Journal of Plant Biology, June 2004, 47(2) : 75-82

Distribution of Elements on Tobacco Trichomes and Leaves under Cadmium and Sodium Stresses

Yong-Eui Choi[1]*, Emiko Harada[1,3], Gwang-Hoon Kim[2], Eui-Soo Yoon[2], and Hiroshi Sano[3]

[1]Division of Forest Resources, College of Forest Science, Kangwon National University, Chunchon 200-701, Korea
[2]Department of Biology, Kongju National University, Kongju 314-701, Korea
[3]Research and Education Center for Genetic Information, Nara Institute of Science and Technology, 8916-5 Takayama, Nara 630-0101, Japan

When tobacco (*Nicotiana tabacum*) plants are exposed to toxic level of cadmium (0.2 mM Cd), their trichomes actively excrete crystals (Choi et al., 2001). In this study, we investigated the distribution of Cd and NaCl on trichomes and leaf surfaces. Energy dispersive x-ray (EDX) analysis revealed that, under toxic Cd stress, crystals exudated from the trichomes contained high amounts of Ca, Mg, and Cd, as well as low levels of P, S, and Mn. Electron spectroscopic imaging (ESI) from trichomes and attached crystals showed that these crystals emitted denser radiation energy for Ca and Cd than did the head cells of the trichomes. However, no Cd was detected on the trichome surface itself or within the leaf epidermis. In contrast, treatment with salt (NaCl) did not stimulate crystal formation; instead, it induced the abnormal expansion of trichome cells. Although Na was not accumulated within the crystals, a considerable amount of both Na and Cl was sequestered within the stalk cells of the long trichomes. Therefore, we believe that tobacco trichomes play an important role in Cd crystal exudation through crystallization, but that, under NaCl stress, the long trichomes sequester those elements within their stalks.

Keywords: crystal, heavy metal, salt, trichome

Many flowering plants bear various types of trichomes derived from the epidermal cell layers of the leaves, stems, petioles, and sepals. These special structures serve as a defense against insect attack, either through spatial hindrance or by exuding toxic chemicals (Duffey, 1986; Wagner, 1991). Glandular trichomes often secrete diverse natural products, e.g., organic acids, polysaccharides, terpenes, nectar, or salt (McCaskill and Croteau, 1999). For example, those in tobacco contain poisonous compounds that interfere with insect movement or feeding to make plants resistant to herbivores. Jones et al. (1985) have demonstrated that nornicotine, a substance about 1000 times more toxic than nicotine, is N-acylated in the trichomes. This chemical is an acknowledged protective agent against herbivore attack (Agrawal, 1998).

Trichomes may play additional or alternative roles in the detoxification of heavy metals and salts, and in response to various other stress conditions. In Indian mustard (Salt and Rauser, 1995), *Alyssum lesbiacum* (Krämer et al., 1997) and *Arabidopsis halleri* (Küpper et al., 2000; Zhao et al., 2000), heavy metals such as cadmium and nickel are preferentially accumulated in the leaf trichomes. Likewise, the epidermal glands in water lily leaves store up heavy metals (Lavid et al., 2001). In some salt-tolerant species, salts are secreted to the plants outer epidermal layer through specialized trichomes, or salt glands, thereby providing resistance to salinity (Luttge, 1971).

In tobacco, two morphologically different types of glandular trichomes -- short trichomes with multicellular heads and long trichomes with a single-cell array -- have been observed (Meyberg et al., 1991). The long trichomes excrete sticky polysaccharides, with terpenoid compounds being the main component (Heemann et al., 1983). In contrast, the short trichomes do not exude such gummy compounds. Therefore, we believe these two types of trichomes may also be functionally different. Previously, we demonstrated that tobacco trichomes excrete Ca/Cd-containing crystals under cadmium stress (Choi et al., 2001). However, variations in Cd-containing crystal formation between the short and long trichomes has not yet been reported. Here, we

*Corresponding author; fax +82-33-250-8316
e-mail yechoi@kangwon.ac.kr

Abbreviations: EDX, energy dispersive x-ray analysis; VP-SEM, variable-pressure scanning electron microscopy; FE-SEM, field emission scanning electron microscopy

focus on the elemental composition and distribution on two types of tobacco trichomes when leaves are exposed to toxic levels of Cd and Na.

MATERIALS AND METHODS

$CdCl_2$ and NaCl Treatments for Tobacco Plantlets

Seed-derived plantlets (~5 cm height) of tobacco (*Nicotiana tabacum* L. cv. Xanthi) were placed in 300-ml glass culture bottles with 1/2-strength MS media (Murashige and Skoog, 1962) that contained 0.6% agar and 1% sucrose, plus 0.00, 0.05, 0.20, or 0.50 mM $CdCl_2 \cdot 2H_2O$. To investigate the response of their trichomes when under salt stress, five plantlets each were transferred to three 300-ml Erlenmeyer flasks containing a 1/2 MS medium supplemented with 0.05 M NaCl. All flasks were then placed in a growth room at 25°C under a 16-h photoperiod provided from 35 μmol $m^{-2}s^{-1}$ (white fluorescent tubes). A total of 15 plantlets were cultured in 3 replicates for each experiment, i.e., testing salt and cadmium stresses. After three weeks, all plantlets were examined for crystal formation.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray analysis (EDX)

We followed the method of Kuboki and Wada (1995), using variable-pressure scanning electron microscopy (VP-SEM) to observe the crystals formed by the trichomes on tobacco leaf surfaces. Segments containing trichomes and crystals were glued onto aluminum stubs and placed on a chamber stage that had been pre-cooled to −20°C. These were then viewed with a VP-SEM (S-3500N, Hitachi, Japan) fitted to an EDX system (EMAX-7000, Horiba, Japan), with a chamber pressure of 30 Pa and an accelerating voltage of 15 KV. To count the number of crystals, we immersed each plantlet in a 50-ml glass bottle that was filled with distilled water and vortexed for 10 s. After the plant materials were removed, the liquid solution was allowed to settle for 2 min before the crystal sediments were collected. These crystals were mounted on aluminum stubs, using double-sided carbon tape, and coated with Pt. The stubs were then placed on the chamber stage and observed under a FE-SEM (Hitachi, S-4700, Japan) fitted with the EDX system described above. X-ray maps and energy dispersive X-ray spectra were also created. To make our crystal observations, tobacco leaves were oven-dried at 60°C, then mounted on aluminum stubs with double-side carbon tape, and coated with Pt. The stubs were placed on the chamber stage and observed under a FE-SEM at 30 Pa chamber pressure and 15 KV accelerating voltage. X-ray maps and energy dispersive X-ray spectra were also taken.

RESULTS

Elemental Distribution in Tobacco Trichomes under Toxic Level of Cadmium

Tobacco plantlets were cultured on 1/2 MS media containing various levels of $CdCl_2$ (0.00, 0.05, 0.20 and 0.50 mM). Those treated with 0.20 mM Cd showed a drastic decline in their growth rate. The yellowing of their lower leaves was also noted after the three weeks of culturing. Using SEM, we identified two morphologically different types of trichomes on the stem and leaf surfaces: 1) long, with a uniserate array (Fig. 1A) short, with a multi-cellular head (Fig. 1B-C). Under toxic levels of cadmium, both types exuded numerous crystals on the surfaces of their head cells (Fig. 1C-F), with the short trichomes showing more active excretions (Table 1). The crystals, with a variety of sizes and shapes (Fig. 1F), were actively formed at a Cd con-

Table 1. Effect of cadmium (Cd) concentration on number of crystals exuded from tobacco trichome heads after three weeks of treatment.

Treatment Cd (mM)	Number of crystals	Size of crystals (μm)	Frequency of crystal formation (%)	
			Long trichome	Short trichome
Free (0.00)	7 ± 2	13 ± 5	0.0	12.2 ± 2.1
0.05	106 ± 24	52 ± 13	1.7 ± 0.2	23.7 ± 3.4
0.20	307 ± 46	83 ± 27	3.6 ± 0.7	34.1 ± 4.1
0.50	203 ± 33	46 ± 14	4.7 ± 0.6	52.5 ± 4.3

Data represent mean values ± standard error of three independent experiments.
Each plantlet was immersed in a 50-ml glass bottle filled with distilled water, then vortexed for 10 s. Following plant removal, the liquid solution was allowed to settle for 2 min, after which the crystal sediments were collected. The size and number of crysta ls were measured under a FE-SEM.

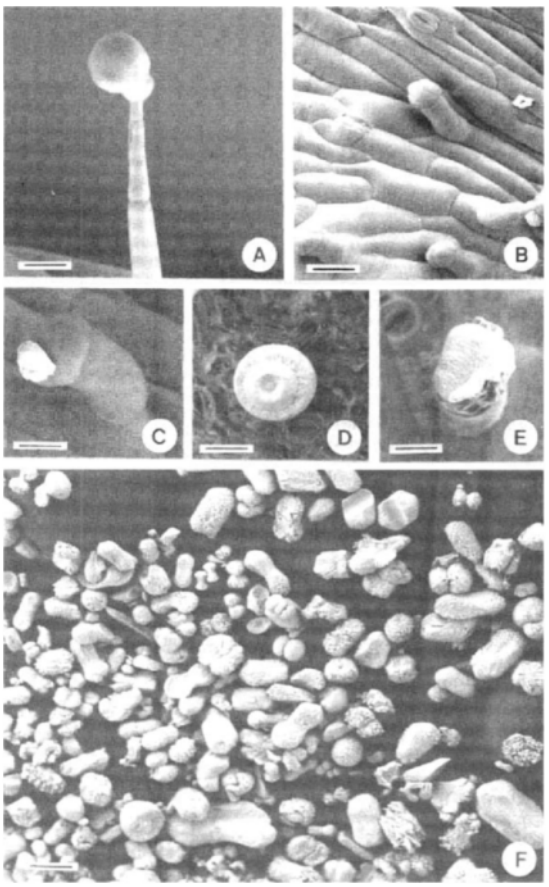

Figure 1. Scanning electron microscope observation of crystal production from tobacco trichomes. **A,** Long trichomes on leaf surface (bar = 250 μm). **B,** Short trichomes with multicellular heads (bar = 120 μm). **C,** Crystal formation from head cells of short trichome after treatment with toxic level of Cd (bar = 50 μm). **D,** Elaborate crystal under toxic Cd (bar = 50 μm). **E,** Crystals formed on head cell of long trichome (bar = 50 μm). **F,** Crystals with various morphology collected from Cd-treated tobacco plantlets after 3 weeks of culture (bar = 80 μm).

centration of 0.20 mM. However, their formation was decreased in severely damaged plantlets cultured in the medium containing 0.50 mM Cd (Table 1). Without cadmium treatment, crystals rarely formed on the trichome heads (Table 1).

Using EDX analysis, we examined the distribution and content of elements in the trichomes and excreted crystals, as affected by treatment with cadmium. On Cd-free media, a small amount of P and K and minor levels of Mg, S, and Cl were detected on the head cells (Fig. 2A); the rarely formed crystals were tiny (=20 μm), and contained a large quantity of Ca and Mg, but only a small amount of K (Fig. 2B). However, under toxic Cd

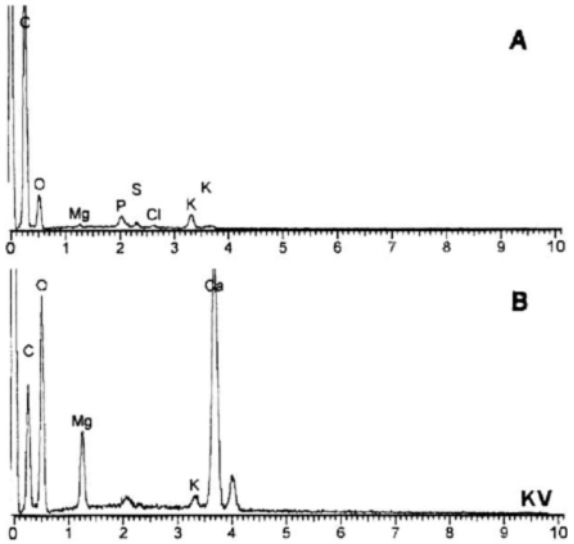

Figure 2. Elemental composition in head cell of trichome and exudated crystals of short trichome on control medium (i.e., without Cd) after three weeks. **A,** Elements detected on surface of trichome head. **B,** Elements detected from crystals formed on trichome head.

stress, we measured high amounts of Ca, Mg, and Cd, as well as low levels of P, S, and Mn in the crystals (Fig. 3B). An elemental mapping of these crystals confirmed that crystals contain Ca, Cd, Mg, and S (Fig. 3C-F). Furthermore, the attached crystals had denser radiation energy for Ca and Mg than the trichome head cells (Fig. 3I, J). A similar pattern was observed with Cd (data not shown). In contrast, the level of K was obviously lower in the crystals than in the head cells (Fig. 3H).

We used the elemental mapping of EDX (Fig. 4A-D) and X-ray spectra (Fig. 5) to analyze the surfaces of leaves treated with toxic level of Cd. Both Cd and Ca were evenly distributed on the leaves (data not shown), except for the region with crystals on the trichome heads. This result indicated that Ca and Cd were not preferentially accumulated on the leaf surface. However, S was accumulated preferentially on the head cells of the short trichomes (Fig. 4B), while Cl and K were significantly accumulated in the stomata (Fig. 4C) and the inter-cellular cavities of the epidermis (Fig. 4D). The energy dispersive X-ray spectra revealed that S and Ca contents in the head cells of the short trichomes increased under Cd treatment compared with the performance of plantlets exposed to Cd-free media (Fig. 5). The high Ca content was a result of the formation of Ca-crystals rather than any accumulation within the trichome. Cl, K, and Na were also preferentially

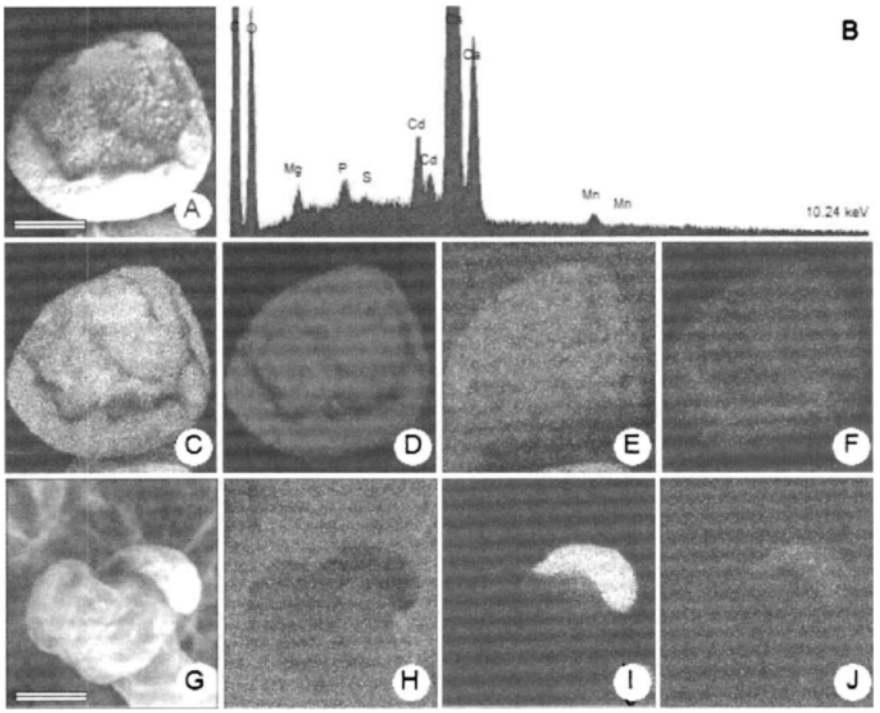

Figure 3. X-ray microanalysis (EDX) of tobacco trichomes and exudated crystals from Cd-treated tobacco plants. **A,** Isolated crystal (bar = 35 μm). **B,** X-ray spectrum of single crystal from **A. C-F,** Elemental distribution of crystal from **A** (Ca, Cd, Mg, and S in **C, D, E,** and **F,** respectively). **G-J,** Elemental mapping of trichome head and attached crystal after Cd exposure. **G,** SEM figure (bar = 20 μm). **H-J,** Distribution of C, Ca, and Mg, respectively.

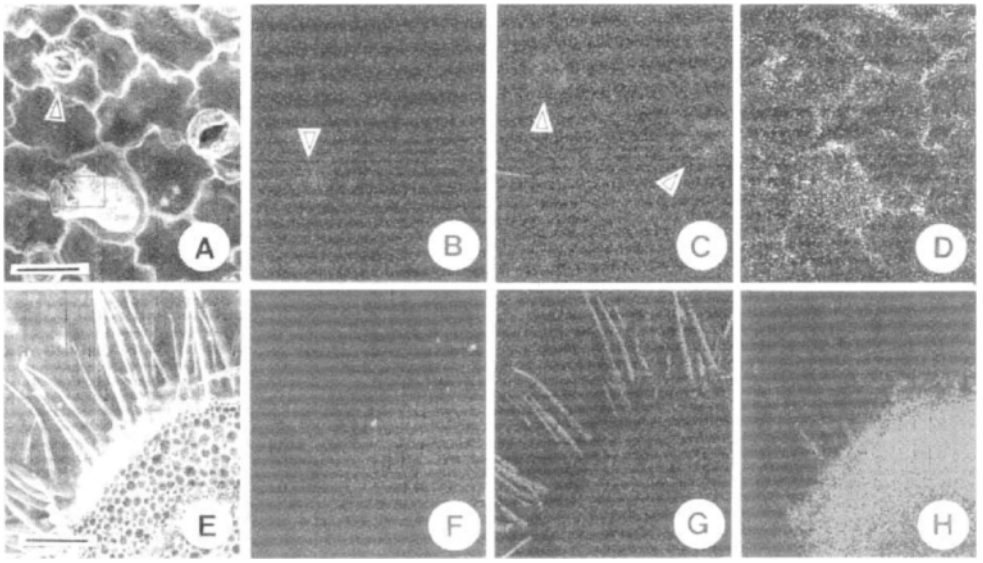

Figure 4. X-ray microanalysis (EDX) of tobacco leaf surface (**A-D**) and cross section of stem segment (**E-G**) after Cd exposure. **A,** SEM figure of leaf surface (bar = 100 μm). **B,** S distribution; note specific accumulation of S in head cells of short trichome (arrow). **C,** Cl distribution; specific accumulation in stomata (arrow). **D,** K distribution; specific accumulation in intercellular spaces. **E,** SEM figure of excised stem segment (bar = 500 μm). **F,** Even distribution of Ca. **G,** Specific accumulation of Na in stalks of long trichomes. **H,** K ion distribution; heavy accumulation in cortical tissue.

accumulated in the stomata (Fig. 5C). Finally, an X-ray spectrum detected no Cd on either the trichome surfaces or the leaf epidermes, except for the excreted crystals (Fig. 3B, 5B, 5D). This observation demon-

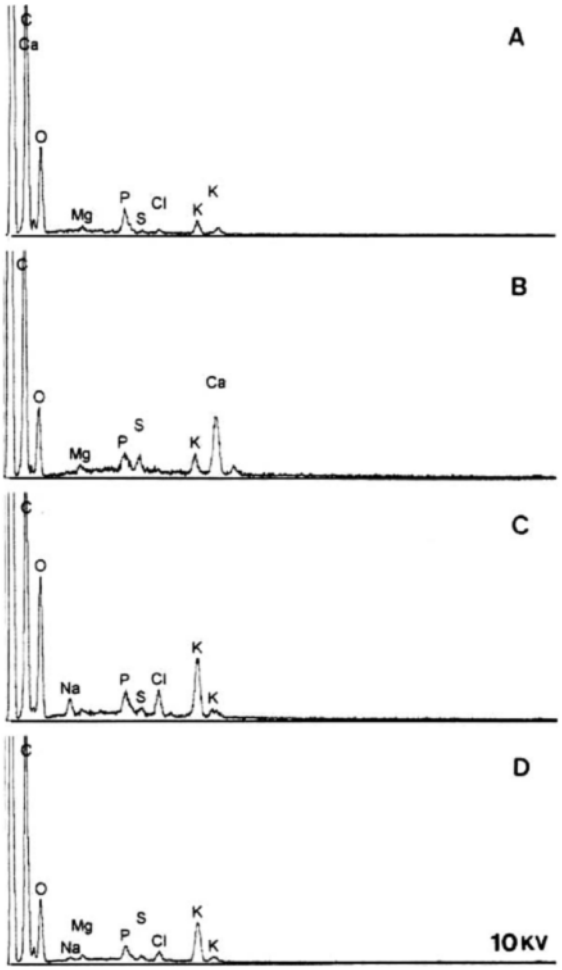

Figure 5. Detection of elements on media after three weeks of culture. **A,** Surface of trichome head without Cd treatment. **B-D,** Surfaces treated with 0.2 mM Cd. **B,** Trichome head. **C,** Stomata. **D,** Intracellular region of epidermis.

strates that the tobacco leaves do not accumulate Cd.

EDX analysis of excised stem segments (Fig. 4E-H) showed that calcium was evenly distributed between the long trichomes and the internal stem tissue (Fig. 4F). A similar pattern was found with Cd, Mg, and S (data not shown). Notably, Na was preferentially accumulated in the cells of the long trichomes (Fig. 4G), while K was significantly stored up in the cortical cells of those stem segments (Fig. 4H).

Elemental Distribution in Tobacco Trichomes under NaCl Stress

When tobacco plantlets were exposed to 0.05 M NaCl, trichomes developed irregularly. In fact, the stalk cells of the trichomes, especially the long ones with a single

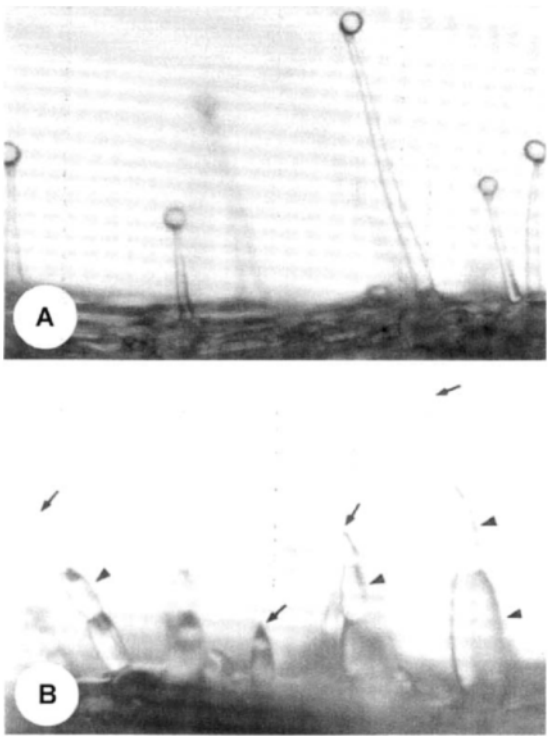

Figure 6. Effect of NaCl on long trichome morphology of tobacco leaf. **A,** Trichome without treatment. **B,** Abnormally enlarged stalk volume of trichome on medium treated with 0.05 M NaCl, after 3 weeks of culture.

array, were abnormally expanded to enlarged cell volumes compared with the weak development seen on the head cells (Fig. 6). The very few crystals (~12 per plant) that were exuded from the trichome head cells were minuscule (23 μm in mean value).

EDX analysis revealed that the head cells of the short trichomes from NaCl-treated tobacco contained some Na and Cl, along with S, K, P, and Mg (Fig. 7A). Interestingly, the stalk cells of the abnormally enlarged long trichomes contained high amounts of Na and Cl (Fig. 7C). Na also accumulated in the stalks of long trichomes from plants treated with Cd (Fig. 4G). Ca and Mg were the main components of the crystals produced from the head cells of the short trichomes; Na and Cl were not detected in those tissues (Fig. 7B).

DISCUSSION

Different Functional Activity Between Short and Long Trichomes of Tobacco

Two types of glandular trichomes have been observed

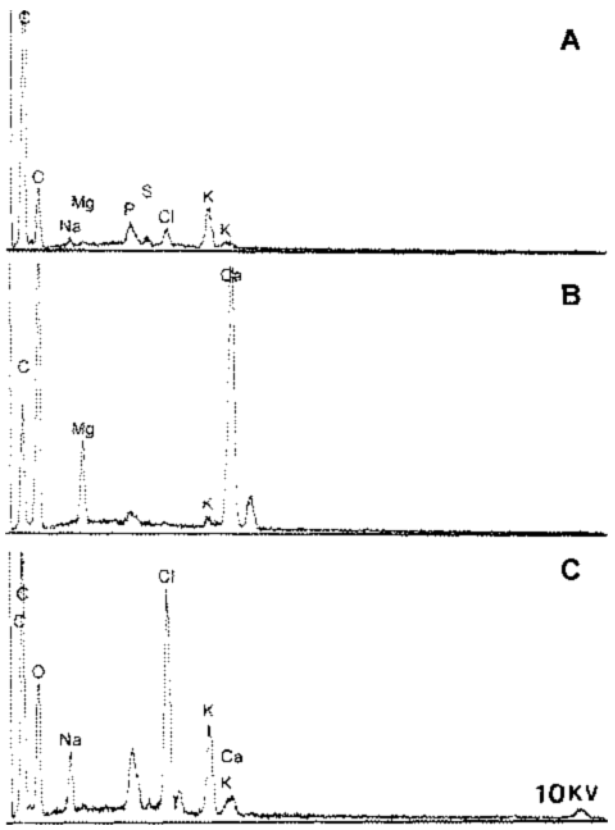

Figure 7. Elemental composition in heads and stalks of trichomes and exudated crystals after three weeks of 0.05 M NaCl treatment. **A,** Surface of head cell from short trichome. **B,** Crystals formed on trichome head. **C,** Stalk cell of long trichome.

in tobacco. In the current study, we found that the short trichomes played a major role in Cd-Ca crystal formation for Cd-detoxification. However, when we exposed the tobacco plants to toxic salt levels, we measured heavy accumulations of Na and Cl in the stalk cells of the long trichomes, rather than the short trichomes, thereby demonstrating the sequestration of salts in those tissues. This phenomenon has been reported in some NaCl-tolerant species, where salts absorbed from the roots are secreted through glands to enhance resistance to salinity (Arisz et al., 1955; Luttge, 1971; Bal and Dutt, 1986). Therefore, we believe that the morphologically different types of tobacco trichomes have separate functions in heavy-metal and salt detoxification.

Elemental Distribution in Tobacco Leaves

The distribution of Ca and Mg was higher in the attached crystals than in the short-trichome heads. This may have been caused by a mechanism for energy-intensive active exudation, although a detailed role for crystal exudation is still required. The high content of calcium in the crystals indicates that those ions play an important function in crystallization and detoxification. Calcium ions are involved in processing toxic heavy metals and salts (Lynch et al., 1989; Ko and Lee, 1995; Rivetta et al., 1997; Epstein, 1998; Liu and Zhu, 1998). Therefore, the addition of Ca strongly decreases the toxicity normally found with cadmium-induced damage while producing a higher number of cadmium crystals (Choi et al., 2001). We also recorded a high concentration of magnesium, which may have been due to the binding affinity that Mg has with calcium crystals, similar to that of Cd.

Elemental mapping of tobacco treated with toxic cadmium showed that Cd and Ca were evenly distributed on the leaf surfaces. However, Cd was not detectable by X-ray spectra for either the trichome heads or the leaf surfaces, perhaps because it was actively excreted outside the leaf via the trichomes or because the leaf surfaces do not accumulate that element.

We also observed that sulfur accumulated preferentially on the head cells of the short trichomes. A close relationship has previously been reported between its metabolism and heavy-metal detoxification. For example, metallothionein-like genes are expressed predominantly in the trichomes of *Arabidopsis* and *Vicia faba* (Foley and Singh, 1994; García-Hernández et al., 1998), while a cysteine synthase gene, Atcys-3A, is highly expressed in *Arabidopsis* trichomes (Gotor et al., 1997; Barroso et al., 1999). Four genes related to glutathione biosynthesis -- i.e., those for O-acetylserine (thiol) lyase, serine acetyltransferase, *r*-glutamylcysteine synthase, and glutathione synthase -- are highly expressed in the leaf trichomes of *Arabidopsis* (Gutiérrez-Alcalá et al., 2000). Therefore, the accumulation of S on the head cells of short trichomes suggests that those cells are active expression sites for the synthesis of sulfur-rich non-protein thiols that detoxify and excrete Cd.

Finally, elemental mapping also revealed that Cl and K significantly accumulated in the stomata. In general, during stomatal opening, the plasma membrane proton pump is stimulated, driving both K and Cl into the guard cells to help increase turgor (Felle et al., 2000).

Heavy Accumulation of Salts in Stalk Cells of Long Trichomes under Stress

Crystal formation was not enhanced by the salt treatment. Instead, the trichomes developed abnormally, gaining extraordinarily enlarged cell volumes.

EDX analysis showed that the stalk cells of the long trichomes had high levels of Na and Cl whereas the crystals exuded from their head cells contained no detectable amounts of either element. Under Cd treatment, Na also was preferentially accumulated in the cells of the long trichomes. Therefore, we believe the accumulation of Na and Cl in those stalk cells aids in sequestering salts in the leaf tissue.

Based on our study results from tobacco, we conclude that Cd is exudated via Ca-Cd crystallization, mainly from the short trichomes. Furthermore, Na is sequestered in the stalk cells of the long trichomes. Both of these actions may possibly serve as detoxification mechanisms for toxic heavy metals and salts.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Research Foundation and the BioGreen 21 Program, Rural Development Administration, and by a Korea Research Foundation Grant (KRF-2000-015-D90358). The authors thank M. Wada and H. Tsuboi (Hitachi Science Systems, Japan) for the VP-SEM analysis and T.S. Bae (Jonju branch of the Korea Basic Science Institute) for performing the FE-SEM.

Received November 6, 2003; accepted January 31, 2004.

LITERATURE CITED

Agrawal AA (1998) Induced responses to herbivory and increased plant performance. Science 279: 1201-1202

Arisz WH, Camphuis IJ, Hckiens H, van Tooren AJ (1955) The secretion of the salt gland in *Limonium latifolium*. Acta Bot Neerl 4: 322-326

Bal AR, Dutt SK (1986) Mechanism of salt tolerance in wild rice (*Oryza coarctata* Roxb). Plant Soil 92: 399-404

Barroso C, Romero LC, Cejudo FJ, Vega JM, Gotor C (1999) Salt-specific regulation of the cytosolic O-acetylserine(thiol)lyase gene from *Arabidopsis thaliana* is dependent on abscisic acid. Plant Mol Biol 40: 729-736

Choi YE, Harada E, Wada M, Tsuboi H, Morita Y, Kusano T, Sano H (2001) Detoxification of cadmium in tobacco plants: Formation and active excretion of crystals containing cadmium and calcium through trichomes. Planta 213: 45-50

Duffey SS (1986) Plant glandular trichomes: Their partial role in defence against insects, *In* BE Juniper, TRE Southwood, eds, Insect and the Plant Surface. Edward Arnold, London, pp 151-172

Epstein E (1998) How calcium enhances plant salt tolerance. Science 280: 1906-1907

Felle HH, Hanstein S, Steinmeyer R, Hedrich R (2000) Dynamics of ionic activities in the apoplast of the substomatal cavity of intact *Vicia faba* leaves during stomatal closure evoked by ABA and darkness. Plant J 24: 297-304

Foley RC, Singh KB (1994) Isolation of a *Vicia faba* metallothionein-like gene: Expression in foliar trichomes. Plant Mol Biol 26: 435-444

García-Hernández M, Murphy A, Taiz L (1998) Metallothioneins 1 and 2 have distinct but overlapping expression patterns in *Arabidopsis*. Plant Physiol 118: 387-397

Gotor C, Cejudo FJ, Barroso C, Vega JM (1997) Tissue-specific expression of *ATCYS-3A*, a gene encoding the cytosolic isoform of O-acetylserine(thiol)lyase in *Arabidopsis*. Plant J 12: 347-352

Gutiérrez-Alcalá G, Gotor C, Meyer AJ, Fricker M, Vega JM, Romero LC (2000) Glutatione biosynthesis in *Arabidopsis* trichome cells. Proc Natl Acad Sci USA 97: 11108-11113

Heemann V, Brummer U, Paulsen CH, Seehofer F (1983) Composition of the leaf surfaces gum of some *Nicotiana* species and *Nicotiana tabacum* cultivars. Phytochem 22: 133-135

Jones D, Jones GA, Hagen T, Creech E (1985) Wild species of *Nicotiana* as a new source of tobacco resistance to the tobacco hornworm *Manduca sexta*. Entomol Exp Appl 38: 157-164

Ko JH, Lee SH (1995) Role of calcium in the osmoregulation under salt stress in *Dunaliella salina*. J Plant Biol 38: 243-250

Krämer U, Grime GW, Smith JAC, Hawes CR, Baker AJM (1997) Micro-PIXE as a technique for studying nickel localization in leaves of the hyperaccumulator plant *Alyssum lesbiacum*. Nucl Instrum Methods Physics Res B 130: 346-350

Kuboki K, Wada M (1995) The variable pressure SEM with the cooling stage. Hitachi Instrument News 27: 20-25

Küpper H, Lombi E, Zhao FJ, McGrath S (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212: 75-84

Lavid N, Barkay Z, Tel-Or E (2001) Accumulation of heavy metals in epidermal glands of the waterlily (Nymphaeceae). Planta 212: 313-322

Liu J, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science 280: 1943-1945

Luttge U (1971) Structure and function of plant glands. Annu Rev Plant Physiol 22: 23-44

Lynch J, Polito VS, Lauchi A (1989) Salinity stress increases cytoplasmic Ca activity in maize root protoplast. Plant Physiol 90: 1271-1274

McCaskill D, Croteau R (1999) Strategies for bioengineering the development and metabolism of glandular tissues in plants. Nature Biotechnol 17: 31-36

Meyberg M, Krohn S, Brümmer B, Kristen U (1991) Ultrastructure and secretion of glandular trichomes of tobacco leaves. Flora 185: 357-363

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497

Rivetta A, Negrini N, Cocucci M (1997) Involvement of

Ca^{2+}-calmodulin in Cd^{2+} toxicity during early phases of radish (*Raphanus sativus* L.) seed germination. Plant Cell Environ 20: 600-608

Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol 107: 1293-1301

Wagner GJ (1991) Secreting glandular trichomes: More than just hairs. Plant Physiol 96: 675-679

Zhao FJ, Lombi E, Breedon T, McGrath SP (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant Cell Environ 23: 507-514