under VP-SEM. The densities of the trichomes were increased in both leaves and stems in transgenic lines (Figure 5A, B, C, D). The numbers of the trichomes on 5 mm^2 of the edge of the leaves were then counted under VP-SEM. The numbers of both long and short trichomes in transgenic plants were 25% higher than that of the wild-type plants under non-stress conditions (Figure 5E). The increased trichomes activated exudation of the Cd, as well as deposited the metals efficiently in trichome top cells, which conferred Cd tolerance to the plants. Thiol accumulation and the dominant expression of genes for sulfur assimilation are reported in trichomes

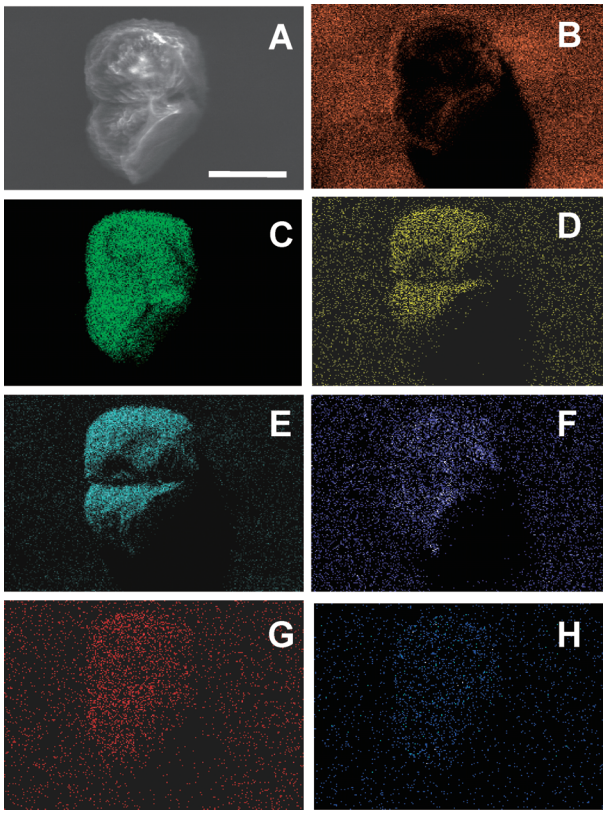

Figure 4. Elemental mapping of grains isolated from Zn treated tobacco plants. (A) Isolated grain. (B–H) Elemental distribution of grain from A (C, Ca, O, Mg, S, Mn and Zn in B, C, D, E, F, G, and in H, respectively). The background contains signals from carbon tape used for the mounting of the grains. Bar=20 μm.

in *Arabidopsis* (Gutiérrez-Alcalá et al. 2000; Lieckfeldt et al. 2008). The activity of cysteine synthase might directly affect the initiation of trichomes. Upon Cd treatment, cysteine synthase activity increased up to 2-fold in wild-type tobacco plants (Harada et al. 2001). After Cd treatment, the numbers of the short trichome were 35.4±2.9, and of the long trichome 24.6±0.5 in wild-type plants. The values were quite similar to those of the unstressed transgenic plants (short trichome 37.4±1.4; long trichome 20.8±2.1; Figure 5E). The relationship between trichome development and cysteine biosynthesis still awaits determination.

In this paper, we showed that the trichomes play important roles in the detoxification of heavy metals in tobacco plants. The metal tolerance in *rcs1* expressing tobacco is due to the high level production of thiols as well as the increased number of the trichomes that sequestrate and excrete heavy metals. The VPSEM-EDX system is a useful tool for observation and analyses of the intact surface of the plants. Finally, we realized the structure of trichomes as well as the ion composition of exudates. Overexpression of cysteine synthase confers stress tolerance to plants, including heavy metal tolerance (Harada et al. 2001; Domínguez-Solís et al. 2001; Kawashima et al. 2004; Moontongchoon et al. 2008), oxidative stress tolerance (Youssefian et al. 2001), and enhanced assimilation of sulfide (Yamaguchi et al. 2006).

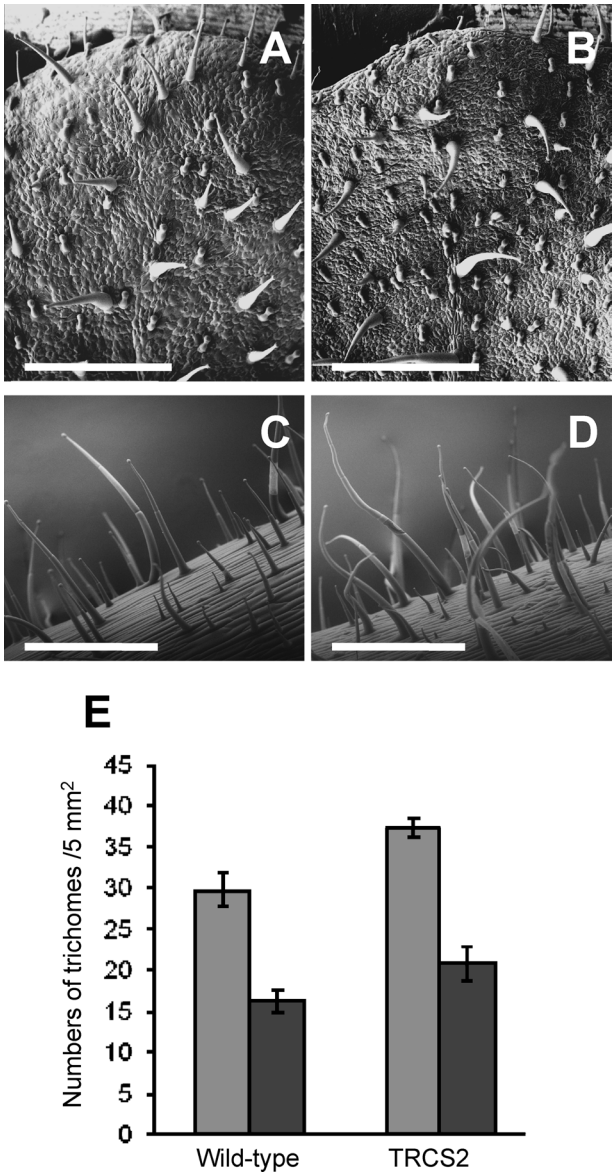

Figure 5. The trichome distribution of (A) leaf of wild-type, (B) leaf of TRCS2, (C) stem of wild-type, and (D) stem of TRCS2. Bars are 1 mm. (E) Number of trichomes on 5 mm^2 of the leaf of wild-type and TRCS2. Data are means±SE, n=5.

Acknowledgements

The authors wish to thank Professor Hiroshi Sano, Dr. Megumi Iwano, Mr. Yohji Morita, Mr. Hideki Tsuboi, and Mr. Masao Wada for their insightful discussions, and Prof. Dee A. Worman (Edit Science) for critical reading of the manuscript. The authors would also like to gratefully acknowledge Hitachi High-Technologies Corporation for kindly providing technical information about the VP-SEM. E.H. was supported by the Brainpool Program (The Korean Federation of Science and Technology Societies, KOFST).

This work was supported financially by the Research for Future Program from the Japan Society for the Promotion of Science (JSPS-RFTF97R16001) and the BK21 program in Korea.

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