

Visible Study of Mercuric Ion in Brassica Juncea Living Cells and Plants Using a Fluorescent Dye

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Keywords: Brassica juncea, Mercuric ion, Fluorescent dye.

Abstract. Concern over mercury cytotoxicity inside live specimens has encouraged the development of efficient methods for the in vivo detection of this heavy metal. In this study, by using the previous developed fluorescence dye EPNP (2,6-bis-(4'-peperazino-N'-butyl-1',8'-naphthalimide) dimethylpyridine) as a highly selective and sensitive probe for mercuric ions (Hg^{2+}), the images of Hg^{2+} distribution and transportation in the living cells and plants of Brassica juncea were obtained. After 10, 50, 100 μM Hg^{2+} exposure, gradually increased fluorescence was detected in B. juncea living cells loaded with EPNP dye. Intracellular Hg^{2+} time-dependent fluorescence enhancement of EPNP was also observed in B. juncea cells. Furthermore, images of confocal microscope shown that Hg^{2+} was found to mostly accumulate in lytic vacuole but not in the nucleus or mitochondria. In addition, a concentration-dependent rise of fluorescence was observed when the EPNP interacted with an increasing concentration of Hg^{2+} in the plants of B. juncea. These results provided direct experimental evidences for the distribution, translocation and transportation of Hg^{2+} in B. juncea. EPNP would be used as a chemical tool for the study of phytoremediation related researches.

Introduction

Mercury, a dangerous and widespread global pollutant, has been increasingly released to the environment by anthropogenic activities. The global contamination by mercury has caused public anxiety [1]. Mercuric ions (Hg^{2+}) are the most stable form of inorganic mercury, and can be accumulated by plants, animals, and finally human beings [2]. However, comparing with methylmercury, the toxicity of Hg^{2+} has not been well investigated. As a strong phytotoxic, Hg^{2+} tends to form covalent bonds with DNA, and induces oxidative stress in several plants. It was also reported that Hg^{2+} could bind to water channel proteins, induce stomata to close and disrupt water flow in vascular tissues [3]. Moreover, plants exposure to Hg^{2+} led to modification of organelles and other physiological processes [4]. Hg^{2+} and Hg^{2+} conjugate might enter the food chain through human or animal uptake. Therefore, plants pose health risks if they accumulated and transported Hg^{2+} . Based on these potential risks, the explorations aimed at developing selective and efficient methods to monitor Hg^{2+} translocation, transportation and distribution in higher plants have been the topic of various studies[5].

Fluorescence imaging is currently the most powerful technique available for this application. Highly selective and sensitive fluorescent dyes have been extensively

investigated for continuous observation of intracellular processes in living cells [6]. In previous studies, a molecular fluorescence dye, EPNP (2,6-bis-(4'-peperazino-N'-butyl-1',8'-naphthalimide)dimethylpyridine) was applied to the cultured cells. The membrane permeability, low toxicity, slow bleaching/fading, and high sensitivity to nanomolar concentrations Hg^{2+} of this fluorescence dye were confirmed in both mammalian cells and plant cells [7]. At the same cell density, fluorescence of EPNP inside live cells in the presence of Hg^{2+} were much higher than those of Ca^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} , Mn^{2+} , and Ni^{2+} , indicating the selectivity of this probe [8].

Since *Brassica juncea* (*B. juncea*) plants have higher biomass and faster growth rates compared to other hyperaccumulator plants for heavy metals like mercury, *B. juncea* has been extensively used for studies on accumulation and phytoremediation of Hg enriched soils [9]. As *B. juncea* is a high-level Hg accumulating plant (accumulates up to 2570 and 27,900 mg kg^{-1} in shoots and roots) [10], is fast growing, and produces high biomass [11], in the present study this species is, therefore, a good candidate for investigation of the physiology and toxicity of Hg^{2+} and Hg^{2+} conjugate following plant exposure. To investigate Hg^{2+} in *B. juncea* at the cellular and tissular levels, EPNP was used to develop a visual technique for real time and online analysis. Our results provided direct evidence for the localization of Hg^{2+} in the lysosomes of *B. juncea* suspension cells. Furthermore, the distribution, translocation and transportation of Hg^{2+} in living plant was visible.

Material and methods

Chemicals and plant material

B. juncea seeds were obtained from Institute of Geochemistry Chinese Academy of Science (Guiyang, China). EPNP was synthesized as follows: 4-Piperazino-N-butyl-1,8-naphthalimide (149 mg, 0.44 mmol) was dissolved in acetonitrile (30 mL), and then, 2,6-dichloromethylpyridine (35.2 mg, 0.20 mmol) was added; after the mixture was stirred and refluxed for 10 h under a nitrogen atmosphere, the mixture was cooled to room temperature to afford a yellow solid after filtration, which was purified by silica gel column chromatography using chloroform/methanol (5:1, v/v) as the eluent to afford EPNP [8]. Nuclei probe (DAPI, 4', 6-diamidino-2-phenylindole) [12], mitochondrial probe (Mito-Tracker), lysosomal probe (Lyso-Tracker) were purchased from Beyotime Biotechnology Inc. (Nanjing, China).

Cell Suspension Cultures

After seeds germination, petiole and leaf segments of *B. juncea* were considered as explants for callus development. Callus growth was monitored by measuring dry weight of the developed callus. Suspension cultures were initiated by inoculation of one gram of callus into a 125 ml Erlenmeyer flask containing 25 ml of liquid Murashige-Skoog (MS) medium. The liquid medium was filtered, and separated cells were used for further experiments.

Visualization of Mercury in *B. Juncea* Suspension Cells

Cells of *B. juncea* were treated with 10 μM EPNP for 30 min and then washed twice with phosphate buffer solution (PBS). Fresh MS medium was added with 0, 10, 50, 100 μM $HgCl_2$. After 0, 0.5, 1, or 3 h of exposure, cells were washed several times with phosphate buffer saline (PBS) until neutral conditions (pH is about 7.0) were approached. Hg^{2+} was visualized in cells using the fluorescence dye EPNP according to

a previous publication . Briefly, the cultured cells were incubated in the presence of 10 μM EPNP for 30 min, washed three times with PBS and observed using a Nikon TE2000 inverted fluorescence microscope (Nikon, Japan) fitted with a wide-band cube U-MWU filter. All these dyeing experiments were performed independently for three times.

In Vivo Fluorescence Imaging of Mercury

The plants of *B. juncea* were grown on solid MS medium containing 0, 10, 50, 100 μM HgCl_2 for different time (0.5 - 3 h). The roots of the plants were then washed with PBS for 3 times. Kodak Carestream Molecular Imaging System (Carestream Health, Inc., USA) was used to observe the translocation, transportation, and distribution of Hg^{2+} in vivo. Three independent experiments were performed.

Results

Concentration- and Time-dependent Hg^{2+} Distribution in Live Cells of *B. juncea*.

The distribution of Hg^{2+} in *B. juncea* suspension cells at various dosages and series of exposure times was shown in Fig. 1 and Fig. 2, respectively. No obvious fluorescence was detected in living cells without Hg^{2+} exposure. Green fluorescence was initially observed in *B. juncea* cells which treated with 10 μM HgCl_2 for 30 min (Fig. 1B). At higher concentrations or longer treatments of HgCl_2 , the fluorescence intensity was obviously increased. Fig. 2D showed the fluorescence image at 3 h after 10 μM HgCl_2 treatment.

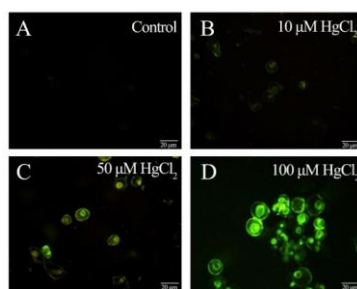


Fig. 1. Concentration-dependent fluorescence microphotographs of HgCl_2 uptake by *Brassica juncea* living cells. Images were taken by an inverted fluorescence microscope. (A) Amplificatory image of control cells which was cultured without HgCl_2 . (B-D) Cells were cultured in with 10 μM EPNP. After 30 min of incubation, cells were washed, and then, indicated doses of HgCl_2 was added, respectively. Images were taken 30 min after HgCl_2 treatment.

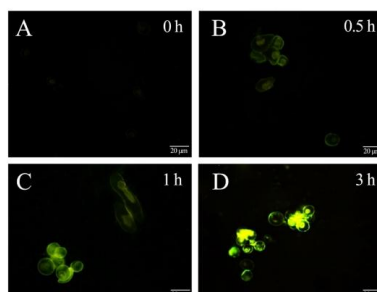


Fig. 2. Time-dependent fluorescence microphotographs of HgCl_2 uptake by *Brassica juncea* living cells. Images were taken by an inverted fluorescence microscope. Cells were cultured in with 10 μM EPNP. After 30 min of incubation, cells were washed, and then, 10 μM HgCl_2 was added. Scanning was taken continuously, (A) before HgCl_2 was added; (B) 0.5 h after exposure to 10 μM HgCl_2 ; (C) 1 h after exposure to 10 μM HgCl_2 ; (D) 3 h after exposure to 10 μM HgCl_2 .

Subcellular localization of Hg^{2+} in *B. juncea* living cells.

To determine the subcellular distribution of Hg^{2+} in *B. juncea* living cells, fluorescence imaging for Hg^{2+} was carried out in living cells using scanning confocal microscopy. According to the results in Fig. 1, concentration-dependent experiments showed that $100\text{ }\mu\text{M}$ Hg^{2+} was sufficient to load cells with stain. DAPI, Lyso-Tracker, Mito-Tracker were used for labeling nuclei, lysosome and mitochondria, respectively. The results were shown in Fig. 3. The overlay images showed significant colocalization of EPNP and Lyso-Tracker. Small extent of colocalization was found between EPNP and DAPI suggesting that Hg^{2+} mostly accumulated in lysosomes. This result indicated that lysosome played an important role in enriching Hg^{2+} in *B. juncea* cells.

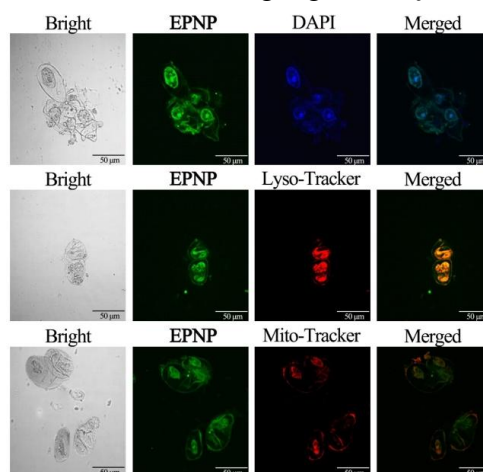


Fig. 3. Laser confocal fluorescence images for the sensors of Hg^{2+} in living *Brassica juncea* cells. *Brassica juncea* cells that were incubated with $10\text{ }\mu\text{M}$ EPNP for 30 min and then incubated with HgCl_2 ($10\text{ }\mu\text{M}$) in MS medium for 30 min. Nucleus (blue), lysosomes (red) or mitochondria (red) was labeled with indicated dyes for 30 min before fluorescence imaging. Confocal fluorescence images, bright-field transmission images and merging images were shown.

Real-time in Vivo Monitoring of Hg^{2+} Translocation and Transportation in the Plants of *B. juncea*.

In order to investigate the translocation and transportation of Hg^{2+} in vivo, the plants of *B. juncea* were grown on MS medium containing a series concentration of HgCl_2 . Then the plants were loaded with $10\text{ }\mu\text{M}$ EPNP dye for 30 min. No obvious fluorescence was detected in control group which was not treated with HgCl_2 (Fig. 4). Significant Hg^{2+} -induced fluorescence was visible from hypocotyl to stem of the plants. Fluorescence intensity was higher in plants that were treated with 50 or $100\text{ }\mu\text{M}$ Hg^{2+} than those treated with $10\text{ }\mu\text{M}$ Hg^{2+} .

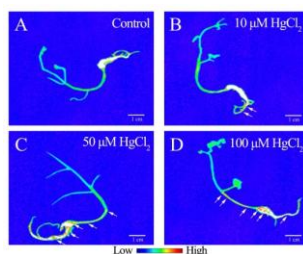


Fig. 4. In vivo imaging of Hg^{2+} in the plants of *Brassica juncea*. Plants treated with (A) $0\text{ }\mu\text{M}$, (B) $10\text{ }\mu\text{M}$, (C) $50\text{ }\mu\text{M}$, (D) $100\text{ }\mu\text{M}$ of HgCl_2 for 30 min. $10\text{ }\mu\text{M}$ EPNP was added for dyeing. Then the roots of the plants were washed with PBS for 3 times. Kodak Molecular Imaging System was used for imaging.

Discussion

The toxic exposure to mercury provide motivation to explore new methods for monitoring aqueous Hg^{2+} from biological and environmental samples [1]. Mercury uptake induced a significant phytotoxicity and reduced in both biomass and leaf relative water content in *B. juncea*. Fluorescent sensors are emerging as key tools for the visualization of Hg^{2+} in live species. In the present study, we performed the real-time analysis of the distribution, translocation and transportation of Hg^{2+} in living cells and plants of *B. juncea*.

At 0.5 to 3 h after treatment with 10 μM HgCl_2 , Hg^{2+} was observed in living cells of *B. juncea* as an increase in fluorescence. Because of the low density of the cultured suspension cells, it was difficult to load these cells with stain. Time- and concentration-dependent experiments were carried out to conform the dyeing concentration and time of EPNP in the suspension cells of *B. juncea*. Our findings in Fig.2 demonstrated the stability of EPNP was maintained within 0.5 to 3 h. It means that the chromophore of this dye was not decomposed in the suspend cells of *B. juncea*, and made it possible to visualize the variation of Hg^{2+} in plant cell by using EPNP as a probe.

Notably, the fluorescence of EPNP concentrated in the middle of cell in Fig. 1 and 2. Nevertheless, due to the marginalization of organelles in mature plant cells we could not hastily conclude that Hg^{2+} was located in the nuclei of *B. juncea* cell. To investigate the subcellular localization of Hg^{2+} , the probes of different organelles were used. EPNP was mostly located at the lysosomes in *B. juncea* living cells, demonstrating that Hg^{2+} was mostly transported into lysosomes. It meant that lysosome played an important role in enriching Hg^{2+} in *B. juncea* cells. Lysosomes are components of the endocytic pathway responsible for storage and processing of digestive enzymes and for terminal degradation as well as absorption of exogenous and endogenous macromolecules. Probably because of the marvelous stability of lysosomal membrane, lysosomes can accommodate quantities of Hg^{2+} in *B. juncea* cells. It also indicated that the stability of lysosomal membrane could be a sensitive stress index in biomonitoring heavy metal pollution.

In conclusion, the fluorescent dye EPNP facilitates the sensitive and easy detection of intracellular Hg^{2+} in *B. juncea* living cells and plants. Therefore, subsequent hyperaccumulator studies of Hg^{2+} could benefit from the use of this fluorescence technique. Because the toxicological and enriched effects of Hg^{2+} are remarkably similar across a range of plant species (i.e., *Chrysopogon zizanioides*, and *Hydrilla verticillata*), examining the distribution, translocation and transportation of Hg^{2+} in *B. juncea* after mercury exposure will advance the current understanding of the mechanisms underlying Hg^{2+} toxicity and enrichment in plants.

Acknowledgement

The authors would like to gratefully acknowledge support from National Natural Science Foundation of China (21502015), and the Doctoral Scientific Research Foundation of Liaoning Province (201501169).

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