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Mercury Distribution in Tobacco (*Nicotiana tabacum*) Cell

Shenghe Chang[1,2], Zhengjing Wu[3], Wei Sun[1,2], Fei Lin[1,2], Jingyi Zhang[3], Qi Zeng[3], Lan Qiao[3], Haiyan Shu[1,2]*

[1]Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Haikou, China
[2]The Key Lab of Hainan Banana Genetics and Breeding, Haikou, China
[3]School of Forestry, Henan University of Science and Technology, Luoyang, China
Email: *shuhy@zzu.edu.cn

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Abstract

Problem: Removing mercury from polluted soil using transgenic plants is an ideal method. However, where mercury was stored in plant cell is not clear until now. **Methods**: Differential centrifugation and laser scanning confocal microscopy were used in this study. **Results and findings**: Results showed that after mercury was absorbed by tobacco plants, most of the mercury accumulated in roots. Mercury content in root was significantly higher than that in shoot. In the seedlings cultured in MS liquid medium containing 5 μmol/L mercuric chloride, the mercury content in cell wall was 6.6 μg/g. The mercury content in cell membrane fraction was 2.1 μg/g. The mercury content in supernatant fraction was 35.1 μg/g. Most of the mercury accumulated in roots located in liquid fraction, about 15% of the mercury was attached by cell wall. Only a small part assembled in cell membrane. Most of the mercury in liquid fraction located in vacuole. This suggested that after the mercury was accumulated in plant root, most of the mercury was transferred into vacuole. There was no important cellular organ in vacuole. The toxicity of mercury in vacuole will be much lower than that in cell membrane or cellular organs. **Recommendation**: These results suggested that much work should be focused on how to transfer the mercury in vacuole into the above-ground tissues of the plants in the future.

Keywords

Mercury, Distribution, Plant, Cell

1. Introduction

Mercury is a heavy metal which is toxic to human body [1] [2]. After mercury

*represents the corresponding author.

was absorbed into human body, human can be poisoned, ocular organs defected, and sometimes died [3] [4] [5]. With the development of industry, more and more mercury was released into the environment [6] [7] [8]. Much agricultural soil and water have been polluted by mercury [6] [7] [8]. The mercury in agricultural soil and water will enter human body through crops. If the mercury in agricultural soil and water is not removed, many people will be harmed. The commonly used methods for removing mercury from soil are physical methods and chemical methods [6] [9] [10] [11]. But the expense of the methods was large [12]. The chemicals used in these methods will be a second pollutant for the environment [12]. Comparatively, phytoremediation is a cheap and green method [12] [13]. Phytoremediation can decrease the harm to the least [12] [13]. No chemicals will be used in phytoremediation and will not bring new pollutant to the environment [12] [13]. More and more scientists have been interested in phytoremediation and much work has been done [4] [14] [15]. However, mercury is an inhibitor for aquaporin in plant [16] [17]. The mercury in plant will be a block for plants' absorbing water [16] [17]. And so, identifying where the mercury was stored in plant cell is very important for understanding the mechanism that mercury inhibited water in plant cell. However, until now, no paper about mercury distribution in plant cell has been published.

2. Materials and Methods

2.1. Materials

Tobacco (zhongyan 100) seeds were donated from Institute of Tobacco Research, Chinese Academy of Agricultural Sciences. The seeds were propagated in the test base (Danzhou, Hainan province, China) of Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences.

2.2. The Method for Preparing Suspension Cells

Tobacco seeds were immersed in 70% ethanol for 30 seconds. And then, the ethanol was removed and the seeds were soaked with H_2O_2 for 10 minutes. After the H_2O_2 was removed, the seeds were washed with sterilized water for five times, one minute each time. Finally, the seeds were cultured on MS medium with 0.3% (w/v) phytogel (pH 5.7). Plates were incubated in dark at 4˚C for three days and then were moved to a growth chamber with controlled temperature (22˚C - 24˚C), relative humidity (75% - 90%), light (750 μEm^{-2}) and photoperiod (16-h day/8-h night) for one month. The fresh leaves were cut from the seedlings in superclean bench and cultured on MAI solid medium (MS, 10 mg/ml maltose, 1 mg/ml 2,4-D, 1 mg/ml IAA, 0.1 mg/ml NAA, 1 mg/ml biotin, 50 mg/ml glutamine, 30 g/l sucrose, 1.3 g/l phytogel, Ph = 5.8). MAI solid medium was placed in dark with controlled temperature (22˚C - 24˚C), relative humidity (75% - 90%). After two months, calli appeared. And then, the calli was cut from the explants and cultured in MAI liquid medium (MS, 10 mg/ml maltose, 1 mg/ml 2,4-D, 1 mg/ml IAA, 0.1 mg/ml NAA, 1 mg/ml biotin, 50 mg/ml

glutamine, 30 g/l sucrose, Ph = 5.8) under 28˚C for three days. The debris in MAI liquid medium was filtered and discarded. And then, tobacco suspension cells were gotten.

2.3. Confocol

Dye reaction buffer was prepared as the following: 0.1 mmol/L $CaCl_2$, 10 mmol/L KCl, 0.5 mol/L sorbitol, 0.05% PVP40, 0.2% BSA, 5 mmol/L MES, Ph = 5.5. 1 ml of suspension cells were transferred from triangular flask into 1.5 ml tube. 1 mg of chemosensor 1 [18] was dissolved with dye reaction buffer. 10 μl of dye was added into the suspension cell mixture. The tube was placed under 28˚C for ten minutes. And then, 100 μl of the suspension cell mixtures were placed on the glass slide and observed using Laser scanning Confocal Microscopy (Olympus, Japan). The wavelength of exciting light was 420 nm. The wavelength of emitted light was 597 nm.

2.4. Differential Centrifugation

Differential centrifugation was performed according to the papers published and modified [19] [20]. Tobacco seeds were cultured on MS plate for one month. And then, the seedlings were cultured in MS liquid medium. After ten days, 10 μmol/L $HgCl_2$ were added into the medium for 2 days. The roots of the seedlings were immersed in 20 mmol/L of Na_2-EDTA for 3 hours. And then, the roots were washed with distilled water for three times, 10 minutes each time. The roots were cut and collected. The roots samples were stored in −70˚C freezer until use. Buffer I was prepared as the following: 0.2 mol/L Tris-HCl, 1 mmol/L PMSF, 1 mmol/L EDTA, 1 mmol/L SDS, 20 mmol/L mercaptoethanol, ph = 8.0. 12 g of the roots sample were ground into powder in liquid nitrogen. The powder was mixed with 100 ml buffer I. The mixture was sonicated for 30 minutes. And then, the mixture was filtered with gauze. The debris was collected and named as D0. The filtered mixture was centrifugated at 500× g. The supernatant was collected and named as S1. The debris was collected and named as D1. D0 and D1 were mixed and ground in liquid nitrogen once again. 100 ml buffer I was added into the powder. The mixture was sonicated for 30 minutes once again. The mixture was filtered with gauze. The debris was collected and named as D01. The filtered mixture was centrifugated at 500× g. The supernatant was collected and named as S11. The debris was collected and named as D11. D01 and D11 were mixed and treated once again following the procedure above. The debris collected from gauze was named as D02. The debris collected from centrifugation was named as D12. The supernatant was named as S12. S1, S11 and S12 were mixed and centrifugated with 10,000× g at 4 oc. The debris was collected and named as F1. The supernatant was centrifugated with 18,000× g at 4 oc. The debris was collected and named as F2. The supernatant was named as F3.

2.5. Mercury Determination

Mercury content in different tissues was measured according to the paper published [21]. After the seedlings were cultured for 15 days, they were harvested, washed thoroughly in distilled water, shoots and roots were separated, and immersed directly in liquid nitrogen. The frozen plants were dried using a freezer-dryer, and the dry weight was determined. After being ground to a fine powder using liquid nitrogen, samples (three replicates of each treatment) were acid-digested by stepwise additions of 70% (v/v) nitric acid, 30% (v/v) hydrogen peroxide, and concentrated HCl. For measuring mercury content of the whole seedling, the procedure was as the same as described above, except that shoots and roots were not separated.

The Hg content in debris was measured following a previously published method [22]. The debris was washed with fresh LB liquid medium three times. And then, the pellets were acid-digested in 70% (v/v) nitric acid, 30% (v/v) hydrogen peroxide and concentrated HCl at 95˚C. The supernatant was collected and put in a tube. 70% (v/v) nitric acid, 30% (v/v) hydrogen peroxide, and concentrated HCl were added into the tube incrementally. The tube was kept at 95˚C for 2 hours. The samples were then analyzed in an AANALYST 200 Perkin Elmer Spectrometer with an MHS-15 Mercury-Hydride System. The Hg content was calculated in μmol/L as the paper published previously [22]. All samples were measured three times, and the average values were used.

3. Results

3.1. Mercury Content in Root Was Significantly Higher than that in Shoot

After tobacco seedlings were cultured in MS liquid medium containing 5 μmol/L mercuric chloride for two days, the mercury content in root and shoot was measured respectively. Results showed that the mercury content in shoot of the seedling was 12 μg/g, while the value in the root of the seedling cultured in the same medium was about 44 μg/g (**Figure 1**, **Figure 2**). The mercury content in roots was about three times higher than that in the shoots. Similar results were also found in seedlings cultured in liquid MS medium containing 2.5 μmol/L mercuric chloride (**Figure 1**, **Figure 2**). These demonstrated that after mercury was absorbed by plants, most of the mercury accumulated in roots. Only a small section of the mercury in plants was transferred into the above tissue.

3.2. Most of the Mercury in Root Accumulated in Liquid Fraction and Cell Wall

To analyze the mercury distribution in plant root further, tobacco roots were ground in liquid nitrogen and different cell fractions were separated. After the mercury content in different fractions were measured, it was found that in the seedlings cultured in MS liquid medium containing 5 μmol/L mercuric chloride, the mercury content in cell wall was 6.6 μg/g (**Figure 3**). The mercury content in cell membrane fraction was 2.1 μg/g (**Figure 3**). The mercury content in

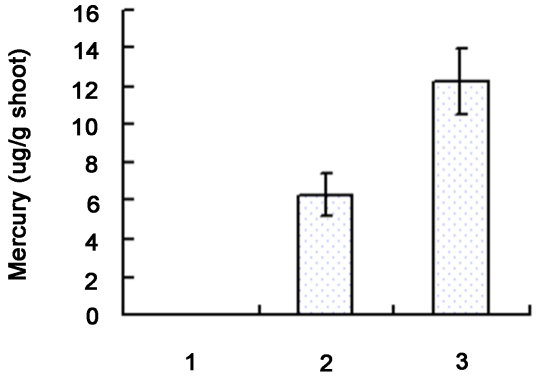

Figure 1. Mercury content in shoot after tobacco seedlings were cultured in medium containing mercuric chloride mercuric chloride for two days. 1 showed the tobacco seedlings cultured in MS medium without mercuric chloride. 2 represented the tobacco seedlings cultured in MS medium containing 2.5 μmol/L mercuric chloride for two days. 3 represented the tobacco seedlings cultured in MS medium containing 5 μmol/L mercuric chloride for two days.

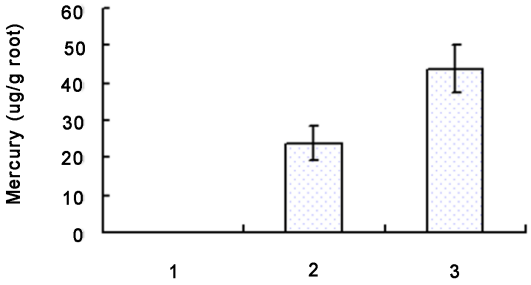

Figure 2. Mercury content in root after tobacco seedlings were cultured in medium containing mercuric chloride mercuric chloride for two days. 1 showed the tobacco seedlings cultured in MS medium without mercuric chloride. 2 represented the tobacco seedlings cultured in MS medium containing 2.5 μmol/L mercuric chloride for two days. 3 represented the tobacco seedlings cultured in MS medium containing 5 μmol/L mercuric chloride for two days.

supernatant fraction was 35.1 μg/g (**Figure 3**). After the mercury contents of fractions from seedlings cultured in MS liquid medium containing 2.5 μmol/L mercuric chloride were measured, it was found that the mercury content in cell wall, cell membrane and supernatant was 3.6 μg/g, 1.2 μg/g, 19.2 μg/g, respectively (**Figure 3**). These demonstrated that in roots, most of the mercury accumulated in liquid fraction, about 15% of the mercury was attached by cell wall. Only a small part assembled in cell membrane.

3.3. Most of Mercury in Liquid Fraction Located in Vacuole

For further identify where the mercury in liquid fraction was distributed, tobacco suspension cell was prepared and mercury was labeled as described in

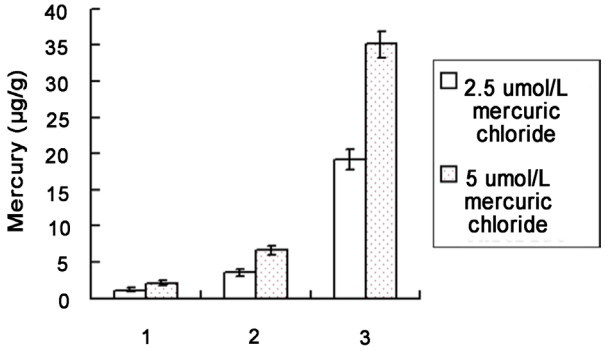

Figure 3. Mercury content in different cellular fractions. 1 showed the mercury content in cell membrane. 2 represented the mercury attached to cell wall. 3 represented the mercury content in supernatant fraction.

materials and methods. After the suspension cells were treated with 0.05 μmol/L mercuric chloride for two hours, the fluorescence indicator was added and cultured in 28˚C for 20 minutes. And then the cells were observed under confocol. It was found that most of fluorescence located in vacuole and cell wall (**Figure 4**). This was consistent with the results gotten from differential centrifugation (**Figure 3**).

4. Discussion

Removing mercury from polluted soil using plants is a green and cheap method. Although clover and mustard had been selected for removing mercury from soil, the biomass of clover and mustard were small and they were not ideal plants for phytoremediation [4] [14]. Comparatively, tobacco is an ideal plant for constructing genetic-modifying plants for accumulating mercury [23]. The biomass of tobacco plant is large. It is self-pollinated and its pollen will not bring pollution to plants around. It grows fast and is easily managed. After tobacco plants were cultured in MS liquid medium containing 2.5 μmol/L mercuric chloride and 5 μmol/L mercuric chloride for two days, it was found that most of the mercury accumulated in the plants is located in roots. These roots containing mercury should be removed from soil. However, the roots of tobacco are large. It will be a hard work to remove all of the roots from soil. If the roots are not removed from soil completely, the mercury stored in the roots will be kept in soil. The aim of removing mercury from polluted soil will not be achieved well. If the mercury stored in the roots can be transferred into the above-ground tissues of the plants, this problem will be resolved. For achieving this aim, the distribution of mercury in plant cell must be studied.

After the clover was cultured in medium containing mercuric chloride and the mercury in roots were analyzed using differential centrifugation, Carrasco-Gil *et al.* found that much mercury in clover roots is located in cell wall [24]. This result was gotten using homogenate and differential centrifugation. The quality of the homogenate will significantly affect the experiment results. If the roots had

Figure 4. Mercury distribution in tobacco suspension cell.

not been ground and broken fully, some cells will not be broken. These cells will be brought into debris after filtering or centrifugation. The mercury in these cells will be regarded as being locating in cell wall. We found that after the roots were treated with liquid nitrogen and sonication for three times, about 85% of the mercury accumulated in tobacco roots was found in liquid fraction. Only about 15% of the mercury accumulated in roots was found in cell wall. These demonstrated that most of the mercury accumulated in tobacco roots is located in liquid fraction, which suggested that such mercury had been transferred into root cell, instead of being attached onto cell wall.

For further identifying where the mercury in tobacco root cell was distributed, chemosensor 1 [18], a mercury-fluorescence indicator was used to label the mercury in cells. For observing the mercury distribution in cell distinctively, suspension cells from tobacco callus were treated with mercuric chloride and chemosensor 1 were observed using confocol. It was found that most of the mercury in cell is located in vacuole. This suggested that after the mercury was accumulated in the plant root, most of the mercury was transferred into vacuole. There was no important cellular organ in vacuole. The toxicity of mercury in vacuole will be much lower than that in cell membrane or cellular organs. This also demonstrated that plants can use vacuole to decrease the toxicity of mercury. This suggested that for constructing genetic-modified plants for removing mercury from polluted soil, further research should be focalized how to transfer the mercury in vacuole in root cells into above-ground tissues.

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