Science of the Total Environment 697 (2019) 134097

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A living plant cell-based biosensor for real-time monitoring invisible damage of plant cells under heavy metal stress

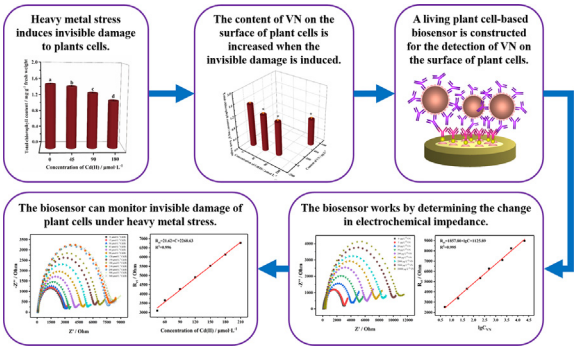
Xiang Wang [a,1], Mengzhu Cheng [a,1], Qing Yang [a], Haiyan Wei [a], Ao Xia [a], Lihong Wang [b], Yue Ben [a], Qing Zhou [b], Zhenbiao Yang [c], Xiaohua Huang [a,*]

[a] National and Local Joint Engineering Research Center of Biomedical Functional Materials, Jiangsu Collaborative Innovation Center of Biomedical Functional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210023, China
[b] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[c] Center for Plant Cell Biology, Institute of Integrative Genome Biology, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA

HIGHLIGHTS

- Heavy metal stress induces invisible damage to plants cells.
- The content of VN on the surface of plant cells can indicate invisible damage.
- A living plant cell-based biosensor is constructed for the detection of VN.
- The biosensor can monitor invisible damage of plant cells under heavy metal stress.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 June 2019
Received in revised form 23 August 2019
Accepted 23 August 2019
Available online 24 August 2019

Editor: Charlotte Poschenrieder

Keywords:
Heavy metals
Living plant cell-based biosensor
Vitronectin-like protein
Plants
Invisible damage

ABSTRACT

Heavy metals inevitably cause invisible or visible damage to plants, leading to significant economic losses. Therefore, it is necessary to develop a method for timely monitoring the damage of plants under the stress of heavy metals. Here, vitronectin-like proteins (VN) on the surface of plant cells is as an important biomarker for monitoring damage of plants under the stress of heavy metals. A living plant cell-based biosensor is constructed to monitor invisible damage of plant cells induced by cadmium [Cd(II)] or lead [Pb(II)]. To fabricate this sensor, L-cysteine was first modified on the glassy carbon electrode followed by the modification of anti-IgG-Au antibody. Then, the living plant cells, incubated with the anti-VN, were modified onto the electrode. The sensor worked by determining the change in electrochemical impedance. Cd(II) and Pb(II) was detected in the linear dynamic range of 45–210 and 120–360 μmol·L^{-1}, respectively. And the detection limit of Cd(II) and Pb(II) of this biosensor was 18.5 nmol·L^{-1} [with confidence interval (95%) 18.4–18.6 nmol·L^{-1}] and 25.6 nmol·L^{-1} [with confidence interval (95%) 25.4–25.8 nmol·L^{-1}], respectively. In both *Arabidopsis* and soybean, when the content of VN increased by about 20 times under the stress of Cd(II) or Pb(II), which means when the electron-transfer resistance increased by 35%, chlorophyll content showed significant decrease about 17%. Therefore, by establishing a quantitative relationship among the content of biomarker, the electron-transfer resistance and chlorophyll

* Corresponding author.
E-mail address: huangxiaohuanjnu@yahoo.com (X. Huang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.scitotenv.2019.134097

content in plant cells, the invisible damage of plants under the stress of heavy metals was detected. These results can provide a reference method for early-onset warning systems for heavy metal pollution in the environment.

1. Introduction

With rapidly industrial development, the pollution of heavy metals (metals with densities > 5 $g \cdot cm^3$) has become a serious global problem (Cai et al., 2019; Guo et al., 2019; Huang et al., 2019; Landrigan et al., 2018; Lee et al., 2017; Pandey et al., 2019; Wan et al., 2018; Zhou et al., 2019). It has been reported that the content of lead (Pb) in atmospheric dust in Beijing (China) reached 167.9 $mg \cdot kg^{-1}$ (Wan et al., 2018). The content of Pb in the atmosphere in Tehran (Iran) reached 81.27 $mg \cdot kg^{-1}$ (Kamani et al., 2017). The content of cadmium (Cd) and Pb in fine particulate matter ($PM_{2.5}$) in Nanjing (China) reached 15.2 and 545.6 $mg \cdot kg^{-1}$, respectively (Qi et al., 2016). Plants are the primary producers in ecosystem. Due to the immobility of plants, plants are constantly exposed to heavy metal pollution, which inevitably causes damage of plants, and finally leads to great disasters to the ecosystem (Han et al., 2015; Hu et al., 2019; Prasad and Hagemeyer, 1999; Zhang et al., 2016).

The adverse impact of heavy metal stress on plants begins with invisible damage of plants, and then the damage enhances after long-term exposure. For example, heavy metal stress decreases the content of chlorophyll in plant leaves before it causes visible damage to plants (Kolotov et al., 2003; Wen et al., 2011). Therefore, the decrease in the content of chlorophyll is widely considered as a sign of invisible damage to plants (Hu et al., 2016a, b; Kolotov et al., 2003; Wen et al., 2011). For this reason, it is essential to develop a method to timely monitor invisible damage to plants caused by heavy metal stress. Nowadays, the common methods for monitoring damage of plants are determining the growth, physiological and biochemical indicators of plants. Specifically, the growth indicators commonly are root modules (Zhang et al., 2018a) and leaf sizes (Wang et al., 2014). The physiological and biochemical indicators usually are cell membrane permeability (Cheng et al., 2018), the content of malondialdehyde (Juknys et al., 2012; Yan et al., 2010), the content of chlorophyll (Wen et al., 2011) and the structure of chloroplasts (Hu et al., 2016a, b; Peng and Zhou, 2009). However, none of these methods can provide rapid, real-time data to reveal invisible damage of plants under the stress of heavy metals.

In recent decades, electrochemical biosensor detection has become a commonly used method in cell biology, clinical science and the early diagnosis of diseases, due to its attractive properties, such as high sensitivity, selectivity, simplicity, fast response, and low cost (Hoon et al., 2019; Pundir et al., 2018). These electrochemical biosensors work based upon detecting various biomarkers. However, due to the presence of cell walls and acidic environment outside the plasma membrane (pH range of 4.4–5.5) in plant cells, it is very difficult to detect the biomarkers in plant cells by using electrochemical biosensors (Buchanan and Gruissem, 2000). Therefore, only a few electrochemical biosensors relating to plants have been reported in the literature (Cheng et al., 2018; Mello et al., 2006). It was recently reported that extracellular vitronectin-like protein (VN, a defensive protein of plant cells for resisting exogenous harmful factors) can serve as the biomarker for detecting the response of plants to lanthanum by using an electrochemical sensor (Leavesly et al., 2013; Yang et al., 2016; Cheng et al., 2018). Can VN be a stable biomarker for real-time monitoring the invisible damage to plants under the stress of heavy metals?

In this study, *Arabidopsis thaliana*, a model plant (Weigel and Glazebrook, 2002), and soybean which was a kind of commercial plant and was recommended by United States Environmental Protection Agency to serve for plant toxicology research (Zhang et al., 2018a), were chosen as the representative of plant species, and Cd(II) and Pb(II) were selected as the typical heavy metals in atmospheric pollutants. Based on the immune-labelling principle, we constructed a bioelectrochemical sensor to detect the content of VN on the cellular surface of plant leaves treated with different heavy metals, and we also determined the content of chlorophyll in plant leaves after same treatments. We confirmed that VN can serve as a biomarker for revealing invisible damage to plants caused by heavy metal stress. By establishing a quantitative relationship among the content of biomarker, the electron-transfer resistance and chlorophyll content in plant cells, the invisible damage of plants was detected under the stress of heavy metals. These results can provide a reference method for early-onset warning for heavy metal pollution in the environment.

2. Materials and methods

2.1. Materials and reagents

Lead chloride ($PbCl_2$, purity > 99.99%), cadmium chloride ($CdCl_2$, purity > 99.99%), *N*-(3-triethylammoniumpropyl)-4-(4-diethylaminophenylhexatrienyl) (FM4-64), Goat Anti-Rabbit IgG (whole molecule)-Gold antibody (anti-IgG-Au, the secondary antibody of VN), VN, and Bovine Serum Albumin (BSA) were obtained from Sigma Aldrich Co., Ltd., China. Anti-vitronectin antibody (anti-VN, the primary antibody of VN) was obtained from Abcam Co., Ltd., China (Fan et al., 2015). All other reagents mentioned in the following sections were of analytical grade and were purchased from Sigma Aldrich Co., Ltd., China.

The cell protoplast wash medium (CPW) was prepared by dissolving 27.2 mg KH_2PO_4, 101 mg KNO_3, 1480 mg $CaCl_2 \cdot 2H_2O$, 246 mg $MgSO_4 \cdot 7H_2O$, 0.16 mg KI and 0.025 mg $CuSO_4 \cdot 5H_2O$ into 1000 mL distilled water. The anti-VN solution was prepared by dissolving 0.8 $mg \cdot mL^{-1}$ anti-VN in 0.05 $mol \cdot L^{-1}$ cacodylic acid sodium salt trihydrate buffer (pH of 7.0, containing 2.5% NaCl, 0.1% BSA and 0.05% Tween 20) in a 1:70 v/v ratio. IgG-Au solution was prepared by dissolving 1.0 $mg \cdot mL^{-1}$ IgG-Au into 0.02 $mol \cdot L^{-1}$ cacodylic acid sodium salt trihydrate buffer (pH of 7.0, containing 2% NaCl, 0.1% BSA and 0.05% Tween 20) in a 1:50 v/v ratio. L-Cysteine (L-Cys) was diluted with anhydrous ethanol to 1 $mmol \cdot L^{-1}$, whereas 0.1 $mol \cdot L^{-1}$ $LiClO_4$ was added to it as the supporting electrolyte.

2.2. Arabidopsis thaliana and soybean culture and treatment

Columbia 0 (Col-0), an ecotype of *Arabidopsis thaliana*, was selected in this study. Seeds were sterilized (5% bleach and 0.1% Triton-X100) for 5 min and washed with sterilized water for at least 5 times. Then they were placed on plates containing MS medium (4.4 $g \cdot L^{-1}$ MS, 1% sugar, 1% agar; pH of 5.7), and incubated at 4 °C for 3 days in dark before being transferred to 22 °C under white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days. Following the seven-day growth incubation, seedlings were planted in nutrient soil (nutrient soil/vermiculite in a 1:1 ratio) for 30 days under the growth conditions: 16 h/8 h light/dark cycle, 60% humidity and 22 °C. After that, all plant treatments were performed as follows: (1) Different doses of Cd(II) and Pb(II) (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, and 360 $\mu mol \cdot L^{-1}$) were uniformly sprayed on leaves till the droplets began to fall and the control seedlings were sprayed uniformly with deionized water. Twelve hours later, leaves in the same leaf position were selected for experiments. (2) Different doses of Cd(II) (60, 120, 210 $\mu mol \cdot L^{-1}$) and Pb(II) (120, 210, 360 $\mu mol \cdot L^{-1}$) in different pH (pH = 5.0, 5.5, 6.0 and 6.5) were uniformly

sprayed on leaves till the droplets began to fall. Twelve hours later, leaves in the same leaf position were selected for experiments. (3) Seedlings were transferred to artificial illumination incubator with different temperature (4 °C, 22 °C and 35 °C). Twelve hours later, leaves in the same leaf position were selected for experiments. (4) Seedlings were sprayed evenly with 0.5 nmol·L^{-1} Cd(II) and 2 nmol·L^{-1} Pb(II) twice a day. Every five days, leaves in the same leaf position were selected for experiments.

In addition, Zhonghuang 25 (Wuxi Seed Co., Ltd., China), which is a kind of soybean, was used in this study. Seeds were sterilized using 0.1% $HgCl_2$ for 5 min and washed with sterilized water for at least 5 times. Then the seeds were macerated in deionized water for 12 h. Seeds were sown in culture dishes with 3-layer cotton gauze and incubated at 22 °C for germination. Deionized water was replenished on time to keep the seeds moist. When the radicle was 3 cm long, every 25 soybean seedlings were transferred to a plastic box (32 cm × 21.5 cm × 10 cm) and fixed with a foam board under growth conditions: 16 h/8 h light/dark cycle, 300 μmol·m^{-2}·s^{-1} and 22 °C. Deionized water was replenished daily into the boxes to maintain the volume of water. When the second true leaf germinated on the main stem, the soybean seedlings were cultured with 1/2 Hoagland nutrient solution (Hoagland, 1920) instead of deionized water, whereas the 1/2 Hoagland nutrient solution was replaced every three days. Following the thirty-day growth, different doses of Cd(II) (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, and 360 μmol·L^{-1}) and Pb(II) (15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, and 480 μmol·L^{-1}) were uniformly sprayed on the leaves till the droplets began to fall. Deionized water was sprayed on the leaves as a control treatment. Twelve hours later, leaves in the same growth position were selected for further experimentation.

2.3. Isolation of protoplasts

The selected *Arabidopsis* and soybean leaves were washed with deionized water and then cut into 2-mm wide strips. The cut segments were placed in an enzymatic hydrolysate solution (1% pectinase and 0.1% cellulose) in the dark at 28 °C for 4 h. Then, the enzymatic hydrolysate mixture was transferred to a centrifuge tube containing a 200-mesh stainless steel filter (Titan, Shanghai Titan Scientific Co., Ltd., China), and centrifuged at 1000 rpm for 1 min. After centrifugation, the supernatant was discarded. The pellet was washed in a CPW solution, containing 0.9 mol·L^{-1} mannitol, and centrifuged at 800 rpm for 3 min. The washing of pellet was repeated thrice to provide clean living plant cells without cell walls (i.e. protoplasts). The morphological properties of protoplasts were observed using a fluorescent inverted microscope (Carl Zeiss Axio Observer. A1, Carl Zeiss Jena Co., Ltd., Germany).

2.4. Modification of electrodes

A glassy carbon electrode (GCE, 3 mm in diameter) was polished using 0.3 and 0.05 μm alumina slurry, and then, ultrasonically cleaned successively in anhydrous ethanol and ultrapure water for 5 min each. The cleaned GCE was dried under a flow of nitrogen, placed in a 1 mmol·L^{-1} L-Cys solution and scanned at a rate of 110 mV·s^{-1} for 10 cycles. The voltage range was −0.41–1.0 V, after which, the GCE-(L-Cys) electrode was formed. The single-layer modified electrode was then carefully washed with ultrapure water to remove the physically adsorbed L-Cys. Then, 5 μL IgG-Au solution was dropped onto the surface of GCE-(L-Cys) electrode, dried at room temperature and washed with ultrapure water, thus forming a GCE-(L-Cys)-(IgG-Au) electrode. Finally, 10 μL of protoplast solution (3500 cells·$μL^{-1}$), which had been incubated with the prepared anti-VN solution for 1 h, was dropped onto the surface of the GCE-(L-Cys)-(IgG-Au) electrode. The electrode was then dried at room temperature and rinsed with ultrapure water, thus forming a GCE-(L-Cys)-(IgG-Au)-VN electrode. The complete procedure for preparing the electrode is shown in Fig. 1A. Some GCE-(L-Cys)-(IgG-Au) electrodes were stood for several days before modified with protoplast in order to test the long-term stability of the biosensor.

2.5. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) of the GCEs was conducted using an RST5000 electrochemical station (Shanghai Electric Science Instruments, Ltd., China). In this assay, 5 mmol·L^{-1} $[Fe(CN)_6]^{4/3}$ was dissolved in 0.1 mol·L^{-1} KCl as the electrolyte. A saturated calomel electrode (SCE), a platinum wire and the post-modified GCE were used as the reference, auxiliary and working electrodes, respectively. Scanning was performed at open circuit potential and recorded within the frequency range of 0.1–100,000 Hz, with a sine wave signal amplitude of 1 mV.

2.6. Quantum chemical computation

The 3D model of VN was built based on the homology modeling, whereas the interacting domains between VN and Cd(II) or Pb(II) were investigated using Discovery Studio 2.5 (DS 2.5, Accelrys Inc. USA). The segment of VN (the amino sequence was 96–100), which could potentially bind to Cd(II) or Pb(II), was selected for further calculations. According to the density functional theory, calculations were performed using Gaussian 09 (Gaussian Inc.), while the geometric structure was optimized using B3LYP at the CEP-121 G level. The experiment data were analyzed using Gauss View 03 and Diamond 3.2 (Crystal Impact Inc. Bonn, Germany).

2.7. Determination of the content of chlorophyll in plant leaves

According to the results of electrochemical impedance spectrum, 45, 90 and 180 μmol·L^{-1} Cd(II), and 120, 180 and 300 μmol·L^{-1} Pb(II), were chosen as representative concentrations in *Arabidopsis* experiments. In soybean experiments, 120, 180 and 300 μmol·L^{-1} Cd(II), and 120, 180 and 300 μmol·L^{-1} Pb(II) were chosen as representative concentrations. Leaves in the same growth position were selected for determining the content of total chlorophyll after treatment with Cd (II) or Pb(II) for 12 h. Furthermore, 0.1 g leaf samples were weighed and transferred to a 15 mL centrifuge tube containing 10 mL 80% acetone. The centrifuge tubes were sealed and shaken gently in the dark to allow the full extraction of chlorophyll until the tissue faded completely. The supernatant was collected and the absorbance was measured at 663 nm and 645 nm (A_{663} and A_{645}) using a UV–vis spectrophotometer (Evolution™ 60S, Thermo Fisher Scientific Inc., US). It is to be noted that 80% acetone was used as the blank. Finally, the concentration of chlorophyll was calculated according to the established equation, as shown in Eq. (1) (Buchanan and Gruissem, 2000):

$$C_T = 8.02A_{663} + 20.21A_{645} \quad (1)$$

where C_T is the concentration of total chlorophyll (mg·L^{-1});

The content of total chlorophyll was calculated by using Eq. (2):

$$\text{Total chlorophyll } (\text{mg} \cdot \text{g}^{-1}\ \text{FW}) = \frac{C_T \times V_T}{\text{FW} \times 1000} \quad (2)$$

where V_T is the total volume of 80% acetone and FW is the fresh weight.

Each treatment group was assessed in triplicates and the significant differences ($p < 0.05$) between each treatment group were analyzed using Duncan's multiple range test module of SPSS (ver. 17.0; IBM, Chicago, IL, USA) software.

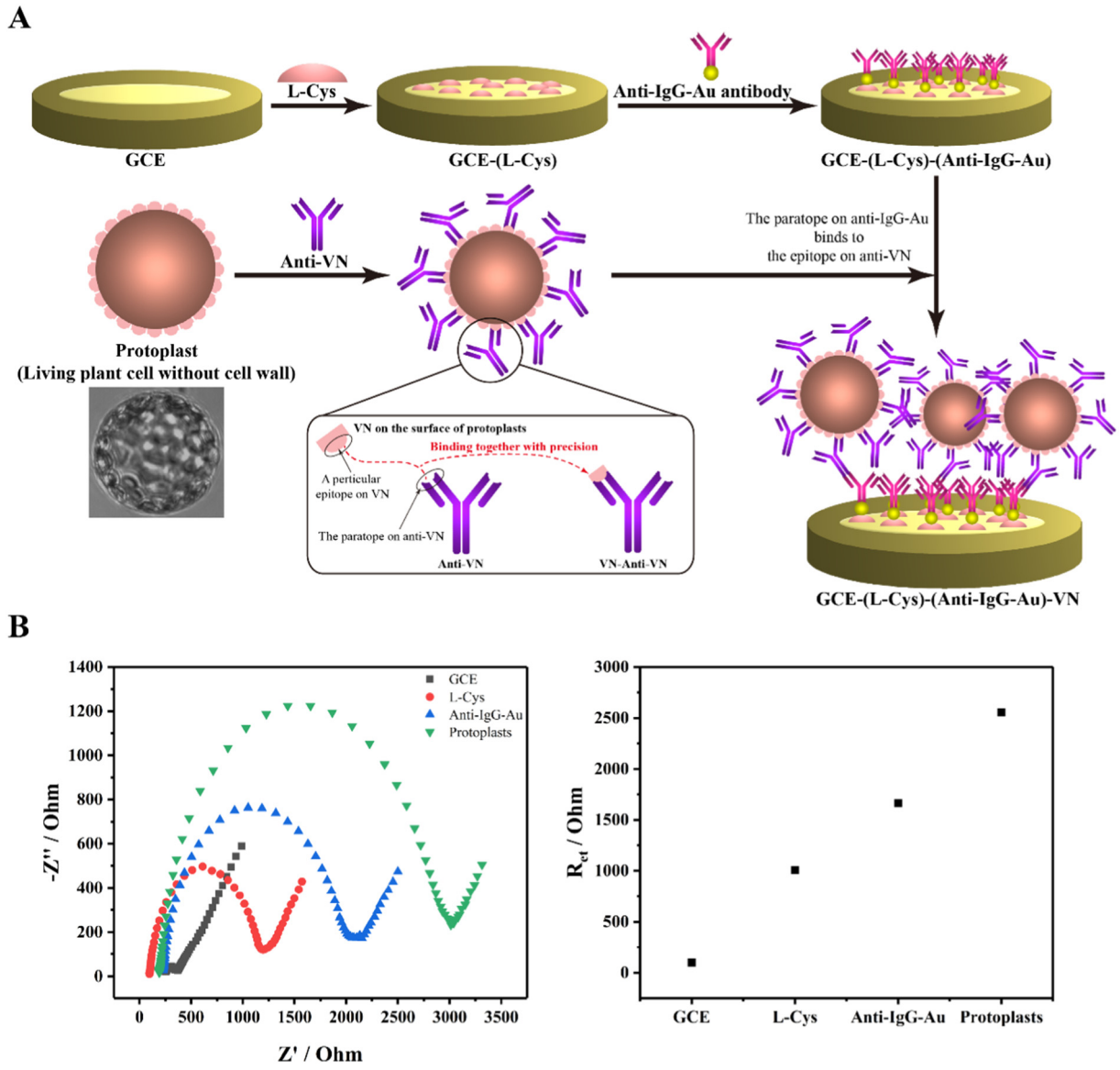


Fig. 1. (A) The principle of constructing the immuno-biosensor (protoplasts: plant cells which have their cell wall entirely removed); (B) the electrochemical impedance spectra of modification.

3. Results and discussion

3.1. The effects of heavy metal pollution on cell morphology

In order to determine the heavy metal threshold concentration that can cause visible damage to plant cells, the morphologies of protoplasts of *Arabidopsis* leaf cells treated with different concentrations of Cd(II) or Pb(II) were observed using a fluorescent inverted microscope (representative images are shown in Fig. 2; all images are shown in Fig. S1). The results showed that protoplasts in the control group were of a regular spherical shape (Figs. 2 and S1). Furthermore, the morphology of protoplasts was also of a regular spherical shape after treatment with Cd(II) (≤180 μmol·L^{-1}) (Figs. 2 and S1) or Pb(II) (≤210 μmol·L^{-1}) (Figs. 2 and S1), and did not display any visible sign of damage. However, after treatment with heavy metals at higher concentrations (up to 360 μmol·L^{-1}), protoplasts were no longer in a regular spherical shape and the proportion of protoplasts having irregular forms increased with the increase in treatment concentrations (Fig. S1). The change in morphology of protoplasts in the Cd(II) treatment group was more significant than in the Pb(II) treatment group (Figs. 2 and S1). In short, heavy metal pollution induced invisible effects on plants within the concentration ranges of 0–180 μmol·L^{-1} for Cd(II) and 0–210 μmol·L^{-1} for Pb(II).

3.2. The construction of a living plant cell-based biosensor for detecting the content of VN on the surface of plant leaf cells

In order to confirm whether VN on the surface of plant leaf cells can be a biomarker for indicating invisible damage to plants caused by heavy metals, a novel immune-biosensor was constructed to detect the content of VN. Firstly, because the primary and secondary amines can covalently bind to the surface of GCE through amino oxidation, L-Cys was modified onto the surface of GCE. Then, anti-IgG-Au was modified onto the surface of GCE-(L-Cys) due to the reason that the sulphydryl group in L-Cys can bind to the nano-Au particle in anti-IgG-Au (Márquez et al., 2017; Wang et al., 2017). After incubating the antibody of VN (anti-VN) (in which is essentially IgG in Y shape) with the protoplasts or commercial VN, the antigen-recognition domain of anti-VN (on the branch part of the antibody in Y shape) was bound to the VN on the surface of protoplasts or commercial VN through electronic attraction, hydrogen bonds, hydrophobic force and Van der Waals's force (Atassi et al., 1984). Finally, the protoplasts or VN

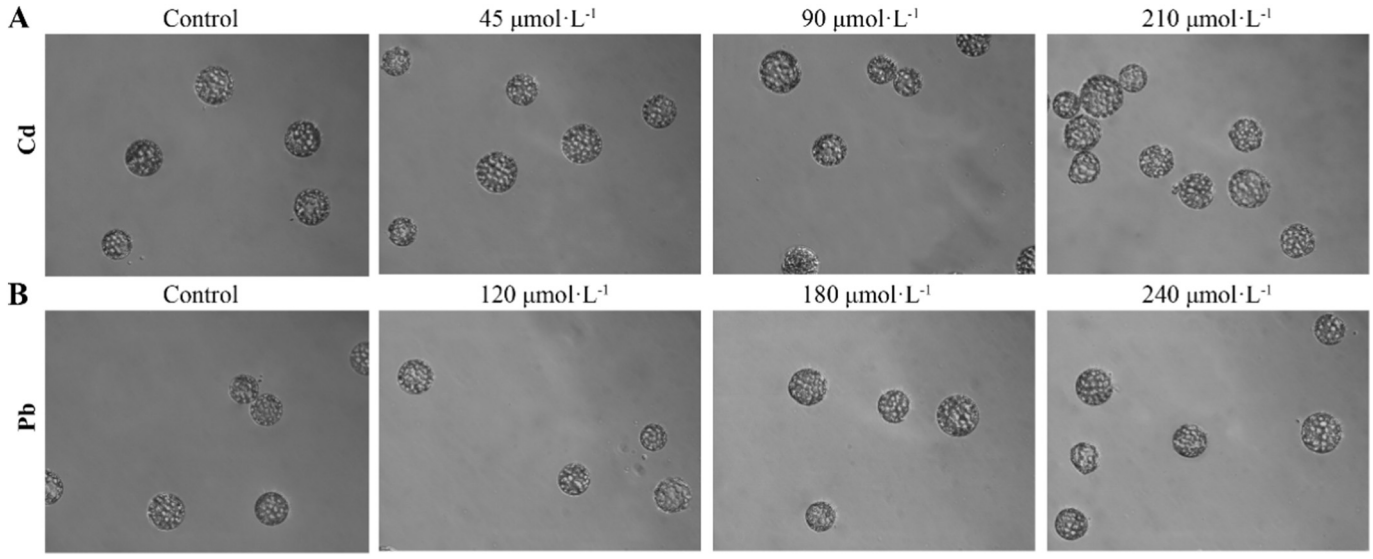


Fig. 2. Representative mages of protoplasts observed under fluorescent inverted microscope. The protoplasts of Col-0 treated with different concentrations of (A) Cd(II) (0, 45, 90 and 210 μmol·L^{-1}); (B) Pb(II) (0, 120, 180 and 240 μmol·L^{-1}). All other images are shown in Fig. S1.

connected with anti-VN was modified onto the surface of GCE-(L-Cys)-(anti-IgG-Au), by which the anti-IgG-Au (a kind of IgG in Y shape) as the antibody of anti-VN bound to the anti-VN connected to the protoplasts with its antigen-recognition domain on the branch part (Figs. 1A and S2). The anti-IgG-Au, anti-VN and VN complexes effectively enhanced the electron-transfer resistance (R_{et}) (Fig. 1B). Meanwhile, the increasing content of VN on the surface of electrode can also lead to an increase in R_{et}.

In order to confirm whether the content of VN on the surface of leaf cells can be detected by using electrochemical impedance, the standard curve of R_{et} was described by modifying a gradient concentrations of VN onto the immune-biosensor, and the corresponding results are shown in Fig. 3. The results showed that, when the concentration of modified VN was in the range of 5–20,000 μg·L^{-1}, there was a linear relationship between R_{et} and lgC(VN) (regression equation was $R_{et} = 1548.09 \times \lg C + 938.40$). The correlation coefficient was 0.995 and the limit of detection (LOD) was 24.35 pg·L^{-1} (defined as the signal-to-noise ratio of 3). These results indicated that the content of VN on the surface of leaf cells could be determined by measuring the R_{et} of the constructed biosensor.

In order to serve as a biomarker for monitoring invisible damage of plants caused by heavy metal stress, the content of VN on the surface of leaf cells must exhibit a stable dose-response to heavy metals stress. Therefore, the electrodes were modified with protoplasts of *Arabidopsis* leaves treated with varying concentrations of Cd(II) or Pb(II), and then, the R_{et} values were determined (Fig. 4). The value of R_{et} increased with increasing the concentrations of Cd(II) and Pb(II) in a linear relationship. And the treatment concentration of Cd(II) or Pb(II) in the linear relationship was the range of 45–210 and 120–360 μmol·L^{-1}, respectively. The regression equations were: R_{et}[Cd(II)] = 21.62 × C + 2268.63 [R^2 = 0.996, LOD = 18.5 nmol·L^{-1} Cd(II), confidence interval (95%) 18.4–18.6 nmol·L^{-1}] and R_{et}[Pb(II)] = 11.22 × C + 1718.01 [R^2 = 0.997 LOD = 25.6 nmol·L^{-1} Pb(II), confidence interval (95%) 25.4–25.8 nmol·L^{-1}], respectively. The sensitivity of this biosensor to Cd(II) was over Pb(II). These results indicated that the content of VN on the surface of plant leaf cells was stably increased with increasing the concentration of Cd(II) or Pb(II). The response sensitivity of VN to Cd(II) and Pb(II) was in accordance with the ranked order of toxicity of heavy metals [Cd(II) > Pb(II)] to *Arabidopsis* (Fischer et al., 2017; Zhang et al., 2018b).

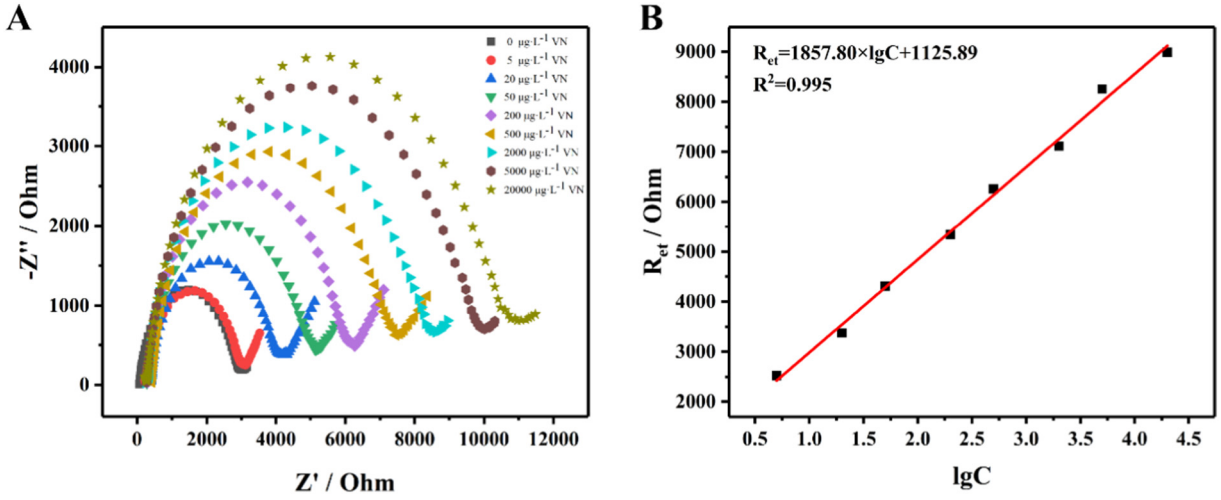


Fig. 3. (A) Electrochemical impedance spectra and (B) linear fitting curve of electron-transfer resistance of GCE-(L-Cys)-(IgG-Au) electrode modified with a gradient concentrations of VN (5, 20, 50, 200, 500, 2000, 5000 and 20,000 μg·L^{-1}), n = 3.

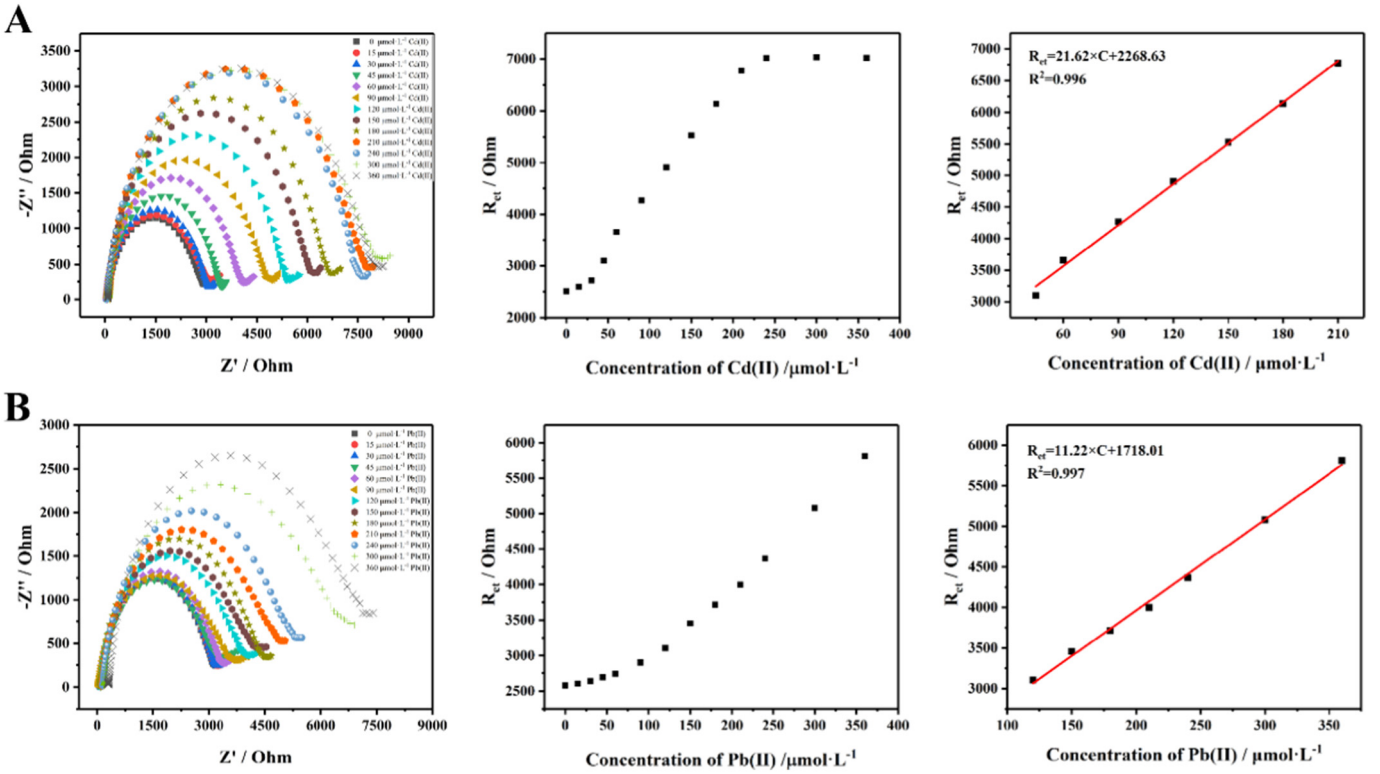


Fig. 4. Electrochemical impedance spectra (left), electron-transfer resistance (medium) and linear fitting curves (right) of GCE-(L-Cys)-(IgG-Au) electrode after *Arabidopsis* leaves treated with a gradient concentrations (0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360 μmol·L^{-1}) of (A) Cd(II) and (B) Pb(II), n = 3.

In order to investigate the interference from other factors such as other non-toxic heavy metals, pH and temperature, and the long-term stability of the biosensor, the following experiments were performed. First, the electrodes were modified with protoplasts of *Arabidopsis* leaves treated with 300 μmol·L^{-1} Zn(II), Fe(III), Ca(II) or Mg(II), and then the R_{et} values were determined. Compared with the control, the change in R_{et} caused by Zn(II), Fe(III), Ca(II) or Mg(II) was < 5% (Fig. S3), suggesting that the change in the content of VN caused by Cd(II) and Pb(II) could not be interfered by non-toxic heavy metals. Second, the electrodes were modified with protoplasts of *Arabidopsis* leaves treated with Cd(II) and Pb(II) in different pH (pH = 5.0, 5.5, 6.0 and 6.5), and then the R_{et} values were determined. The results showed that the change in R_{et} caused by the changes in the pH value was also < 5% (Fig. S4). Therefore, pH ranging from 5.0 to 6.5 showed no interference to this biosensor. Third, the electrodes were modified with protoplasts of *Arabidopsis* leaves that had grown under different temperature (4, 22 and 35 °C), and then the R_{et} values were determined. The results showed that there was almost no change in R_{et} value between different groups (Fig. S5), suggesting that this biosensor was also not interfered by the changes in the temperature. Moreover, the long-term stability of this biosensor was evaluated. The results showed that the change in R_{et} value was < 5% within 7 days (Fig. S6), indicating that this biosensor had a good stability within 7 days.

In addition, due to the fact that plants are continuously exposed under atmospheric concentration of Cd(II) and Pb(II), we investigated the effects of long-term exposure under low concentrations of Cd(II) and Pb(II) on the content of VN. We found that the effects of long-term exposure under low concentrations of Cd(II) and Pb (II) were similar to that of short-term exposure under high concentrations (Fig. S7). Therefore, all of these results indicated that VN could be a biomarker for monitoring invisible damage of plants caused by heavy metal stress.

3.3. Molecular basis of VN as a biomarker

In order to deeply understand why the content of VN on the surface of leaf cells was increased with increasing the concentration of heavy metals, the interaction between VN and Cd(II) or Pb(II) was studied using computer simulation, respectively. Firstly, based on the amino acid sequence of VN in the *Arabidopsis* database, 3D model of VN was constructed using homology modeling. It has been reported that the pH value between the cell wall and plasma membrane ranged between 4.4 and 5.5 (Buchanan and Gruissem, 2000). Therefore, the charge distribution on the surface of VN was calculated (Yang et al., 2016). A segment of VN (the amino sequence was 96–100) in the negatively charged area was selected, and the interaction between the segment and Cd(II) or Pb(II) was calculated using quantum chemical calculations (Fig. 5 and Table S1). According to the results, Cd(II) formed a bond to a carboxyl group oxygen atom (O) in VN, forming a 2.2799 Å Cd—O bond. Meanwhile, the hydrogen atom (H) in the coordination water of Cd(II) formed a bond to the adjacent O and formed two hydrogen bonds with the lengths of 1.6682 Å and 1.6496 Å, respectively (Fig. 5B). Similarly, Pb(II) bound to VN and formed a 2.3112 Å Pb—O bond and 2.3991 Å and 1.2513 Å hydrogen bonds (Fig. 5C). These results suggested that Cd(II) and Pb(II) could form stable bonds with VN. With the increase in the concentrations of Cd(II) and Pb(II), the content of VN on the surface of leaf cells was not sufficient to defend against exogenous Cd(II) or Pb(II). Therefore, as a protective defense method, plant cells increased the synthesis of VN and recruited VN onto the plasma membrane to bind more Cd(II) and Pb(II) (Cheng et al., 2018; Yang et al., 2016).

3.4. The effect of heavy metal pollution on the content of total chlorophyll

In order to confirm whether the increase in the content of VN on the surface of leaf cells can reflect invisible damage to plants caused

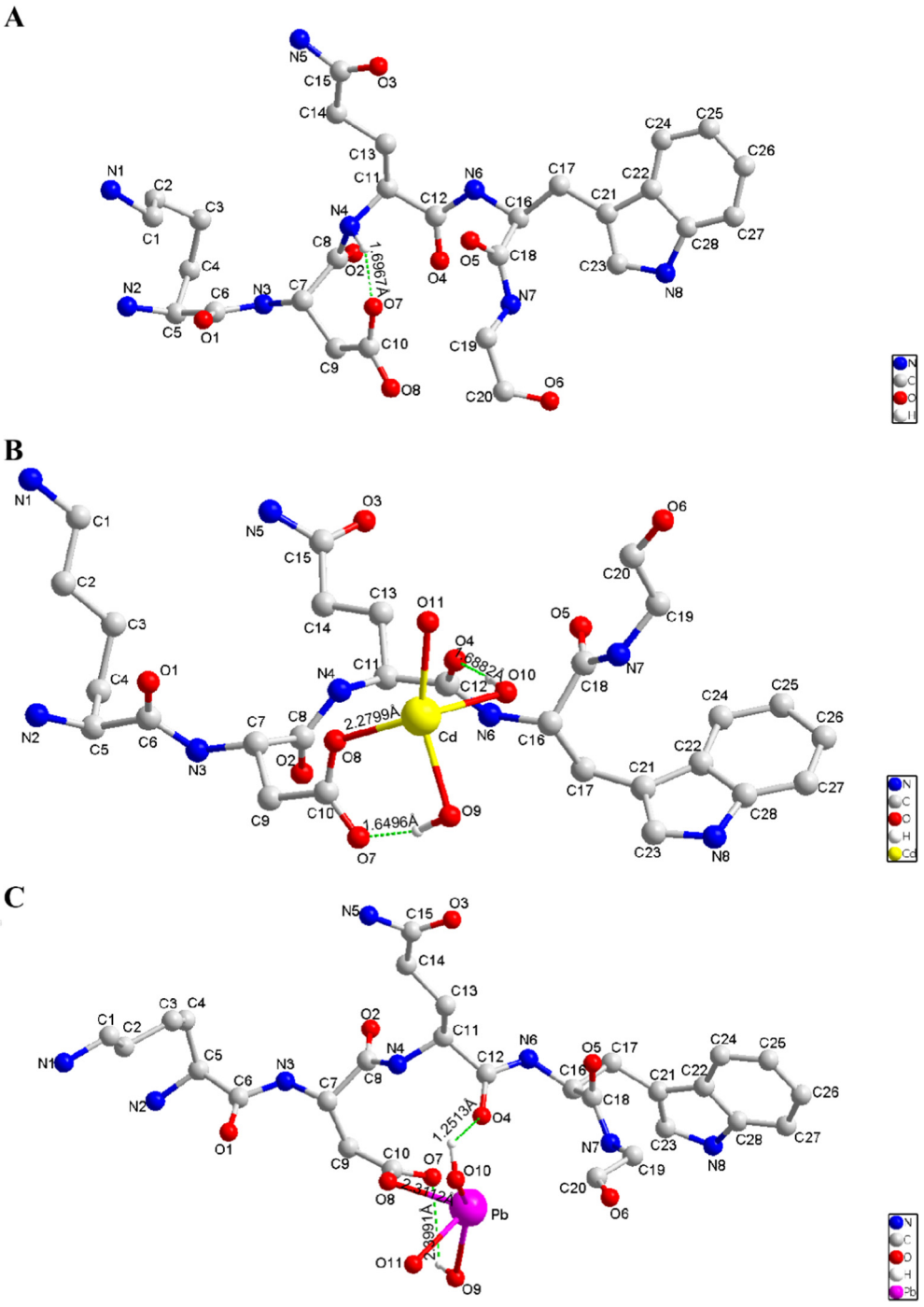


Fig. 5. The results of quantum chemical computation used to simulate the interaction between the metal cation [Cd(II), Pb(II)] and a part in VN model. (A) The part of VN; (B) and (C) The interaction between the metal cation-Cd(II) and Pb(II) and the part of VN, respectively. The amino sequence of VN is 96–100.

by heavy metal stress, the content of total chlorophyll in *Arabidopsis* leaves treated with various concentrations of Cd(II) or Pb(II) was determined (Fig. 6). Compared with the control group, the protoplasts did not show any signs of visible damage after treatment with 45 $\mu mol \cdot L^{-1}$ Cd(II), although the content of VN on the surface of leaf cells increased by about 14 times (R_{et} increased by about 29%), and the content of total chlorophyll decreased significantly about 13% (Fig. 6A). With increasing the concentrations of Cd(II), the content of VN further increased, while the content of total chlorophyll was further decreased (Fig. 6A). The contents of VN and total chlorophyll in the Pb(II) treatment group (Fig. 6B) were similar to those obtained from the results of Cd(II) treatment group. However, when the content of total chlorophyll decreased significantly, the increase in VN on the surface of leaf cells became different. In the Pb(II) treatment group, when the content of total chlorophyll decreased significantly about 20%, the content of VN increased by about 23 times (R_{et} increased by about 45%) that of the control group in the experiment of *Arabidopsis* leaves. These effects may be due to different tolerances of plants to Cd(II) and Pb(II) (Fischer et al., 2017; Prasad and Hagemeyer, 1999; Zhang et al., 2018b). In short, these results suggest that, when the content of VN was increased by about 20 times (R_{et} increased by about 35%) under the stress of Cd(II) or Pb(II), the

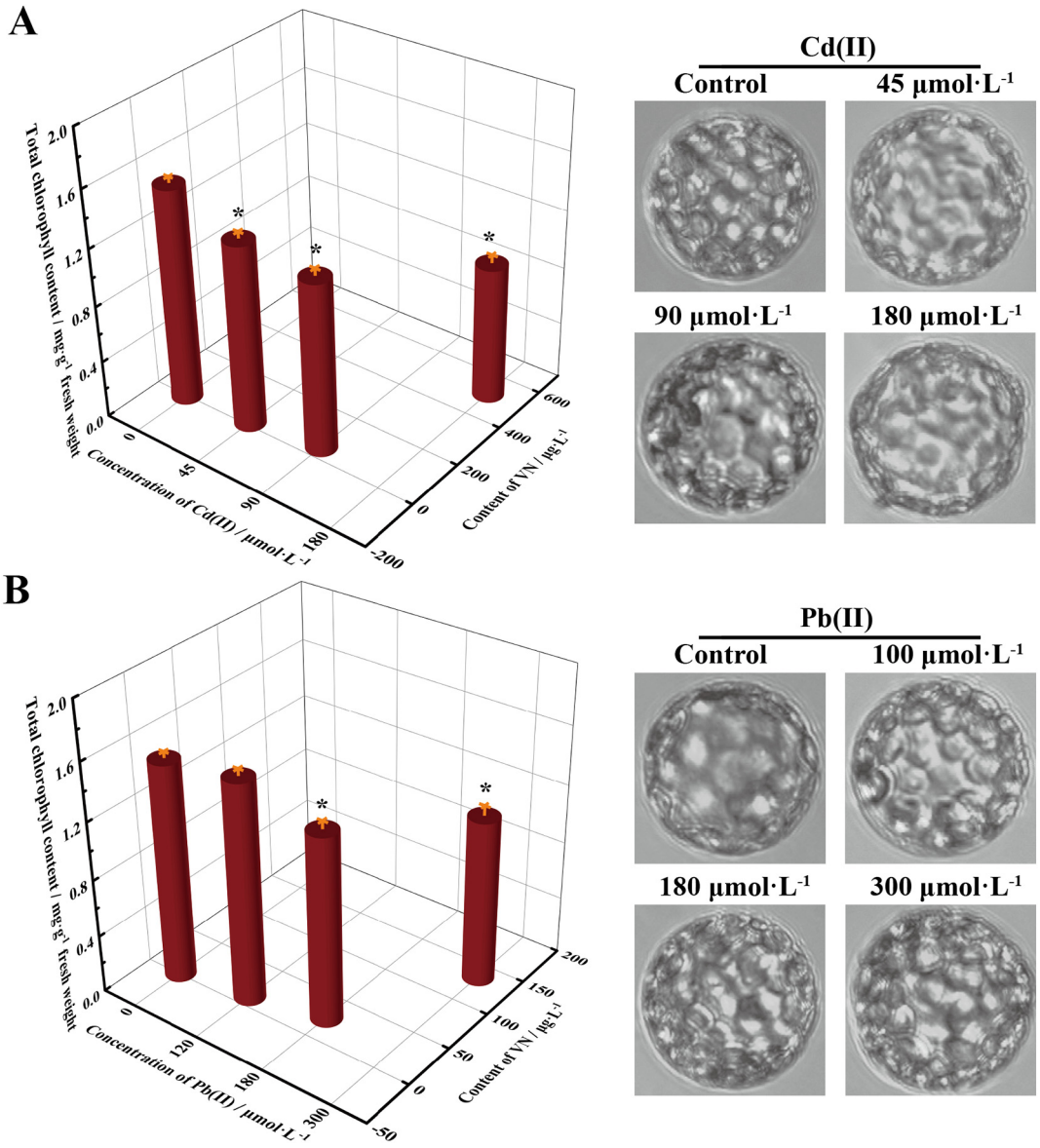


Fig. 6. The content of total chlorophyll in *Arabidopsis* leaves and the corresponding content of VN on the surface of leaf cells and images of protoplast morphology after treatment with different concentrations of (A) Cd(II) (0, 45, 90, 180 μmol·L^{-1}) and (C) Pb(II) (0, 120, 180, 300 μmol·L^{-1}), n = 3 and significant differences at $p < 0.05$ were denoted with "*".

content of total chlorophyll in *Arabidopsis* leaves was significantly reduced, indicating that invisible damage to *Arabidopsis* caused by heavy metal stress.

3.5. The application of the biosensor in monitoring invisible damage to soybean caused by heavy metal stress

In order to test the practicability of this biosensor, such as monitoring invisible damage of plants with different morphological properties and physiological characteristics, and its application in agriculture, soybean, which is an important commercial crop, was selected as another representative plant for detecting its invisible damage caused by Cd (II) and Pb(II) using our biosensor. The results showed that a linear relationship also existed between R_{et} and the Cd(II) and Pb(II) treatment concentrations within the range of 120–300 and 120–480 μmol·L^{-1}, respectively (Fig. S8). Additionally, the regression equations were found to be: $R_{et}[Cd(II)] = 20.93 \times C + 773.29$ [$R^2 = 0.995$ LOD = 18.5 nmol·L^{-1} Cd(II), confidence interval (95%) 18.4–18.6 nmol·L^{-1}]; and $R_{et}[Pb(II)] = 10.96 \times C + 1801.52$ [$R^2 = 0.990$ LOD = 25.7 nmol·L^{-1} Pb(II), confidence interval (95%) 25.4–25.8 nmol·L^{-1}].

In addition, the trend of the content of total chlorophyll treated with varying doses of Cd(II) and Pb(II) was similar to the results of *Arabidopsis* experiments (Fig. S5). When the content of VN on the surface of leaf cells increased by about 14 times (R_{et} increased about 27%) in Cd(II) treatment group and 25 times (R_{et} increased by about 45%) in Pb(II) treatment group, the content of total chlorophyll significantly decreased by about 14% (Fig. S9A) and 21% (Fig. S9B), respectively. However, the thresholds of Cd(II) and Pb(II) treatment concentrations were different when the content of total chlorophyll significantly decreased. The reason was that there were discrepant tolerances to Cd (II) or Pb(II) in different plants.

In short, VN can serve as a universal biomarker for monitoring invisible damage of plants under the stress of heavy metals. The LOD of this biosensor for Cd(II) and Pb(II) is close to that of previously reported biosensors using in animal research (Liu et al., 2009; Xiang et al., 2010). Meanwhile, we establish a quantitative relationship among the content of biomarker, the electron-transfer resistance and chlorophyll content in plant cells, which can provide a theoretical basis for further building biosensor for various biomonitoring.

4. Conclusions

In summary, VN on the surface of plant leaf cells can serve as a biomarker for indicating invisible damage of plants caused by heavy metal stress. An immune-electrochemical biosensor is successfully constructed which can monitor invisible damage of plants by detecting the content of VN on the surface of leaf cells. When the increase in the content of VN exceeds about 20 times under the stress of Cd(II) or Pb(II), namely, an increase in the electron-transfer resistance exceeds about 35% and a decrease in the content of chlorophyll exceeds about 17%, the invisible damage of plant cells occurs. These results can provide a reference method for early-onset warning systems for heavy metal pollution in the environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank X.W. Deng (Peking University), C.H. Huang (Peking University), G.B. Jiang (Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences) and H.X. Ju (Nanjing University) for their valuable suggestions on the project. This work was supported by the National Natural Science Foundation of China (21371100, 21501068, 31170477), Ph.D. Programs Foundation of Ministry of Education of China (20130093120006), the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134097.

References

Atassi, M.Z., van Oss, C.J., Absolom, D.R., 1984. Molecular immunology. Marcel Dekker Press, New York.

Buchanan, B., Gruissem, W., 2000. Biochemistry & Molecular Biology of Plants. John Wiley & Sons, Rockville.

Cai, L.M., Wang, Q.S., Luo, J., Chen, L.G., Zhu, R.L., Wang, S., Tang, C.H., 2019. Heavy metal contamination and health risk assessment for children near a large Cu-smelter in central China. Sci. Total Environ. 650, 725–733. https://doi.org/10.1016/j.scitotenv.2018.09.081.

Cheng, M.Z., Wang, L.H., Yang, Q., Huang, X.H., 2018. A detection method in living plant cells for rapidly monitoring the response of plants to exogenous lanthanum. Ecotoxicol. Environ. Saf. 158, 94–99. https://doi.org/10.1016/j.ecoenv.2018.04.021.

Fan, L.S., Li, R.L., Pan, J.W., Ding, Z.J., Lin, J.X., 2015. Endocytosis and its regulation in plants. Trends Plant Sci. 20, 388–397. https://doi.org/10.1016/j.tplants.2015.03.014.

Fischer, S., Spielau, T., Clemens, S., 2017. Natural variation in *Arabidopsis thaliana* Cd responses and the detection of quantitative trait loci affecting Cd tolerance. Sci. Rep. 7, 3693. https://doi.org/10.1038/s41598-017-03540-z.

Guo, Z.Q., Ni, Z.X., Ye, H.Z., Xiao, J., Chen, L.Z., Green, I., Zhang, L., 2019. Simultaneous uptake of Cd from sediment, water and diet in a demersal marine goby *Mugilogobius chulae*. J. Hazard. Mater. 364, 143–150. https://doi.org/10.1016/j.jhazmat.2018.09.045.

Han, M.Y., Guo, W.L., Liang, Z.S., Yang, D.F., Yan, X.J., Zhu, Y.H., Liu, Y., 2015. Effects of cerous nitrate on growth and tanshinone production in salvia miltiorrhiza hairy roots. J. Rare Earths 33, 1228–1235. https://doi.org/10.1016/S1002-0721(14)60549-4.

Hoagland, D.R., 1920. Optimum nutrient solutions for plants. Science 52, 562–564. https://doi.org/10.1126/science.52.1354.562.

Hoon, B.S., Woo, K.M., Yoeng, P.C., Chang-Sun, C., Kumar, K.S., Pil, P.J., Jung, P.T., 2019. Development of a rapid and sensitive electrochemical biosensor for detection of human norovirus via novel specific binding peptides. Biosens. Bioelectron. 123, 223–229. https://doi.org/10.1016/j.bios.2018.08.064.

Hu, H.Q., Wang, L.H., Li, Y.L., Sun, J.W., Zhou, Q., Huang, X.H., 2016a. Insight into mechanism of lanthanum (III) induced damage to plant photosynthesis. Ecotoxicol. Environ. Saf. 127, 43–50. https://doi.org/10.1016/j.ecoenv.2016.01.008.

Hu, H.Q., Wang, L.H., Zhou, Q., Huang, X.,.H., 2016b. Combined effects of simulated acid rain and lanthanum chloride on chloroplast structure and functional elements in rice. Environ. Sci. Pollut. Res. 23, 8902–8916. https://doi.org/10.1007/s11356-015-5962-9.

Hu, C.Y., Yang, X.L., Gao, L.J., Zhang, P.D., Li, W.T., Dong, J.Y., Li, C.J., Zhang, X.M., 2019. Comparative analysis of heavy metal accumulation and bioindication in three seagrasses: which species is more suitable as a bioindicator? Sci. Total Environ. 669, 41–48. https://doi.org/10.1016/j.scitotenv.2019.02.425.

Huang, Y., Wang, L.Y., Wang, W.J., Li, T.Q., He, Z.L., Yang, X., 2019. Current status of agricultural soil pollution by heavy metals in China: a meta-analysis. Sci. Total Environ. 651, 3034–3042. https://doi.org/10.1016/j.scitotenv.2018.10.185.

Juknys, R., Vitkauskaitė, G., Venclovienė, J., 2012. The impacts of heavy metals on oxidative stress and growth of spring barley. Cent. Eur. J. Biol. 7, 299–306. https://doi.org/10.2478/s11535-012-0012-9.

Kamani, H., Mahvi, A.H., Seyedsalehi, M., Jaafari, J., Hoseini, M., Safari, G.H., Dalvand, A., Aslani, H., Mirzaei, N., Ashrafi, S.D., 2017. Contamination and ecological risk assessment of heavy metals in street dust of Tehran, Iran. Int. J. Environ. Sci. Technol. 14, 2675–2682. https://doi.org/10.1007/s13762-017-1327-x.

Kolotov, B.A., Demidov, V.V., Volkov, S.N., 2003. Chlorophyll content as a primary indicator of the environment degradation due to contamination with heavy metals. Dokl. Biol. Sci. 393, 550–552. https://doi.org/10.1023/B:DOBS.0000010321.42939.1d.

Landrigan, P.J., Fuller, R., Hanrahan, D., Sandilya, K., Acosta, N.J.R., Adeyi, O., Arnold, R., Basu, N.N., Balde, A.B., Bertollini, R., Bose-O'Reilly, S., Boufford, J.I., Breysse, P.N., Chiles, T., Mahidol, C., Coll-Seck, A.M., Cropper, M.L., Fobil, J., Fuster, V., Greenstone, M., Haines, A., Hunter, D., Khare, M., Krupnick, A., Lanphear, B., Lohani, B., Martin, K., Mathiasen, K.V., McTeer, M.A., Murray, C.J.L., Ndahimananjara, J.D., Perera, F., Potocnik, J., Preker, A.S., Ramesh, J., Rockstrom, J., Salinas, C., Samson, L.D., Sly, P.D., Smith, K.R., Steiner, A., Stewart, R.B., Suk, W.A., van, S.O.C.P., Yadama, G.N., Yumkella, K., Zhong, M., 2018. The lancet commission on pollution and health. Lancet 391, 462–512. https://doi.org/10.1016/S0140-6736(17)32345-0.

Leavesly, D.I., Kashyap, A.S., Croll, T., Sivaramakrishnan, M., Shokoohmand, A., Hollier, B.G., Upton, Z., 2013. Vitronectin-master controller or micromanager? IUBMB Life 65, 807–818. https://doi.org/10.1002/iub.1203.

Lee, S., Han, C., Shin, D., Hur, S.D., Jun, S.J., Kim, Y.T., Byun, D.S., Hong, S., 2017. Characteristics of elemental and Pb isotopic compositions in aerosols (PM10-2.5) at the Ieodo Ocean Research Station in the East China Sea. Environ. Pollut. 231, 154–164. https://doi.org/10.1016/j.envpol.2017.08.007.

Liu, G.L., Wang, J.F., Li, Z.Y., Liang, S.Z., Wang, X.N., 2009. Immunoassay for cadmium detection and quantification. Biomed. Environ. Sci. 22, 188–193. https://doi.org/10.1016/s0895-3988(09)60044-1.

Márquez, A., Jiménez-Jorquera, C., Domínguez, C., Muñoz-Berbel, X., 2017. Electrodepositable alginate membranes for enzymatic sensors: an amperometric glucose biosensor for whole blood analysis. Biosens. Bioelectron. 97, 136–142. https://doi.org/10.1016/j.bios.2017.05.051.

Mello, L., Hernandez, S., Marrazza, G., Mascini, M., Kubota, L., 2006. Investigations of the antioxidant properties of plant extracts using a DNA-electrochemical biosensor. Biosens. Bioelectron. 21, 1374–1382. https://doi.org/10.1016/j.bios.2005.05.012.

Pandey, L.K., Park, J., Son, D.H., Kim, W., Islam, M.S., Choi, S., Lee, H., Han, T., 2019. Assessment of metal contamination in water and sediments from major rivers in South Korea from 2008 to 2015. Sci. Total Environ. 651, 323–333. https://doi.org/10.1016/j.scitotenv.2018.09.057.

Peng, Q., Zhou, Q., 2009. Influence of lanthanum on chloroplast ultrastructure of soybean leaves under ultraviolet-B stress. J. Rare Earths 27, 304–307. https://doi.org/10.1016/S1002-0721(08)60238-0.

Prasad, M.N.V., Hagemeyer, J., 1999. Heavy Metal Stress in Plants. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim https://doi.org/10.1002/9783527628964.ch8.

Pundir, C., Jakhar, S., Narwal, V., 2018. Determination of urea with special emphasis on biosensors: a review. Biosens. Bioelectron. 123, 36–50. https://doi.org/10.1016/j.bios.2018.09.067.

Qi, L., Chen, M.D., Ge, X.L., Zhang, Y.F., Guo, B.F., 2016. Seasonal variations and sources of 17 aerosol metal elements in suburban Nanjing, China. Atmosphere 7, 153. https://doi.org/10.3390/atmos7120153.

Wan, D.J., Yang, G.L., Yang, J.S., Zhan, C.L., 2018. Ecological risks and spatial distributions of heavy metals in Beijing atmospheric dust. Pol. J. Environ. Stud. 27, 881–887. https://doi.org/10.15244/pjoes/76285.

Wang, L.H., Li, J.G., Zhou, Q., Yang, G.M., Ding, X.L., Li, X.D., Cai, C.X., Zhang, Z., Wei, H.Y., Lu, T.H., Deng, X.W., Huang, X.H., 2014. Rare earth elements activate endocytosis in plant cells. Proc. Natl. Acad. Sci. U. S. A. 111, 12936–12941. https://doi.org/10.1073/pnas.1413376111.

Wang, L.H., He, J.F., Yang, Q., Li, X.D., Wei, H.Y., Chen, D.D.Y., Huang, X.H., 2017. A preliminary study on the effects of lanthanum (III) on plant vitronectin-like protein and its toxicological basis. Ecotoxicol. Environ. Saf. 145, 227–234. https://doi.org/10.1016/j.ecoenv.2017.07.039.

Weigel, D., Glazebrook, J., 2002. *Arabidopsis*: a laboratory manual. Eur. J. Soc. Theory 10, 339–356. https://doi.org/10.1177/1368431007080699.

Wen, K.J., Liang, C.J., Wang, L.H., Hu, G., Zhou, Q., 2011. Combined effects of lanthanumion and acid rain on growth, photosynthesis and chloroplast ultrastructure in soybean seedlings. Chemosphere 84, 601–608. https://doi.org/10.1016/j.chemosphere.2011.03.054.

Xiang, J.J., Zhai, Y.F., Tang, Y., Wang, H., Liu, B., Guo, C.W., 2010. A competitive indirect enzyme-linked immunoassay for lead ion measurement using mAbs against the lead-DTPA complex. Environ. Pollut. 158, 1376–1380. https://doi.org/10.1016/j.envpol.2010.01.002.

Yan, L., Li, X.R., He, M.Z., Zhao, X., Liu, Y.B., Yan, C., Pan, Y.X., Tan, H.J., 2010. Seedlings growth and antioxidative enzymes activities in leaves under heavy metal stress differ between two desert plants: a perennial (*Peganum harmala*) and an annual (*Halogeton glomeratus*) grass. Acta Physiol. Plant. 32, 583–590. https://doi.org/10.1007/s11738-009-0436-7.

Yang, Q., Wang, L.H., He, J.F., Li, X.D., Tong, W.J., Yang, Z.B., Zhou, Q., Huang, X.H., 2016. Vitronectin-like protein is a first line of defense against lanthanum (III) stress in

Arabidopsis leaf cells. Environ. J. Exp. Bot. 130, 86–94. https://doi.org/10.1016/j.envexpbot.2016.05.011.
Zhang, X.B., Wang, L.H., Zhou, A.H., Zhou, Q., Huang, X.H., 2016. Alterations in cytosol free calcium in horseradish roots simultaneously exposed to lanthanum(III) and acid rain. Ecotoxicol. Environ. Saf. 126, 62–70. https://doi.org/10.1016/j.ecoenv.2015.12.014.
Zhang, F., Li, Y.H., Shi, Y., Wang, L.H., Zhou, Q., Huang, X.H., 2018a. A novel evaluation of the effect of lanthanum exposure on plant populations. Chemosphere 202, 377–386. https://doi.org/10.1016/j.chemosphere.2018.03.086.
Zhang, X., Li, M., Yang, H.H., Li, X.X., Cui, Z.J., 2018b. Physiological responses of Suaeda glauca and *Arabidopsis thaliana* in phytoremediation of heavy metals. J. Environ. Manag. 223, 132–139. https://doi.org/10.1016/j.jenvman.2018.06.025.
Zhou, X.W., Qu, X.Y., Zheng, N., Su, C.Y., Wang, J.Q., Soyeurt, H., 2019. Large scale study of the within and between spatial variability of lead, arsenic, and cadmium contamination of cow milk in China. Sci. Total Environ. 650, 3054–3061. https://doi.org/10.1016/j.scitotenv.2018.09.094.