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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 (PrB) 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.
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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 et al. [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

Abbreviations: AtCKX2, *Arabidopsis thaliana* cytokinin dehydrogenase isoform 2; PrB, Prussian Blue, $K_3[Fe(CN)_6]$.

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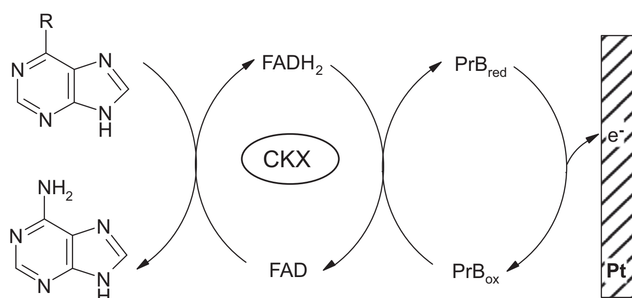

Scheme 1. Mechanism of the cytokinin biosensor.

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.

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 (PrB) 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

2.1. 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 Ref. [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).

2.2. 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 *P. pastoris* SMD1168 (Invitrogen) genome. His$^+$ transformants were grown on MD plates (1.34% yeast nitrogen base without amino acids (Difco™, Detroit, MI, USA), 4×10^{-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 °C 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 h cultivation at 28 °C 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].

2.3. 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 *ckx*2 and *aox*1 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.

2.4. High cell density fermentation and protein purification

Fermentation experiments were performed in a 15 L, 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™), 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

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