Prussian Blue acts as a mediator in a reagentless cytokinin biosensor

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ABSTRACT

An electrochemical biosensor for detection of the plant hormone cytokinin is introduced. Cytokinin homeostasis in tissues of many lower and higher plants is controlled largely by the activity of cytokinin dehydrogenase (CKX, EC 1.5.99.12) that catalyzes an irreversible cleavage of N^6-side chain of cytokinins. Expression of *Arabidopsis thaliana* CKX2 from *Pichia pastoris* was used to prepare purified AtCKX2 as the basis of the cytokinin biosensor. Prussian Blue was electrodeposited on Pt microelectrodes prior to deposition of the enzyme in a sol-gel matrix. The biosensor gave amperometric responses to several cytokinins. These responses depended on the presence of both the enzyme and the Prussian Blue. Thus Prussian Blue must act as an electron mediator between the FAD centre in CKX2 and the Pt surface.

Keywords: phytohormone, electrochemistry, oxidase, dehydrogenase, quantitation, electrode

Abbreviations:

AtCKX2, *Arabidopsis thaliana* cytokinin dehydrogenase isoform 2

PrB, Prussian Blue, $K_3[Fe(CN)_6]$

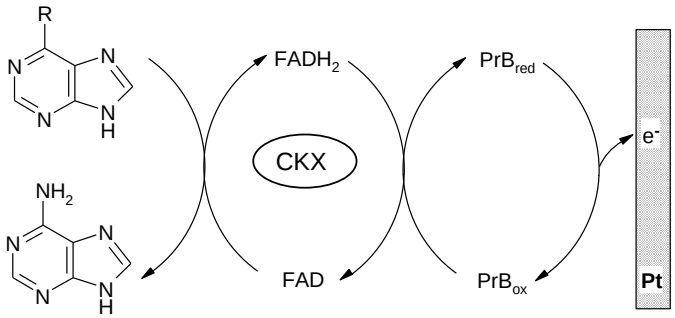
1. INTRODUCTION

If we are to understand the timing, direction and amplitude of plant responses to hormonal stimuli we need to capture quantitative information about each hormone from living, responding tissues. Most traditional phytohormone detection methods have tended to be post-event, time fractionated measurements such as by gas chromatography [1,2], capillary electrophoresis [3], HPLC [4], ELISA [5,6] and radioimmunoassay [7,8]. Moreover many require elaborate sample work-up, radioactive chemicals and are time-consuming. Other assays like genetic biosensors using promoter-reporter constructs, though very helpful, remain largely qualitative and post-event with little or no temporal resolution. Therefore, exploring new, simple, low cost methods for real-time hormonal quantification is of high interest.

Good biosensors offer operational simplicity, low expense of fabrication and high selectivity. Many are single-use, single record devices, but there is a developing interest in real time detection. The first electrochemical biosensor was introduced nearly fifty years ago [9] and since then quantitative biosensors have become widely used in numerous areas of biology and medicine. The most common enzymes used for electrochemical biosensors include peroxidases and alkaline phosphatase [10]. Typically, an electrochemical biosensor contains a redox enzyme specific for the analyte of interest. The redox centre is recharged by electron-carrying intermediates which are, in turn, regenerated by oxidation or reduction at the electrode surface where a current can be measured. Alternative, affinity-based sensors have also been developed for particular analytes, such as antibody- or oligonucleotide-based sensors [11]. Naturally-occurring selectivities found in enzymes also remain attractive qualities for sensor development. To keep enzymes highly active close to the electrode surface different immobilizing techniques are applied including nafion membranes [12], polypyrrole films [13], cross-linking with chitosan [14-16] or different sol-gel techniques [17-19].

We decided to prepare a microbiosensor for detection of the important plant hormones, cytokinins. Cytokinins promote cell division and serve as signaling molecules [20]. In 2003 Li and co-workers [21] fabricated an amperometric immunosensor for one cytokinin, N^6-(Δ^2-isopentenyl) adenosine (iPR). The sensor utilized horseradish peroxidase entrapped in a polypyrrole/poly(*m*-phenylenediamine) multilayer with $K_4Fe(CN)_6$ on a glassy carbon electrode. On this modified surface staphylococcal protein A was adsorbed and this, in turn, was used to bind anti-iPR IgG. The assay was then a competitive immunoassay with the sample containing free iPR and an aliquot of iPR-labelled glucose oxidase. In the presence of glucose, any bound glucose oxidase produced H_2O_2, which was then reduced by peroxidise and the regeneration of the ferrocyanide mediator was recorded amperometrically. Apart from the complexity of creating multilayered electrodes, there was a need for considerable sample clean-up and concentration before measurement and the electrode was not designed for real-time analyses.

In order to develop a more versatile biosensor for detection of a range of cytokinins cytokinin dehydrogenase (CKX, EC 1.5.99.12) has been used. CKX catalyzes irreversible degradation of these phytohormones by cleaving the N^6-side chain of cytokinins to form adenine and a side-chain-derived aldehyde [22]. CKX is a flavoprotein with covalently bound FAD [23]. Importantly, it prefers electron acceptors other than molecular oxygen as the primary electron acceptor [24]. Thus, no H_2O_2 is produced in the catalytic cycle, making it necessary to find an alternative modality for electrical coupling of the sensor enzyme to the electrode.



Scheme 1. Mechanism of the cytokinin biosensor.

We chose the most abundant CKX enzyme in *Arabidopsis thaliana*, AtCKX2. This isoform has been expressed heterologously in *Sacharomyces cerevisiae* and well characterized [25]. However to obtain more efficient expression we chose to prepare AtCKX2 in a fermentor using *Pichia pastoris* constitutive expression system. For biosensor fabrication the purified enzyme was immobilized in sol-gel film on the surface of a Prussian Blue-modified platinum electrode. The principle of cytokinin detection is represented in scheme 1 which shows the redox reactions between CKX, cofactor FAD, Prussian Blue and the electrode. The results show biosensors with a fast response, fair sensitivity and selectivity and, notably, the activity of PrB as a direct electron mediator in this configuration to give a reagentless biosensor.

2. EXPERIMENTAL

Construction of expression vector

RNA was isolated from the leaves of transgenic tobacco overexpressing *AtCKX2* [26] using Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was carried out with RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania). Specific primers were designed (pGAP2-fw: 5'-GGAATTCCATATGATTAAAATTGATTTACCTAAAT-3', pGAP2-rev: 5'-GCTCTAGATCAAAAGATGTCTTGCCC-3') so that resulting amplicons would be

missing an *N*-terminal fragment of 66 nucleotides predicted to be a signal sequence (SignalP 3.0 Server, [27]). A substitute signal peptide would be added from the expression vector. The *AtCKX2* gene was amplified with Phusion DNA Polymerase (Finnzymes, Espoo, Finland). A TGradient Thermocycler (Biometra, Goettingen, Germany) was programmed as follows: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 60 s at 55 °C, 30 s at 72 °C; and terminated by 10 min at 72 °C. The gene was further cloned into the pGAPZαA$(His)_{10}$ shuttle vector, carrying an additional *N*-terminal His-tag sequence (preparation described in [28]). Plasmid constructs were transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV and transformants were selected on the basis of zeocin resistance. *Pichia* transformation and subsequent selection of transformants was done according to the pGAPZαA manual (Invitrogen).

Preparation of pPIC9K vector under control of constitutive GAP promoter

The plasmid construct pGAPZαA$(His)_{10}$::AtCKX2 and pPIC9K vector (Invitrogen) were subjected to partial digestion with *BglII* (Takara) and *BshTI* (Fermentas). Digestion products of the expected size (approx. 8 kb for pPIC9K and 2.4 kb for pGAPZαA$(His)_{10}$::AtCKX2) were ligated and transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV. Selected plasmid constructs pPIC9K::AtCKX2 were linearized with *AvrII* (NEB) before integration into *Pichia pastoris* SMD1168 (Invitrogen) genome. His^+ transformants were grown on MD plates (1.34% yeast nitrogen base without amino acids (Difco™, Detroit, MI, USA), $4 \cdot 10^{-5}$% biotin, 2% D-glucose, 2% agar). Screening for multicopy inserts was carried on YPD plates (1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar) containing various concentrations (from 0.5 to 3 mg mL^{-1}) of Geneticin® (G-418 sulfate) (Calbiochem, Merck, Darmstadt, Germany). Selected transformants were picked and grown for one day in 2 mL of YPD medium (2% peptone, 1% yeast extract, 2% glucose) with appropriate concentration of Geneticin at 30 ºC and shaking at 230 rpm. Subsequently, the pPIC9K::AtCKX2 transformants were transferred into 50 ml of YPD medium without antibiotic buffered to pH 7.2 with 0.1 M potassium phosphate buffer. After 48 hours cultivation at 28 ºC with 230 rpm shaking, yeast cells were removed by centrifugation at 5000*g* for 10 min and CKX activity measured in the cell-free medium [28].

Estimation of AtCKX2 gene copy number

To establish how many copies of *AtCKX2* gene was integrated into pPIC9K vector a real-time PCR experiment was designed. Yeast genomic DNA isolated with the use of MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and digested with *NcoI* (Fermentas) served as a template. Primers for *ckx2* and *aox1* genes were designed using Primer

Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The real-time reaction mixtures contained diluted DNA samples, POWER SYBR Green PCR Master Mix and 300 nM of each primer. All DNA samples were run in four technical replicates on the StepOne-Plus Real-Time PCR System using a default program (Applied Biosystems). Cycle threshold values were normalized with respect to the alcohol oxidase 1 gene.

High cell density fermentation and protein purification

Fermentation experiments were performed in a 15 litre, R'ALF Plus Duet fermenter (Bioengineering AG, Wald, Switzerland) with a 10 L working volume and control modules for pH, temperature and dissolved oxygen. The inoculum was grown in flasks at 30 °C with orbital shaking at 230 rpm, in 200 ml of medium containing 13.4 g L^{-1} of yeast nitrogen base without amino acids (Difco^{TM}), 0.1 M potassium phosphate buffer (pH 7.2) and 2% D-glucose. After 24 - 40 h cultivation, until the cell density reached an OD_{600} of >10, the cells from the flask were used to inoculate afermenter containing the same medium but at pH 6.5 with 1% glycerol as a carbon source and 0.02% defoamer KFO 673 (Emerald Performance Materials, Cheyenne, WY, USA). The process temperature was maintained at 30 °C and pH was controlled by the addition of 5 M KOH. The pH was measured with a Mettler Toledo pH electrode 405-DPAS-SC-K8S/325 (Urdorf, Switzerland). The impeller speed was set to 800 rpm and the air flow was 300 L h^{-1}. The oxygen concentration was monitored with a Mettler Toledo InPro® 6950/6900 O_2 Sensor. Fed-batch fermentation was initiated after about 40 h, when a dissolved oxygen spike appeared indicating the depletion of the initial glycerol. The fed-batch medium consisted of (per litre of deionized water): 500 g D-glucose, 2.4 mg D-biotin, 0.2% defoamer and 4 ml trace salts solution (per litre of deionized water: H_3BO_3 0.02 g, $CuSO_4 \cdot 5H_2O$ 2 g, KI 0.1 g, $MnSO_4 \cdot H_2O$ 3 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, $ZnSO_4 \cdot 7H_2O$ 17.8 g, $CoCl_2$ 0.92 g) and it was fed at a rate of 0.2 ml/min. In order to monitor culture density and CKX activity samples were taken over time. The fermentation process was stopped after about 50 hours of feeding and yeast cells were removed by centrifugation at 4600*g* for 40 min at 4 °C. The cell-free medium was concentrated to about 60 ml by ultrafiltration in a VivaFlow 50 system (Sartorius Stadius Biotech GmbH, Goettingen, Germany) with 30 kDa membrane cut-off. Ultrafiltration was repeated three times to exchange the buffer to 20 mM Tris/HCl (pH 8.2). The concentrated AtCKX2 was loaded on a High Q hydrophobic column (Bio-Rad; 18 x 1.4 cm) connected to BioLogic LP chromatograph equipped with UV and conductivity detector (Bio-Rad). The column was washed with a linear gradient of KCl (up to 1 M). Fractions showing enzyme activity were pooled and concentrated to 2 ml using the ultrafiltration device with 30 kDa membrane cut-off (Millipore) and the buffer was exchanged for 50 mM potassium phosphate (pH 7.4) containing 0.5 M NaCl. CKX samples were applied to a Ni Sepharose HP (GE Healthcare; 9.5 x 1 cm) equilibrated with the same buffer. His-tagged proteins were eluted from the column by a gradient of imidazole to 50 mM. Active fractions were collected, concentrated by

ultrafiltration with buffer exchange to 20 mM Tris/HCl (pH 8.0) and stored at -20 °C.

Protein content in enzyme samples was measured according to Bradford [29] with bovine serum albumin as a standard.

Fed-batch production of recombinant AtCKX2

In order to prepare AtCKX2 for expression in *Pichia* and secretion into growth medium the native secretion signal of the protein was replaced by the 85 amino acid α-factor prepro peptide from *S. cerevisiae*. This signal peptide has proven to be a potent and easily removed secretion signal [30,31] and resulted in efficient accumulation of AtCKX2 protein in the growth medium. An auxotrophic and protease-deficient *Pichia* strain SMD1168 (*his4*, *pep4*) was chosen to reduce degradation of recombinant proteins in high cell density culture in fermentor [32]. The expression cassette of the new *HIS4*-based vector contained a constitutive GAP promoter, a polyhistidine tag and *AtCKX2* gene. *Pichia* transformant demonstrating highest activity was selected on 1.75 mg mL^{-1} of Geneticin® and was shown to have 4 copies of the *AtCKX2* gene. It was selected for large scale expression in a fermenter that was carried in fed-batch mode with 50% glucose containing biotin, defoamer and trace salts. Cell yield was between 70 - 180 g L^{-1} dry cell weight. The CKX activity began to increase shortly after commencing feeding and continued to grow till the end of the fermentation process. Purification of AtCKX2 by means of liquid chromatography resulted in 80+% pure protein (10-fold purification, 35% recovery) with an activity of 293 nkat mg^{-1} with 250 μM iP at pH 6.5.

Reagents and instrumentation for biosensor preparation

All inorganic salts were purchased at highest purity. Cytokinins and silanes were commercially obtained from Sigma–Aldrich. Fresh $K_3Fe(CN)_6$ and $FeCl_3$ solutions were prepared just before use. Potassium chloride (0.1 M, pH 5.0) was used as electrolyte in amperometric detection experiments. Each aqueous solution was prepared with 18.2 MΩ deionized water.

For cyclic voltammetry and amperometric experiments a CHI 660B workstation was used. Sol–gel electrodeposition was carried using a PG580 potentiostat–galvanostat (Uniscan instruments). A three electrode cell equipped with a platinum foil counter electrode and a Ag/AgCl (saturated KCl) reference electrode was used. In all experiments platinum microelectrodes (obtained from Sycopel International Ltd.; with a diameter of 50 μm, a length of 0.5 mm and a surface area of 7.85×10^{-4} cm^2) were employed as the working electrode. Amperometric measurements were carried in a flow system at room temperature.

Preparation of biosensors

The Pt microelectrode was etched in a saturated NaCl solution and coated with Prussian Blue (PrB) in a solution containing 4 mM $K_3[Fe(CN)_6]$ and 4 mM $FeCl_3$. The supporting electrolyte was 0.1 M KCl with HCl. For electrodeposition a potential of 0.4 V was applied for 360 s, followed by cycling over the potential range from 0 to 0.5 V at the scan rate of 50 mV s^{-1} until the cyclic voltammetry (CV) curve was stable.

A silicate layer was enzymatically deposited on top of the PrB layer by galvanostatic electrodeposition using methods previously described [34,36]. A smooth, transparent silica layer was formed on the surface of Pt microelectrode. To ensure uniformity of the PrB coating after gel film deposition, an oxidation potential of 0.6 V was applied for 60 s. Afterwards, the electrode was cyclic scanned again from 0 to 0.5 V at 50 mV s^{-1} until the CV curve was stable. The cytokinin biosensors were stored in 0.1 M KCl pH 5.0 at 4 °C, ready for use.

3. RESULTS

Preparation of cytokinin biosensor

The enzyme cytokinin dehydrogenase degrades cytokinins very efficiently in the presence of electron acceptors (other than oxygen) that withdraw two electrons from the enzyme's flavin cofactor [25]. Therefore, the use of CKX for biosensor preparation requires an exogenous electron mediator. PrB has been proved to act as an "artificial peroxidase" in glucose biosensors [12,33], although it is poisoned by Na^+ ions. As plant sap does do not contain high concentrations of Na^+, PrB is a promising candidate surface-bound mediator for the CKX reaction on the electrode.

Microelectrodes were modified with PrB by electrodeposition, optimizing the reaction time to obtain a thick and uniform layer that was further stabilized by cyclic scanning in 0.1 M KCl. Subsequently, a sol-gel film was formed with CKX incorporated according to the method described previously [34]. The gel layer is characterized by high porosity that allows diffusion of small molecules throughout the sol-gel film thus enabling fast responses to changing analyte concentrations [34, 35].

The CVs of gel coated microelectrodes in 0.1 M KCl (pH 5.0) demonstrate lower currents than the PrB modified electrodes and the peak currents are slightly shifted, each to slightly lower potentials (Figure 1). This suggests that the gel deposition has degraded the PrB layer somewhat. Once formed, the microelectrodes were tested for optimal operating potential. Cyclic voltammograms of freshly prepared microelectrodes were run in a perfusion system maintaining 50 μM iP as substrate. The response was recorded within the potential range from 150 mV to 310 mV (Figure 2). The highest response was observed on the reducing cycle at 180 mV (*vs*. Ag/AgCl, saturated KCl) and this was

chosen for further analyses. Comparison of Figure 2 with data from other PrB-based electrodes indicates that performance is context-specific with examples both of response currents rising with operating potential [36, Yin] and declining past an optimum [this work and 37,38].

Performance of cytokinin biosensor

In order to determine the dose-response relationship of the biosensor, concentrations of iP were flowed across the electrode. The response clearly increases with iP concentration from 5 μM to 75 μM in 0.1 M KCl, pH 5.0. The corresponding calibration plot (Figure 3) demonstrates a linear dependence within that concentration range with a limit of detection of about 5 μM. The regression equation was I (μA cm^{-2}) = 0.0361C (μM) + 1.2294 and R^2=0.995.

Since iP is one of the most abundant cytokinins in plants it was used in all experiments as our working standard. However in order to verify the sensitivity of prepared biosensors to different cytokinins 25 μM iPR (isopentenyladenine riboside; aliphatic side-chain with ribosylated purine), *t*-Z (*trans*-zeatin; hydroxylated aliphatic side-chain), ZR (mixture of *cis*- and *trans*-isomers of zeatin riboside) and K (kinetin, aromatic side-chain) were each prepared in 0.1 M KCl, pH 5.0. Representative response curves from both the null electrode (with no gel-trapped enzyme) and resulting biosensor (Figure 4) illustrate selectivity of the cytokinin biosensor. The response to iP was slightly greater than for the other cytokinins, which each gave similar signals. The null sensor gave no response to cytokinins in the same system and under the same conditions.

The response time of the biosensor was rapid, showing immediate rises in current on addition of substrates and reached a steady value within another 20 s – 30 s (Figure 4) which then persisted. Perfusion times in the experiment were 120 s. The signal also ceased immediately on withdrawal of the substrate, cytokinin.

When not in use, the cytokinin biosensors were stored in 0.1 M KCl pH 5.0 at 4 °C. No decrease of the initial response of the enzyme electrode to 50 μM iP was observed after 5-7 days of storage.

4. DISCUSSION

Many of the most suitable electrochemical sensor enzymes are dioxygenases, or are coupled to dioxygenases, because they generate H_2O_2 which can be detected readily on noble metal electrodes. Unfortunately, these surfaces are not selective for peroxide under oxygen and many workers have sought alternatives to improve specificity. Prussian Blue has been exploited widely as an ‘artificial peroxidase’ on electrochemical biosensors [12,38] and shown to offer many advantages over electroreduction of peroxide directly on the electrode surface at low operating potentials where non-

specific interferences are unlikely to contribute to any signal. However, as Na^+ ions do not fit readily into the lattice structure of PrB they poison PrB and this reduces the viability of this mediator in many animal and clinical sensing situations. The analogue Ruthenium Purple, which tolerates the presence of Na^+ has proved successful in these contexts, for example [39]. However PrB is suitable for use in plants where the extracellular concentration of Na^+ is very low. Our use of PrB in this context is rather novel as we are not employing it as an artificial peroxidase as CKX does utilize O_2 as an electron acceptor to produce H_2O_2. Instead, PrB must directly interact with the FAD redox centre of the enzyme.

The cytokinins are purine-based phytohormones all carrying N^6-side chains. A family of enzymes catalyzes the irreversible cleavage of these N^6-side chains from CKs, the CKXs. The CKXs are flavoproteins classified as cytokinin dehydrogenases (EC 1.5.99.12) and they are of interest in that, although originally described as oxgenases, molecular oxygen is found to be a very poor substrate [40-42]. Instead, *in planta*, it is likely that quinones act as electron mediators. In vitro, the CKXs were tested to establish that alternative electron transport intermediates were also active [43] and, in this work electrodeposited PrB has been shown to act as a satisfactory mediator for microbiosensors. This demonstration raises the prospect of reagentless biosensors for CKs. For CK biosensors to be valuable *in vivo*, some efficiency improvements still need to be made, but reagentless biosensors are an extremely attractive experimental proposition. This would avoid the need to perfuse the site of sensor placement with high concentrations of quinones, for example, which would be unsatisfactory.

The CK biosensor was sensitive to micromolar concentrations of CK, the response time was rapid and certainly sufficient to detect the rates of change of CK anticipated *in planta*. The responsiveness demonstrated to a range of different CKs does not fully correspond to previous *in vitro* studies on AtCKX2, which indicated that K was a poor substrate (relative activity to iP was 2.9%) and *t*-Z was the best substrate (relative activity to iP was 289.1%) [25]. Clearly, the reaction conditions were different with Frébortová *et al* measuring specific activity with Q_0 as an electron acceptor at pH 7.0. The conditions used for evaluating the CK biosensor were set to be mildly acidic in order to represent likely physiological conditions in plant samples for which the apoplastic pH is typically between 5-6. Other observations have indicated that K remains a poor substrate in acidic conditions, and *t*-Z a stronger substrate than iP (Galuszka and Kowalska, unpublished). It is possible that entrapment of AtCKX2 in silica changes the enzyme's selectivity, although other explanations also remain possible. A broadened substrate selectivity could be helpful, allowing the opportunity to record generic CK concentrations (rather than just iP-type CKs). Future work will focus on the improvement of sensor's characteristics, validation of the sensor against traditional batch-fed assays and its application to *in vivo*, real-time monitoring of phytohormone levels.

5. CONCLUSIONS

The constitutive expression system presented in this paper allows safe handling of the *P. pastoris* production system and avoids the hazardous use of methanol, which is especially appreciated in large scale protein production. Yields were adequate for the fabrication of a series of microelectrodes. For higher yields further optimization of the cultivation conditions will be needed, possibly moving to continuous fermentation [44].

A reagentless CK biosensor has been developed based on the activity of purified AtCKX2 enzyme. PrB proved to be an efficient electron mediator between the enzyme and the electrode allowing galvanometric quantitation of a broad range of cytokinins at micromolar concentrations. The response to substrate was fast and stable within seconds. The long-term stability of the electrodes still needs to be tested. We conclude that the cytokinin microbiosensor holds the promise of a fast, real-time detection method for cytokinins in plants.

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403 **Figure captions**

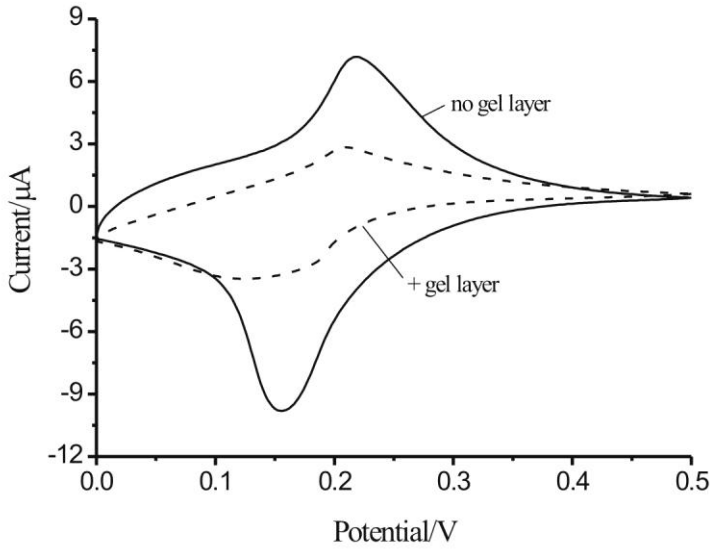
404 Figure 1. Cyclic voltammograms of a PrB modified Pt electrode before and after sol-gel/CKX film
405 deposition (solid and dashed line, respectively); scan rate 50 mV/s; 0.1 M KCl pH 5.0.
406

407 Figure 2: Determination of the optimal operating potential for the CKX2 microbiosensor. Amplitude
408 of amperometric responses to 50 μM iP at different operating potentials (vs Ag/AgCl).
409

410 Figure 3. Calibration of the cytokinin microbiosensor responses to iP. The linear regression equation
411 is included. Operating potential 180 mV (Ag/AgCl, saturated KCl) in 0.1 M KCl, pH 5.0.
412

413 Figure 4. Response of the null electrode (above: before enzyme deposition) and microbiosensor
414 (below) to different cytokinins: iP, iPR, tZ, ZR, and K. Substrate concentrations were 25 μM.
415 Operating potential 180 mV *vs*. Ag/AgCl (saturated KCl).
416

417 Fig 1



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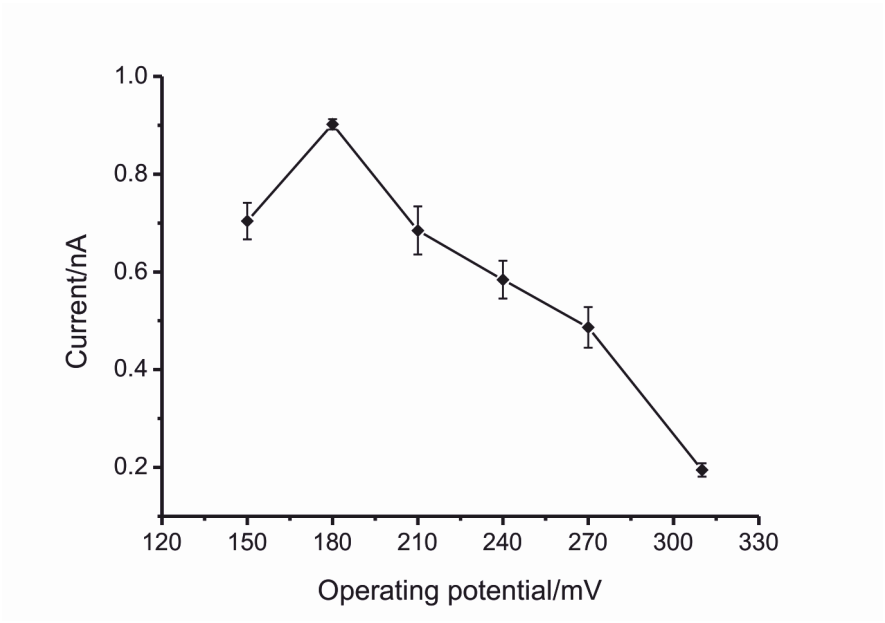
420 Fig 2



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423 Fig 3

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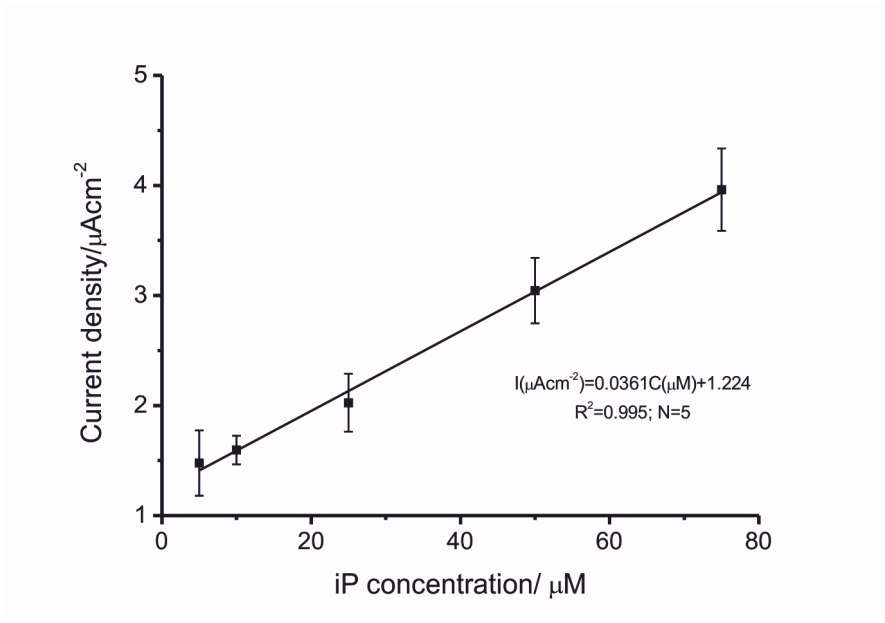

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427 Fig 4

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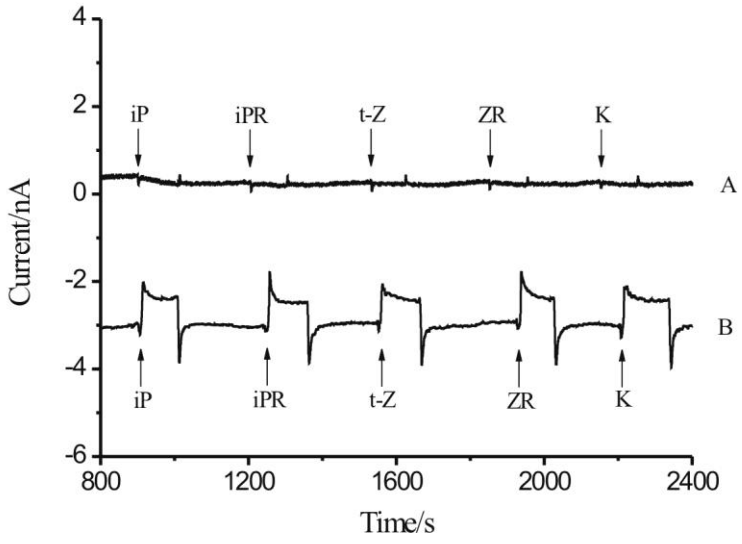

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