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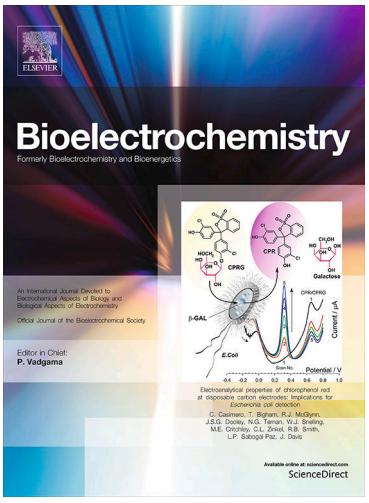

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Electrochemical biosensor for cytokinins based on the CHASE domain of *Arabidopsis* histidine kinases 4

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Abstract: In this study, An AHK4 CHASE domain was used to construct an electrochemical cytokinin biosensor using ferrocene as the electrochemical mediator. Upon addition of cytokinin, the binding of cytokinin and AHK4 led to dimerization, which blocked electron transfer between ferrocene and the electrode, and the redox peak current of ferrocene was gradually reduced. Cytokinin was detected by recording the change of the ferrocene redox peak current. The biosensor shows a linear range of 50 to 400 nM with a linear regression equation of $i_p = 0.0086\ c + 0.732$ ($R^2 = 0.993$) with i_p in μA and c in nM and a detection limit (LOD) of 1.5 nM (S/N = 3). The biosensor exhibits excellent performance that avoids interference of other types of plant hormones and was successfully applied to the detection of cytokinins in bean sprouts.

Key words: Cytokinin; *Arabidopsis* histidine kinases 4; Ferrocene; Electrochemical biosensor

1. Introduction

Plant hormones regulate the growth and development of plants with high efficiency, and plant organs show high sensitivity to the hormones [1]. At present, people widely use plant hormones and derivatives to control agricultural production. Effective qualitative and quantitative methods of detection of plant hormones provide the foundation for studying their functional mechanism. However, the detection of phytohormones is difficult due to the structural complexity of these compounds, the extremely low concentrations (generally ng / g level) and their chemical instability. Thus, the developed method for the detection of phytohormones should have high sensitivity and high specificity [2].

Cytokinins(CKs) are one kind of the seven major natural plant hormones, which are derivatives of adenine molecular [3]. CKs regulate the activity of shoot meristem and play a key role in the degree of ear branching [4]. In addition, CKs play a key role in plant development, such as regulation of apical dominance, promotion of flower and fruit development, acceleration in chloroplast metabolic synthesis and inhibition of elongation of the main root [5]. Cytokinins exist widely in plants, and they are categorized into many types by their similar impacts on plants. Trans-zeatin (tZ) and isopentenyl adenine (iP) are the two most common natural cytokinins.

Various methods for the detection of cytokinins have been developed in the past several decades, including immunoassay, chromatography, mass spectrometry, and

electrochemical analysis [6]. Weiler has developed a zeatin nucleoside (tZR) antibody using the radio immunoassay method that accurately detects zeatin nucleosides (tZR) with a detection limit of 40 FM (15 pg) and a linear range of 0.06 - 30 PM (0.02 - 10 ng) [7]. Maldiney has developed the biotin-avidin system coupled with the ELISA method to detect zeatin nucleoside (ZR) in tomato with a detection limit of 3-5 pg and a linear range of 10 - 1000 pg [8]. The immunoassay method to detect cytokinins is fast and reliable, but the antibody preparation cost is high. Recently, high-performance liquid chromatography (HPLC) coupled with mass spectrometry has been widely used to detect cytokinins [9]. Cao et al. developed a method based on HPLC−MS to detect six cytokinin nucleotide monophosphates in coconut flesh. The quantification ranged from 0.2 to 1.0 ng/mL with the limits of detection 0.3 ng/mL [10]. HPLC−MS methods have achieved the required sensitivity and specificity, but the samples need pretreatment and purification, which are time-consuming. Chemical biosensors are widely applied to analyze CKs. Li et al. used electropolymerization to make multilayer films for immobilization of horseradish peroxidase (HRP) and electron mediators. N^6-(Δ2-isopentenyl) adenosine (iPA), glucose oxidase-labeled iPA and unlabeled iPA were electrostatically adsorbed on the electrode and reacted with the antibody competitively. Glucose was catalytically reduced to form hydrogen peroxide by HRP and electrons were captured by the electron mediator to detect the iPA content. The linear range of this biosensor was 5-300 μg/mL [11]. Tian et al. have developed a Pt microelectrode which immobilized cytokinin dehydrogenase from Zea mays with electron mediator 2,6-dichlorophenolindophenol (DCPIP) to detect isopentenyl adenine (2-iP). The linear

dynamic range was from 10 nM to 10 mM with a detection limit of 3.9 nM [12]. Daudu et al. have developed a selective and highly sensitive yeast biosensor based *Malus domestica* CHASE histidine kinase receptor 2 (MdCHK2) for the application of cytokinin detection in bacterial samples. The biosensor can detect 0.22 nM 2-iP in biological samples[13].

The electrochemical biosensor for the detection of cytokinin mainly uses biologically active substances (enzymes, receptors, etc.) as recognition elements. When the enzyme immobilized on the electrode recognizes and reacts with the CKs, the electrode can convert the biochemical signal into an electrical signal to realize the detection of the CKs. The receptors of plant hormones are the natural sensors to sense the signals. Thus, the receptors are good alternatives to develop plant hormone biosensors.

CK signal transduction is based on a two-component signaling system (TCS) that utilizes the mediation of phosphate groups to achieve continuous signal transmission. In 2001 Inoue demonstrated that *Arabidopsis* histidine kinases 4 (AHK4) from *Arabidopsis thaliana* was the cytokinin receptor firstly. Studies have shown that AHK4 has a signal transduction function in a single cell system. When CK is added, AHK4 can be transiently expressed in protoplasts and the AHK4 acts as a direct receptor which regulates the transduction pathway of CK rapidly and accurately [14]. The structure of AHK4 is well studied. It contains a histidine kinase module in the cytoplasm and an about 270 amino acid residue sensor domain in the membrane that bind the cytokinins directly. This is called CHASE domain (cyclases/histidine kinases associated sensory extracellular domain) [15].

It was reported that isopentenyl adenine (2-iP) belonged to the natural CKs in bacteria produced by tRNA degradation. The 2-iP in *Escherichia coli* bound with the AHK4 CHASE domain in the nanomolar affinity and was co-purified with the AHK4 protein [16]. Therefore, 2-iP was used as an ideal substrate for AHK4 CHASE domain. AHK4 itself had no redox signal on the electrode, ferrocene was chosen as an electrochemical probe for the interaction between the AHK4 protein and the substrate 2-iP. Ferrocene (Fc) is an aromatic metal compound with high enrichment electrons, good thermal stability, easy modification of structure and has the ability to participate in redox reactions [17]. Furthermore, the electrochemical properties of ferrocene and its derivatives are not easily affected by changes in oxygen concentration, and its electron transfer rate is fast. Therefore, ferrocene and its derivatives are widely used in the detection of biologically active small molecules [18]. In this experiment, the AHK4 protein was immobilized on the surface of the electrode by the cross linking of glutaraldehyde and bovine serum albumin (BSA). Ferrocene carboxylic acid (FCA) was immobilized on the electrode by the covalent bond with the amino group of BSA. The addition of 2-iP caused the dimerization of AHK4 [19], which blocked the electron transfer between the ferrocene and the electrode, resulting in the decrease of the redox current of the ferrocene (Schematic 1). The relationship of the current change of ferrocene with the concentration of the addition of 2-iP was investigated, and it was used to detect 2-ip.

2. Materials and methods

2.1. *Materials*

The gene *AHK4* CHASE domain and gene *SUMO* were synthesized by Wuhan Tsingke Innovation Biotechnology Co. Ltd, China. *E. coli* DH5α, *E. coli* BL21 (DE3) strain and plasmid pET-26b (+) were preserved in this laboratory. The restriction enzymes *Nde*I and *Hind* III, T4 DNA ligase, DNA marker, protein marker were purchased from Takara, Japan. Ferrocenecarboxylic acid (FCA) was bought from Shanghai Dibo Chemical, China. Bovine serum albumin, isopentenyl adenine, brassinolide, abscisic acid and methyl jasmonate were purchased from Sigma, USA.

2.2 Construction of the prokaryotic expression vector pET26b-SUMO-AHK4

The prokaryotic expression of AHK4 protein was found to be an inclusion body. To enhance the soluble amount of the expressed AHK4, the small ubiquitin-related modifier from *Saccharomyces cerevisiae* (*SUMO*, Sequence ID: NM_001180818.1) was fused with *AHK4* to construct the prokaryotic expression vector. The *SUMO* gene and the AHK4 CHASE domain (aa 127-514) were amplified by polymerase chain reaction (PCR). Then the two sequences were fused by overlapping PCR. Finally, the fused fragment was ligated into the vector pET-26b (+) to construct the prokaryotic expression vector pET26b-SUMO-AHK4. The recombinant vector was sequenced and the positive transformant was stored at -80 °C for the following use.

2.2 Prokaryotic expression and purification of the fusion protein SUMO-AHK4

2.2.1 Expression of the SUMO-AHK4 fusion protein

The constructed vector pET26b-SUMO-AHK4 was transformed into *E. coli* BL21 (DE3) by heat-transforming method. Then the transformed bacterial cells were cultured on LB solid medium containing 30 μg/mL kanamycin overnight at 37 °C. Single colony was inoculated into the LB liquid medium containing 30 μg/mL kanamycin and

cultured at 37 °C. The bacterial LB solution was diluted into the TB medium with 30 μg/mL kanamycin at a volume ratio of 1:100. Then the bacteria was cultured in TB at 37°C for about 2 hours until the OD_{600} reached 0.6. The SUMO-AHK4 protein was induced to express with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 160 rpm and at 28°C for 24 h.

2.2.2 Purification of SUMO-AHK4 Fusion Protein

The cells were collected by high-speed refrigerated centrifugation. Then the lysozyme and phosphate buffer were added together to resuspend the bacterial cells. The cells were incubated on ice for 30 min to hydrolyze the outer membranes and lysed thoroughly in an ice water bath with a sonicator for 20 min. Then they were centrifuged at 15000 g / min for 20 min. The resulting spheroplasts were recovered and applied into the Ni column. The column was washed with different concentration of imidazole in phosphate buffer, and the fractions were collected. After the pool was analyzed by SDS-PAGE, salt ions in protein were dialyzed to be removed. Then the SUMO-AHK4 fusion protein was concentrated by a ultrafiltration system, and its concentration was analyzed by BCA method.

2.3 Electrochemical workstation and detection procedure

A CHI660C electrochemical workstation (CHI Instruments, Shanghai, China) was used for electrochemical measurements with the classic three electrodes system. The modified gold electrode (diameter 3 mm) was used as a working electrode with saturated calomel electrode (SCE) as the reference electrode and platinum wire as a counter electrode. Phosphate buffer solution (PBS, pH 7.0) was used as the support

electrolyte.

A Model RDE 5 Bi-potentiostat (Princeton Applied Research, USA) and a Model 5210 Lock-in Amplifier interfaced to an EG&G 273 A Potentiostat/Galvanostat (Princeton Applied Research, USA) were used for EIS experiments. The impedance experiments were done in the supporting electrolyte 0.1 M KCl with equimolar of 5 mM redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The EIS measurements were performed at the formal potential at 0.20 V with an amplitude of 5.0 mV (rms) in a wide frequency range of 100 KHz to 0.1 Hz.

For the detection of isopentenyl adenine, the modified electrode was placed in a measuring cell with 10 mL of PBS solution, and a different concentration of isopentenyl adenine (2-iP) was added. The solution was mixed with a magnetic stirrer and then linear sweep voltammetry was performed at a sweep rate of 500 mV/s. The differential pulse voltammograms of the modified gold electrodes were recorded in a potential range of 0.2 V - 0.7 V.

2.4 Preparation of the modified electrode

The gold electrode was thoroughly polished with 0.05 μm alumina powders on the suede material and then sonicated in deionized water twice for 3 minutes each and ethanol once for 2 minutes. The surface was let dry at room temperature. Based on the current signal for 2-iP, the amount of glutaraldehyde solution, bovine serum albumin solution, and AHK4 and ferrocene carboxylic acid solution were optimized to modify the gold electrode as the following. Three μL of 2.5% (w/v) glutaraldehyde solution (GA) and three μL of 2.5% (w/v) bovine serum albumin solution (BSA) and four μL

AHK4 were mixed. ten μL of the mixture was dropped on the dry surface of the gold electrode first. Then eight μL of 0.05 mol/L ferrocene carboxylic acid solution (FCA, dissolved by acetone) was cast to the surface of the bold gold electrode. After the modified electrode was dry, it was immersed in a stirred solution containing 5 mM of EDC and NHS in 0.1M MES buffer pH 5 for 30 min to activate the COOH groups of ferrocene carboxylic acid and to form an amide bond with the amino group of BSA. The modified electrodes were thoroughly washed with PBS buffer and stored at 4°C overnight. The modified electrode was denoted as FCA-AHK4-BSA-GA/Au. The FCA-BSA-GA modified electrode was prepared similarly without protein AHK4 as the control.

2.5 Morphological characterization of the electrode by Atomic force microscopy

Atomic force microscopy (AFM) operating in tapping mode (Shimadzu SPM-9700HT) was used to characterize the morphology of the electrode in air at room temperature. The procedure for AHK4 attachment on the gold plates was the same on the gold electrode above. After the modified gold plates were dry, they were immersed in a stirred solution containing 5 μM 2-iP in pH 7 0.1M PBS for 10 min to let 2-iP bind with AHK4.

2.6 Selectivity of the FCA-AHK4 -BSA-GA modified gold electrode

The modified electrode was placed properly in a measuring cell with 10 mL of 0.1M PBS solution. Five kinds of plant hormones including isopentenyl adenine(2-iP), trans-zeatin(t-Z), jasmonic acid methyl ester(MeJA), abscisic acid (ABA) and brassinolide

(BR) were electrochemically analyzed under the experimental methods and conditions as indicated above.

2.7 The analysis of bean sprouts samples

The bean sample was ground to a powder in a clean mortar by adding an appropriate amount of liquid nitrogen. 10 mL 80 % methanol was added and mixed uniformly. After it was extracted at 4°C for 10 h, it was centrifuged at 4000 r/min for 20 min. The residues were extracted twice with 80 % methanol. The total 30 mL of the extraction was combined and evaporated to obtain the yellow coagulum by a rotary evaporator. Then 5 mL of methanol was added to dissolve the residues, and it was filtered through a 0.22 μm syringe filter and stored at 4°C in the dark. The sample was determined by the standard addition method in parallel for 3 times.

3. Results and discussion

3.1. Expression and purification of fusion protein SUMO-AHK4

The theoretical molecule weight of fusion protein SUMO-AHK4 is about 48 kDa. From Figure S1A, it can be seen that there is an obvious band between 45 kDa and 66 kDa in the samples of recombinant *Escherichia coli* cells containing pET26b-SUMO-AHK4 when compared with samples containing empty vector pET26b. The experimental results indicated that the fusion protein was successfully expressed. It was found that the protein mainly existed as an inclusion body. When the expression time was 24 h, no soluble protein was detected. However, when the expression time was extended to 48 h, the amount of expressed protein increased, and the target protein appeared in the supernatant. It indicated that the soluble amount of the fusion protein

was enhanced with longer induction time.

Figure S1B shows a SDS-PAGE analysis of the protein fractions after purification and dialysis. It was found that the impurity protein could be removed by eluting the nickel affinity chromatography column with a volume of different concentrations of imidazole buffer. A relative purity target protein was obtained as shown in lane 7 and lane 8.

3.2. Characterization of the FCA-AHK4-BSA-GA modified gold electrode with cyclic voltammetry and electrochemical impedance spectrometry.

Figure S2A compares the CV responses of a bare gold electrode with that of two modified gold electrodes in 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KCl at 100 mV /s. Quasi-reversible one-electron redox behavior of ferricyanide ion is observed on the bare gold electrode (Figure S2A a) with a peak separation (ΔEp) of 80 mV at 100 mV/ s. After the electrode is modified with AHK4-BSA-GA, an obvious decrease in the peak current and a larger ΔEp are observed (Figure S2A b). AHK4-BSA-GA composite film hinders the diffusion of ferricyanide ion toward the electrode surface due mainly to the blocking behavior of the composite protein film. For the FCA-AHK4-BSA-GA modified electrode (Figure S2A c), the peak current increases and the ΔEp becomes smaller compared with the AHK4-BSA-GA modified electrode. The decrease in ΔEp is related to the increase of electron transfer ability of FCA. The voltammograms demonstrates that FCA-AHK4-BSA-GA film is effectively immobilized on the gold electrode surface and provides the necessary receptor between the cytokinin and the electrode surface.

The impedance changes of the electrode surface during the modification process can be investigated by EIS. Figure S2 B shows the results of EIS for the bare gold electrode and the two modified electrodes in the presence of equimolar $Fe(CN)_6^{3-/4-}$. The EIS includes a semicircular part and a linear part. The semicircular part at higher frequencies and the linear part at lower frequencies correspond to the electron transfer limited process and the diffusion process, respectively. The Randles circuit (inset of Figure S2 B) is chosen to fit the obtained impedance data. The values of the simulation are listed in Table S1, and the fit plot is displayed in Figure S2 C. The Rct for the bare gold electrode in $Fe(CN)_6^{3-/4-}$ is 148 Ω. The Rct for the AHK4-BSA-GA modified electrode is increased to 9496 Ω. The results for FCA-AHK4-BSA-GA modified electrodes are shown in Figure S2B c. Compared with the AHK4-BSA-GA modified electrode, Rct decreases to 2683 Ω. These results indicatethat the ferrocene groups are successfully coupled with BSA and mediate the redox of ferricyanide ion.

3.3 The morphological characterization of the electrode by atomic force microscopy.

Fig. S3 A and B shows the AFM morphology of AHK4 on the gold electrode in absence and presence of 2-iP, respectively. As can be seen, the gold surface is covered by an uneven compact film of globular particles with widths ranging from 40-50 nm and heights 10-30 nm(Fig. S3 A). After being bound with 2-iP, AHK4 on the surface of the gold appears to aggregate and more of the globular particles are presented (Fig. S3 B). However, as the gold plate surface is almost totally covered, it is difficult to characterize the dimerization of AHK4.

3.4 The electrochemistry characteristic of the FCA-AHK4-BSA-GA modified gold electrode

Figure 1 displays the cyclic voltammograms of gold electrodes coated with different films in PBS buffer at a scan rate of 500 mV/s in the potential range of 0.2 V - 0.7 V. The FCA-BSA-GA modified gold electrode (curve a) distinctly shows one pair of waves with anodic peak potential (Epa) at 0.48 V and cathodic peak potential (Epc) at 0.42 V. The peak potential separation (Δ Ep) is about 60 mV. It indicates that the coupled ferrocene does not affect its electrochemical reversibility. The FCA-AHK4-BSA-GA modified gold electrode (curve b) shows the CV with anodic peak potential (Epa) at 0.5 V and cathodic peak potential (Epc) at 0.4 V. The peak potential separation (Δ Ep) is about 100 mV. Compared with curve a, the ΔEp has increased and the redox peak current has decreased. The reason is that the fixation of the AHK4 protein on the surface of the electrode increases the film thickness of the electrode surface and blocks electron transfer between the ferrocene and the electrode. For the control, the AHK4-BSA-GA modified gold electrode has been investigated and no peaks are observed (curve c).

Figure S3 shows the cyclic voltammograms of the FCA-AHK4-BSA-GA modified gold electrode at different scan rates in 0.1 M PBS (pH 7.4) buffer solution. As the scan rate increases, the redox peak current increases, indicating that the electron transfer rate of the FCA bonded in the AHK4-BSA-GA composite film is faster and maintains good electrochemical activity. The peak current is proportional to the scan rate in the range of 100 -1000 mV·/ s (Figure S3), and the peak potential does not change with different

sweep rates, indicating that the electrode reaction process is a surface control process. The relationship between the redox peak current and the different sweep rates is shown in the inset in Figure S3 with the regression equation of oxidation peak of y = 21.21x + 6.06(R^2 = 0.997) and the regression equation of reduction peak of y = -16.02 x - 3.68 (R^2 = 0.994).

For the diffusionless electrochemical system the ks of ferrocenecarboxylic acid (n = 1) was determined to be 38.95 / s according to the equation for diffusionless CV with $n\Delta Ep < 200mV$:

$$ks = nF\nu / (RTm^{-1}) \quad (1)$$

the transfer coefficient α was taken as 0.5, m for ferrocenecarboxylic acid in this work is 0.5 according to reference [20]. The symbols R, T and F have their usual electrochemical meanings.

3.5 Electrochemical detection of isopentenyl adenine by the FCA-AHK4-BSA-GA modified gold electrode

The continuous addition of 2-iP caused the redox peak current of ferrocene to be gradually reduced, and the redox peak potential remained simultaneously unchanged. This indicated that the binding of 2-iP and AHK4 CHASE domain led to the dimerization of AHK4[19], which hindered the electron transfer between the ferrocene and the electrode surface. Therefore, the detection of 2-iP can be achieved by recording the change of the ferrocene redox peak current. The electrochemical response of 300 nM 2-iP at the FCA-AHK4-BSA-GA modified gold electrode in 0.1 M phosphate buffer with different pH values was studied using differential pulse voltammetry (DPV).

The results showed that the change value of ferrocene oxidation peak current increased along with a pH change from 3.0 to 7.0. However, the peak currents change began to decrease as the pH further increased from 8.0 to 10.0. Thus, a phosphate buffer with a pH of 7.0 was used to determine 2-iP.

Differential pulse voltammetry of FCA-AHK4-BSA-GA modified gold electrode was carried out by adding 0 - 1.2 μM 2-iP, and 3 parallel tests were performed for each concentration. The results are shown in Figure 2A. The relationship of the changed value of ferrocene oxidation peak current with the concentration of 2-iP is shown in Figure 2B. It can be seen from Figure 2B that the change in the oxidation peak current gradually decreases as the concentration of 2-iP increases. When the concentration of 2-iP increases from 0 to 400 nM, the decrease in oxidation peak current is significant. In the range of 500 -1200 nM, the decrease in oxidation peak current tends to be stable. This phenomenon is characteristic of the enzymatic reaction. As displayed in the Figure 2C, when the concentration of 2-iP is in the range of 50-400 nM, the relationship between the decrease of oxidation peak current and the concentration of 2-iP is linear. The linear regression equation is: $\Delta I = 0.0086\ c + 0.732$ ($R^2 = 0.993$, i_p in μA, c in nM) , the limit of detection (LOD) is 1.5 nM (S/N=3) and the sensitivity is 0.03 μA $nM^{-1}cm^{-2}$. The relative standard deviation (RSD) of 3.5% for 200 nM 2-iP (n = 7) shows good reproducibility. The linearity and LOD for 2-iP by this electrochemical biosensor were compared with other biosensor methods, such as anti-iPA IgG based immunosensor [11], cytokinin dehydrogenase based electrochemical biosensor [12] and mutated *sln1Δ Saccharomyces cerevisiae* biosensor [13]. The results are listed in

Table 1. It indicates that the presented method has the advantages of wide linearity range and, simultaneously, very low detection limitfor 2-iP. Generally, the endogenous cytokinin concentration is about 0.05 nmol/g of fresh weight in plants [21]. Compared with the sensitivity result of this electrochemical biosensor, it indicates this biosensor could be applied to detect cytokinins in plant samples.

The stability of FCA-AHK4-BSA-GA modified gold electrode was evaluated by measuring the current responses at a fixed 2-iP concentration of 200 nM in a standard working solution over a period of 3 weeks. The FCA-AHK4-BSA-GA modified gold electrode was used daily and stored in the 4°C refrigerator. The experimental results indicated that the current responses deviated only 5.8%, revealing that FCA-AHK4-BSA-GA modified gold electrode fabricated by this method possesses long-term stability. The stability of FCA-AHK4-BSA-GA modified gold electrode was also examined in real bean sprout samples. The current response in seven tests showed a standard deviation of 10.8%. Compared with the standard working solution, the high deviation attributes to the contamination or adsorption from the real samples on the surface of the electrode.

3.6 Selectivity of the FCA-AHK4 -BSA-GA modified gold electrode

The selectivity of the modified electrode for different hormones is another important aspect of this research. Whether the detection method has a high selectivity is quite crucial. In this research, (trans-zatin)t-Z, methyl jasmonate(MeJA), abscisic acid(ABA), and brassinolide(BR) were used as the interference hormones. The experimental

conditions and the final concentration of all the three hormones are the same as the above experiments. The results are shown in Figure 4. Being similar to 2-ip, the addition of t-Z caused the obvious change of the FCA redox peak current. It is consistent with the report that AHK4 in *Arabidopsis thaliana* can bind with trans-zeatin and isopentenyl adenine [22]. However, the addition of MeJA, ABA and BR did not significantly change the FCA redox peak current (Figure 3a, 3b, 3c, respectively), indicating that the AHK4 CHASE domain could not bind to these interfering hormones.

Keeping the concentration of 2-iP, t-Z, MeJA, ABA and BR at 50, 400 and 500 nM, the corresponding ferrocene oxidation peak current change is plotted, as shown in Figure 4. In contrast to 2-iP t-Z, MeJA, and ABA, the addition of BR causes a slight increase of ferrocene. It may be that BR can slightly destroy the crosslinking of GA and BSA, but the real reason needs further study. It can be seen from Figure 4 that the current change led by t-Z and 2-iP is slightly larger than that of the other three hormones, being 7.55 times that of ABA and 2.65 times that of MeJA. When the concentration is maintained at 400 nM, the current difference between t-Z and 2-iP increases rapidly, and the other hormones have weak electrochemical signals. The current difference caused by 2-ip is 20 times and 7.5 times that of ABA and MeJA, respectively. When the concentration increases to 500 nM, the electrochemical signals of the four hormones increase less. The reason is that the binding reaction has reached to saturation condition, and the current change led by 2-ip is 6.98 and 3.5 times that of ABA and MeJA, respectively. The ferrocene oxidation peak current change caused by 2-ip is much larger than that of the other hormones. AHK4 CHASE domain is the receptor of cytokinin.

Therefore it does not bind with other electroactive species present in a sample. The selectivity of AHK4 CHASE domain and the relative low working potentials lead to the strong anti-interference ability of the electrochemical biosensor.

3.7 Analysis of bean sprout samples

The FCA-AHK4-BSA-GA modified gold electrode was used to detect the CK content in the roots of fresh mung bean sprouts. The experimental data was recorded by repeating experiments for 3 times to reduce errors. The concentration of CK in the tested bean sprout roots was calculated to be 107 ± 1.56 nM based on the calibration curve obtained with PBS, and the results are listed in Table 2. The recovery and RSD are excellent, indicating that this method can be used in the practical sample analysis. The amount of cytokinins present in the root of the fresh mung bean sprouts was calculated to be 105 ng/g.

4. Conclusion

In this study, the pET26b-*SUMO-AHK4* prokaryotic vector was constructed for the expression of *Arabidopsis* histidine kinases 4 chase domain in *Escherichia coli*. The fusion of SUMO was introduced to increase the solubility of AHK4 in prokaryotic expression. After the protein samples were processed, the supernatant protein samples, the whole cell lysis samples, and the protein samples in the debris were investigated by polyacrylamide gel electrophoresis analysis. The SDS-PAGE results showed that the target protein existed in the supernatant when the cells were cultured at 28 °C for 48 h with 0.5 mM IPTG induction. The supernatant protein samples were purified with Ni

column, and the salt ions in the protein sample were removed by dialysis. The fusion protein SUMO-AHK4 was obtained with high concentration by ultrafiltration. The AHK4 could specifically recognize and bind to CK. However, it could not produce an electrochemical signal. Thus, ferrocene was chosen as the electron mediator to monitor the binding interaction of AHK4 with cytokinins by electrochemical method. Using the strong cross-linking ability of BSA and glutaraldehyde, the AHK4 was immobilized on the Au electrode surface, and the biosensor successfully achieved electrochemical detection of cytokinins. The biosensor had strong specificity for cytokinins and could avoid interference from other plant hormones. Its application of real samples demonstrated that the developed biosensor could be used to detect cytokinin with accuracy, rapidity, and specificity. The limitations of the method lie in that the loading amount of AHK4 CHASE domain on the electrode is limited, which leads to the limited detection concentration range of cytokinins.

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Table 1. The linearity and LOD for cytokinins by various biosensor method.

Analyte	Linearity	LOD	Ref.
N6-(Δ2-isopentenyl) adenosine	5-300 μg/mL	/	[11]
isopentenyl adenosine	10 nM to 10 μM	3.9 nM	[12]
isopentenyl adenosine	/	0.22 nM	[13]
isopentenyl adenosine	50-400 nM	1.5 nM	This work

Table 2 Analysis of cytokinin content in roots of bean sprouts (n =3)

Sample	C_{ck}/nM				RSD / %	Recovery / %
	Found before adding	Added	Expected	Found after adding		
1	107 ± 1.56	50	157	162 ± 1.38	4.5	103.2
2	107 ± 1.56	100	207	210 ± 1.16	3.6	101.4
3	107 ± 1.56	200	307	315 ± 1.32	2.5	102.6

Figure Legends:

Schematic 1. Schematic illustration of the immobilization of AHK4 and ferrocenecarboxylic acid with BSA and glutaraldehyde (above); The principle of the cytokinins detection of the electrochemical biosensor(below).

Figure 1. Cyclic voltammograms of FCA-BSA-GA (a), FCA-AHK4-BSA-GA (b) and AHK4-BSA-GA (c) modified Au electrode in 0.1 M PBS (pH 7.0) at scan rate of 500 mV/s

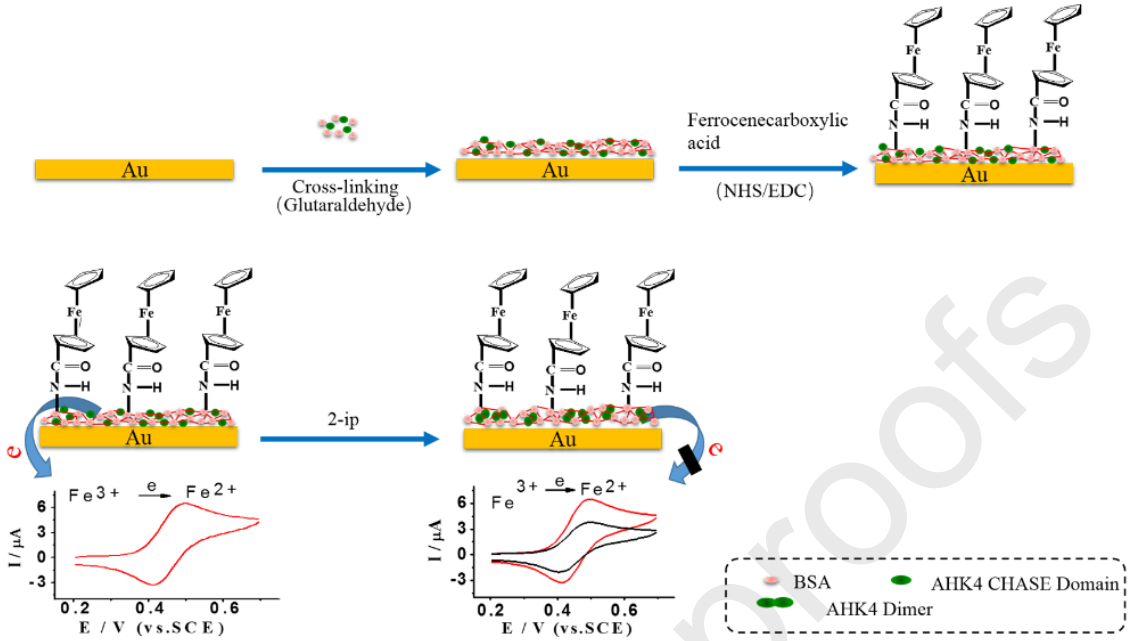
Figure 2A Differential pulse voltammograms of FCA-AHK4-BSA-GA modified Au electrode in presence of different concentration of 2-iP (from outer to inner: 0, 50, 100, 200, 300, 400, 500, 600, 800, 1000, 1200 nM, respectively) in 0.1 M PBS at scan rate of 500 mV/s.

Figure 2B The relationship between the concentration of 2-ip and the change of the oxidative peak current of FCA.

Figure 2C: The linear relationship between the concentration of 2-ip and the change of the oxidative peak of FCA with the linear regression equation of $\Delta I = 0.0086\ c + 0.732$ ($R^2 = 0.993$, i_p in μA, c in nM).

Figure 3 Cyclic voltammograms of FCA-AHK4-BSA-GA modified Au electrode at different concentration of ABA(a), BR(b) and MeJA(c) (0, 50, 100, 200, 300, 400, 500, 600, 800, 1000 nM from curve 1-11, respectively) in 0.1M PBS at scan rate of 500 mV/s.

Figure 4 Selectivity of FCA-AHK4 -BSA-GA modified Au electrode to 2-ip vs.t-Z, ABA, MeJA and BR.



Schematic 1.

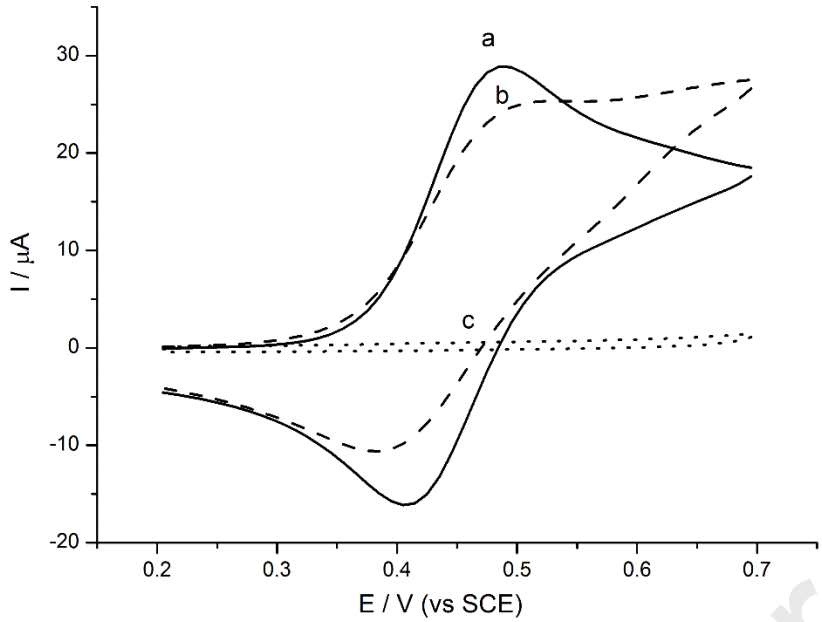


Figure 1.

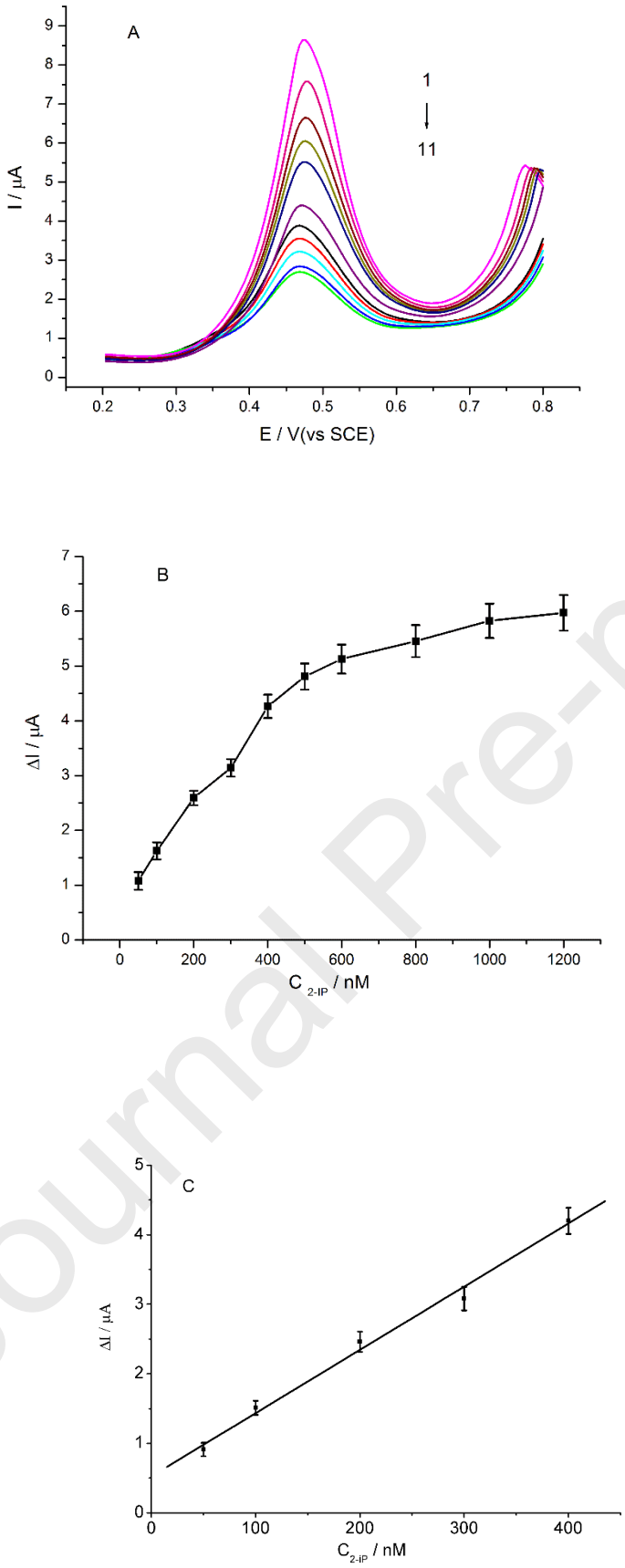


Figure 2.

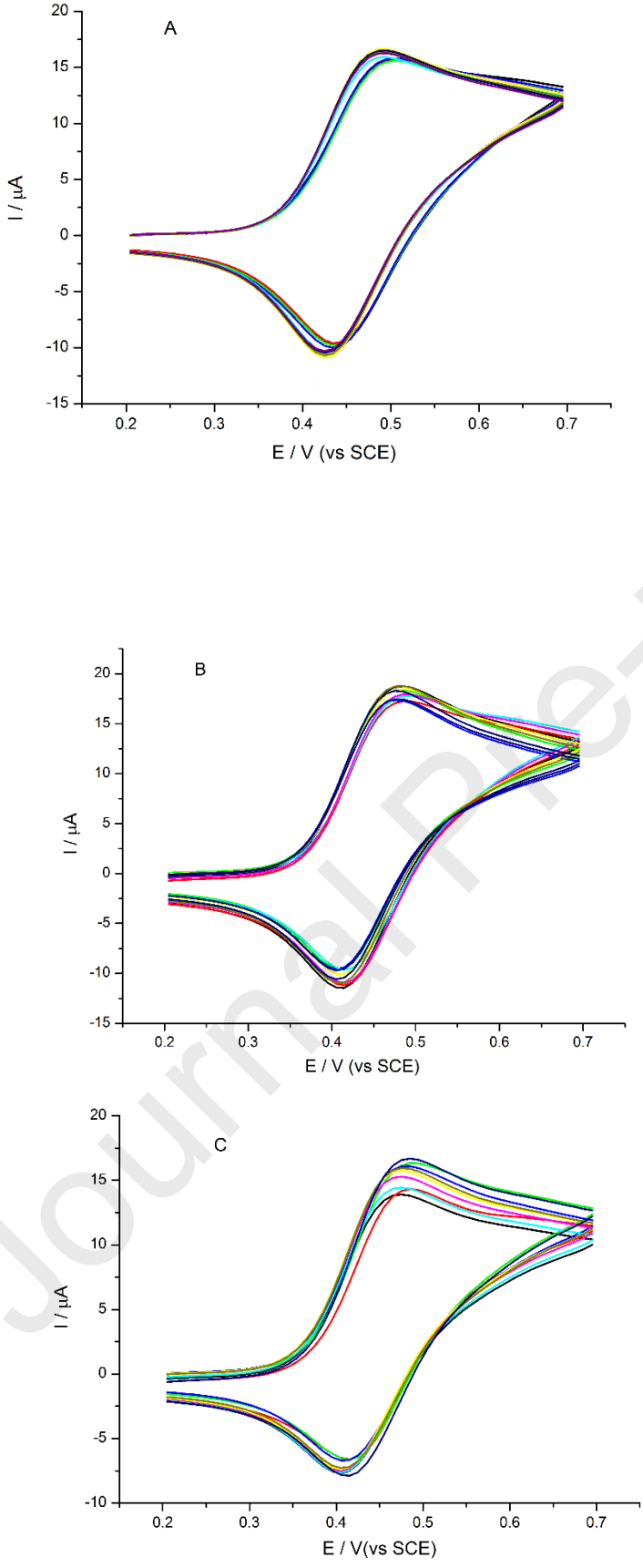


Figure 3.

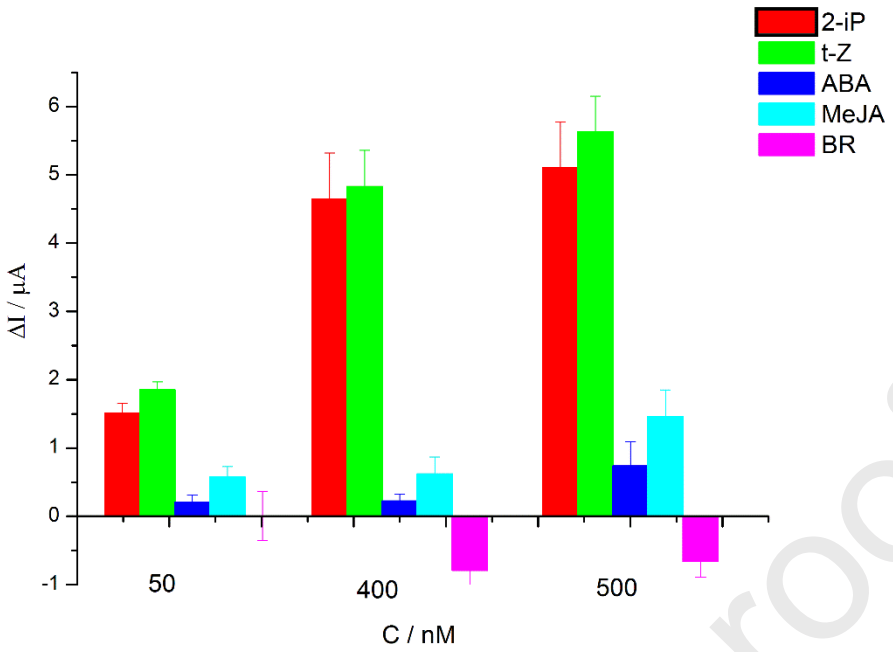


Figure 4.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "Electrochemical biosensor for cytokinins based on the CHASE domain of *Arabidopsis* histidine kinases 4".

Highlights

- AHK4 CHASE domain was used to construct an electrochemical cytokinin biosensor.

- Cytokinin was detected by recording the change in ferrocene redox peak current.
- The biosensor avoided interference of other plant hormones.