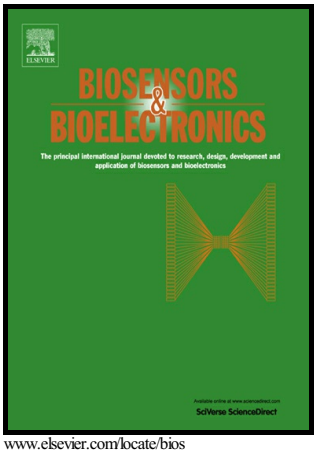

www.elsevier.com/locate/bios

Integrated Electrochemical Chip-On-Plant Functional Sensor for Monitoring Gene Expression Under Stress

Richa Pandey, Orian Teig-Sussholz, Silvia Schuster, Adi Avni, Yosi Shacham-Diamand

PII: S0956-5663(18)30476-7
DOI: https://doi.org/10.1016/j.bios.2018.06.045
Reference: BIOS10565

To appear in: *Biosensors and Bioelectronic*

Received date: 22 April 2018
Revised date: 6 June 2018
Accepted date: 23 June 2018

Cite this article as: Richa Pandey, Orian Teig-Sussholz, Silvia Schuster, Adi Avni and Yosi Shacham-Diamand, Integrated Electrochemical Chip-On-Plant Functional Sensor for Monitoring Gene Expression Under Stress, *Biosensors and Bioelectronic,* https://doi.org/10.1016/j.bios.2018.06.045

Integrated Electrochemical Chip-On-Plant Functional Sensor for Monitoring Gene Expression Under Stress

Richa Pandey[a*], Orian Teig-Sussholz[b], Silvia Schuster[b], Adi Avni[b], Yosi Shacham-Diamand[a,c*]

[a]Department of Physical Electronics, School of Electrical Engineering, Faculty of Engineering, Tel Aviv University, Tel-Aviv 69978, Israel.

[b]Department of Molecular Biology and Ecology of Plants, Tel-Aviv University, Tel-Aviv, Israel

[c]Department of Materials Science and Engineering, Faculty of Engineering, Tel Aviv University, Tel-Aviv 69978, Israel.

Abstract

The ability to interact with plants, both to sense and to actuate, would open new opportunities for precision agriculture. These interactions can be achieved by using the plant as part of the sensing system. The present work demonstrates real-time monitoring of β-glucuronidase (GUS) expression in transgenic tobacco plants using its activity as a biomarker for functional sensing. As "proof of concept", we demonstrated GUS enzyme biosensing under constitutive expression in Msk8 tomato cells and transgenic tobacco plants and in heat shock inducible BY2 tobacco cells and tobacco plants. The sensing was done using a three-electrode microchip in Msk8 or BY2 cell culture or in tobacco plant leaves. The electrode microchip was used to transduce the expression of the GUS enzyme by chronoamperometry to a measurable electrical current signal. For the constitutive expression of GUS in Msk8 cells, the system sensitivity was 0.076 mA/mM-cm^2 and the limit of detection was 0.1 mM. For the heat shock inducible BY2 cells the GUS enzyme activity was detected 12-26 hours after the heat shock was applied (40 °C for 2 hours) using two different substrates: p-nitrophenyl-β-glucuronide (with sensitivity of 0.051mA/mM-cm2) and phenolphthalein-β-glucuronide (with sensitivity of 0.029 mA/mM-cm^2).

Keywords: Plant cell sensor, Integrated chip, Precision agriculture, Electrochemical biosensing.

1. Introduction

Feeding the rising global population is a topic of concern and discussion(Alexandratos and Bruinsma, 2012). Several stages of the food-supply chain, from field to consumer, need to be monitored in order to improve the overall yield. This work deals with sensor technology for the first stage, in the field, where sensors of all kinds will eventually provide critical information that enables the optimization of food production from the field to the packaging and distribution stage. Using low cost and abundant sensing platforms in the field will allow "closing the loop" with "almost real time" control over a farm's resources in supplying the essentials to the field. This in turn will greatly help in optimizing harvest and preparation for next season. To maximize sensor utilization and optimize their economic contributions, sensors should be designed and developed with the requirements of farmers in the field(López et al., 2012) and the subsequent stages of harvesting, storage, and transportation in mind.

The main applications of sensors in agriculture today are in soil water content and nutrient analysis(Gmur et al., 2012; Wilczek et al., 2012), weed control(Perez-Ruiz et al., 2013), pest/micro-organism control(López et al., 2012), and plant physiology(Kim et al., 2013; Navarro et al., 2012). The current approaches are indirect: The sensors rely on information obtained from the environment, such as soil and air, rather than information obtained by monitoring of the plants themselves. Environmental data are used as proxies for plant wellbeing and/or condition. In this paper we describe an alternative approach, using

whole-cell functional sensing, that takes information from the plant itself, transducing it to an electrical signal and distributing the data using advanced communication over on the internet. The data can then be translated to decisions and action at the field (e.g., irrigation, nutrients dispensing, pest control). We refer to this new approach as the "internet of plants" paraphrasing the concept of the "internet of things".

The internet of plants is a very nascent ideology that has been recently conceived from the concept of the internet of things. Plants have an inherent ability to communicate with other plants using their roots(Bais et al., 2004). Signals sent from roots can be decoded and mimicked with the application of sensors, wireless networking, and cloud technology in the field of agriculture. Such systems can be also integrated into the internet of plants to enhance the inter-plant information exchange and make this information accessible to the farmers. Transcending the knowledge generated by sensors employed, on or around, plants to the internet has already been demonstrated for both agriculture and the food industries. Both conventional and functional sensors, as the one described here, will play a key role in precision agriculture providing important information allowing improving overall food production.

There are various ways in which currently available sensing technology can be amalgamated with plants. Using a direct biochip interface, as described here, is a step forward from current remote functional sensing of whole plants or specific parts of the plant like fruits or leaves that detects, for example, changes of color, shape, or photoluminescence. One approach uses internal functions of the plant to drive the biocatalysis needed for bio-electrochemical sensing of a particular analyte. The first potentiometric sensor in plant tissue was performed in yellow squash by Kuriyama et al. in 1981(Kuriyama and Rechnitz, 1981). A thin squash tissue slice expressed glutamate decarboxylase, a biocatalyst in the process of glutamic acid detection. Sidwell et al.(Sidwell and Rechnitz, 1985) demonstrated dopamine

sensing using banana pulp expressing polyphenol oxidase mounted on Clark's oxygen electrode and called it the "Bananatrode". Other electrodes have been developed for use on corn kernels(Kuriyama et al., 1983) and cucumber leaves(Smit and Rechnitz, 1984) for sensing pyruvate and cysteine, respectively.

Another very interesting approach is when transgenic plants are used as sensors for the desired analyte. This approach relies on genetic modification of the plant by fusing a reporter gene, for example, the gene encoding green fluorescent protein (GFP), to the promoter of a gene that responds to a particular environmental cue. Manak et al. reported that a measurable GFP signal was induced by expression from the alcohol dehydrogenase promoter under hypoxia and high salt conditions(Manak et al., 2002). Naturally occurring plants and trees have also served as qualitative biosensors for mines or minerals(Oliphant et al., 2017).

The class of sensors that directly interact with plants is based on a novel methodology that is still in its embryonic stages, but an increasing number of ideas have emerged in recent years. One of the most noteworthy is the transistor-based sensor. This type of sensor has been demonstrated for sensing nutrients in the sap fluid(Coppedè et al., 2017) through impedance measurement of conducting textiles that are inserted into the stem. Although innovative, this approach is invasive and may affect the plant functions. Another work studied plant water status measured as a function of leaf thickness and leaf electrical capacitance(Afzal et al., 2017). This approach poses some challenges since leaf thickness changes with time and the associated leaf electrical capacitance could be attributed to various factors such as pathogen attack or water toxicity.

β-glucuronidase (EC 3.2.1.31) (GUS) enzyme is encoded by the *uidA* gene. GUS is an acid hydrolase enzyme that cleaves a wide variety of β-glucuronic acids, including 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) which gives rise to an easily detectable blue colored

product. The use of *uidA* as a reporter gene was first demonstrated in tobacco plants by Jefferson et al.(Jefferson et al., 1987a, 1986).. Additionally, β-glucuronidase substrates are commercially available and can generate electroactive products that can be electrochemically oxidized or reduced.

Here we describe a novel approach for non-invasive and stimulus-specific direct plant sensing. We assayed the activity of GUS expressed constitutively in Msk8 tomato cells and induced by heat shock in BY2 tobacco cells PhG and pNPG. We also demonstrated real-time *in vivo* detection of GUS activity in transgenic tobacco plants by integrating a simple three-electrode chip into the plant leaf and connecting it to a portable potentiostat.

2. Materials and Methods

2.1 Cell culture and plant

Suspension-cultured cells derived from plant tissue have allowed research on highly homogenous and rapidly dividing cell populations saving time and the need for growing a large number of individual plants to be examined. In this work we employed two different plant cell cultures: tomato (*S. lycopersicum* cv Mill.; line Msk8) and tobacco (*N.tabacum* BY2). Cultures were grown as described by Felix et al.(Felix et al., 1991) for tomato and Van Leeuwen et al.(van Leeuwen et al., 2007) for tobacco and used 4 to 6 days after weekly sub culturing.

In order to achieve a plant or a plant cell culture expressing a reporter gene, we used the ability of *Agrobacterium tumefaciens* to transfer DNA (gene of interest) to plant cells for the purpose of plant genetic transformation (Caplan et al., 1983).Msk8 and BY2 cells were transformed using *A. tumefaciens*.strains EHA105 and GV3101, respectively, harboring plasmids for expression of *uidA*, the gene encoding GUS (Deng, 1996). For constitutive expression of *uidA* the pBIS-N1 vector (Narasimhulu et al., 1996) was used, whereas for

heat-activated expression pBINPLUS vector was used (Strange and Petolino, 2012; van Engelen et al., 1995).

Nicotiana tabacum plants were genetically transformed using *Agrobacterium* strain GV3101 harboring the *uidA* gene encoding for the GUS enzyme and driven by the constitutively expressing 35S promoter in the vector pPCV702 (Koncz et al., 1987) or driven by the *Arabidopsis thaliana* heat shock inducible promoter Hsp 18.2 (Takahashi and Komeda, 1989) in the pBINPLUS vector .

2.2 GUS staining and heat induction

Leaf disks or plant cells were stained with 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc), the substrate for the GUS assay reaction,at 37 °C overnight. Once the substrate is cleaved by the GUS enzyme (which being a bacterial enzyme is active at 37 °C) a blue color is observed. For heat induction, cell aliquots or whole plants were incubated at 40 °C for 2 hours, then returned to the original growing conditions. GUS staining was performed 48 hours after heat induction.

2.3 Electrochemical chip fabrication

Three-electrode electrochemical chips (Figure 1a) were fabricated on 4-inch silicon wafers (p-Si [1 0 0]) with 500-nm thick thermal oxide layers (University Wafer, Inc.) in a cleanroom using a combination of photolithography and sputtering techniques(Vernick et al., 2011). Briefly, using photolithography the patterned wafers were sputtered with 15-nm Ti and 150-nm Au films without breaking the vacuum. A lift off was performed to obtain the final pattern of seven chips per wafer. Each chip consisted of a planar gold working electrode (3.14 mm^2), the gold counter electrode (6.28 mm^2), and Ag/AgCl open reference electrode (1 mm^2). The reference electrode was made by electroplating Ag and later forming AgCl(Popovtzer et al., 2006).

2.4 Electrochemical characterization

The Msk8 cells and BY2 cells grown in Murashige and Skoog (MS) media were suspended in the 0.1 M phosphate buffer (PB) of pH 5.8 and 7.1 respectively for all the experiments. All the electrochemical studies with the cells were performed using a VSP BioLogic potentiostat. The cyclic voltammetry (3 cycles; sweeping voltage three times from E1 to E2) was performed on the three electrode chips for the PB (control), substrate phenolphthalein beta-D glucuronide (PhG, 0.1 M, Sigma Aldrich) and p-nitrophenyl beta-D glucuronide (pNPG, 2 mM, Sigma Aldrich), commercial enzyme β-D-glucuronidase (GUS, 0.1 M, Sigma Aldrich), product phenolphthalein (0.1 M, Sigma Aldrich), MS cell medium (Murashige and Skoog, 1962) and BY2 or Msk8 cells with a voltage sweep from 1V to -1V with a scan rate of 100 mV/s. Next, the cyclic voltammetry was performed using 30 µl of GUS-expressing or wild-type Msk8 cells in the presence of the substrate. Chronoamperometry studies for different PhG concentration and pNPG concentration was performed at 0.7 V and -0.4 V vs. Ag/AgCl, respectively. These potentials were selected after conducting cyclic voltammetry in the same system configuration in the presence of respective substrates. The sensitivity was calculated from the slope of the calibration curve and the limit of detection (LOD) was calculated from the concentration obtained by extrapolating the current value of background solution onto the calibration curve.

For the experiments in tobacco plants, chronoamperometry was performed using a portable Palmsense® potentiostat (Palm Instruments BV) at 0.7 V vs. Ag/AgCl after injection of 0.1 ml PhG or PB into the back of the leaf.

3. Results and Discussion

Briefly, our approach has two parts: i) an enzyme-catalyzed reaction involving a specific substrate inside the plant cell and ii) electrochemical reaction on a three-electrode chip

(Figure 1). The substrates used were phenolphthalein-β-glucuronide (PhG) and p-nitrophenyl-β-glucuronide (pNPG). Each substrate is a conjugate of β-glucuronide with another moiety "P" (phenolphthalein and p-nitrophenol, respectively). The substrate diffuses inside the cell and GUS catalyzes the breakdown of the conjugate chemical into β-glucuronide and P, where P is electrochemically active. The electroactive product transverses out of the plant cell and is oxidized on the working electrode under a voltage bias similar to or above to its oxidation potential and is measured versus a quasi-electrode composed of Ag/AgCl.

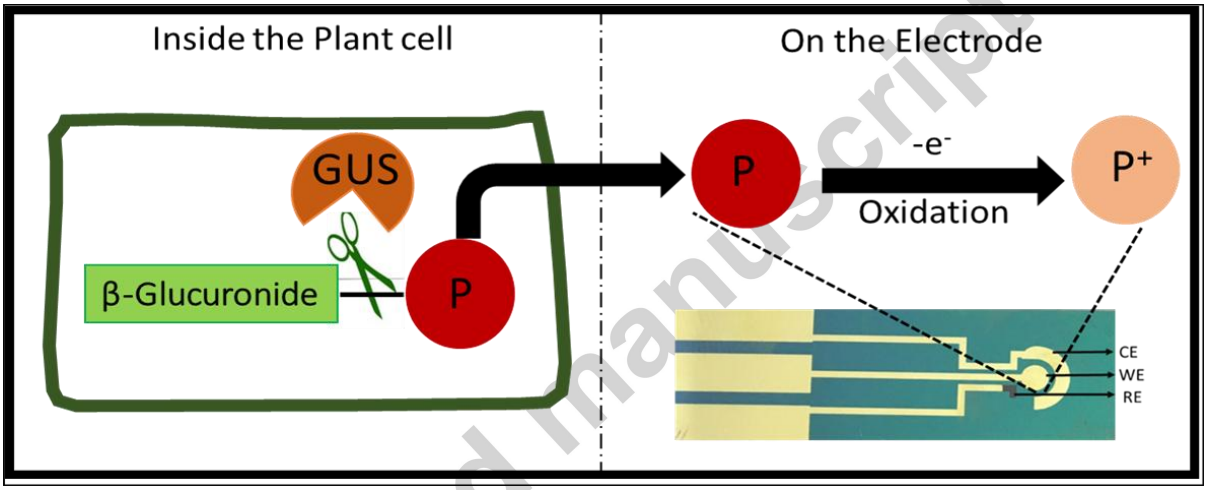

Fig 1. Schematic of GUS enzyme reaction inside the cell and oxidation of the enzyme product (P, either phenolphthalein or p-nitrophenol) onto the three-electrode chip. The electrodes were the Au working electrode (WE), the Au counter electrode (CE), and the Ag/AgCl reference electrode (RE).

3.1 Biosensing of constitutively expressed GUS

3.1.1 Plant cell-based biosensing

The Msk8 cell line was stably transformed with the pBIS-N1 vector for constitutive expression of GUS. The GUS assay using the X-Gluc stain (Jefferson et al., 1987b) is particularly efficient for plants since the un-transformed (wild type) species do not exhibit detectable GUS activity, eliminating background noise and allowing easy evaluation of the transformed plants. Following this GUS assay, the cells showed a strong blue color, resulting

from the X-gluc hydrolysis by the GUS enzyme, thus indicating stable expression of the enzyme, whereas non-transformed cells did not develop a blue color as mentioned above (Figure 2a)). We sought out to test our experimental system both in tomato and tobacco in order to validate that it is not plant-specific but has the potential to work in different plants, including an agricultural important crop such as tomato. Hence, we first explored sensing of the GUS enzyme expressed constitutively in Msk8 cells and in tobacco plants using our electrochemical sensing device. It is very important for efficient biosensing that the signal due to other electroactive components is low and does not interfere with signal to be measured. Therefore, our preliminary goal was to establish the absence of any electrochemically active species in the suspension solution and/or the cell culture media. Cyclic voltammogram (CV) of solutions of phosphate buffer (PB) and PB solutions of cell medium, a commercial GUS enzyme, or PhG substrate did not show significant peaks that interfered with the expected signal (Figure 2b).

To validate our approach in cells, we performed a control experiment with the substrate PhG using the wild-type (GUS^-) cells lacking the GUS enzyme and transgenic (GUS^+) cells. In the CV, the GUS^- cells treated with substrate yielded a minor oxidation signal near 0.7 V, which was attributed to phenolphthalein present in the commercial substrate (the substrate contains <0.1% free phenolphthalein). GUS^+ cells yielded a much higher current response, by approximately 0.1 mA, than GUS^- cells (Figure 2c). To confirm that the peak at 0.7 V was due to the oxidation of phenolphthalein, we performed CV of commercial phenolphthalein. The resultant CV demonstrated a significant oxidation potential peak at 0.7 V (Figure S1a), indicating that in GUS^+ cells with PhG as substrate the peak observed was due to oxidation of the added substrate. We also observed unique reversible redox peaks at 0.15 V and -0.15 V in the CV of GUS^+ cells incubated with PhG. We did not observe the presence of these peaks in the CV of the substrate, product, enzyme, cell medium, or PB. Hence, we performed another

CV of GUS$^+$ cells in PB to investigate the occurrence of these redox peaks. We observed similar peaks, suggesting they could be due to the cellular production of electroactive components that undergo a redox reaction at lower potentials (Figure S1b).

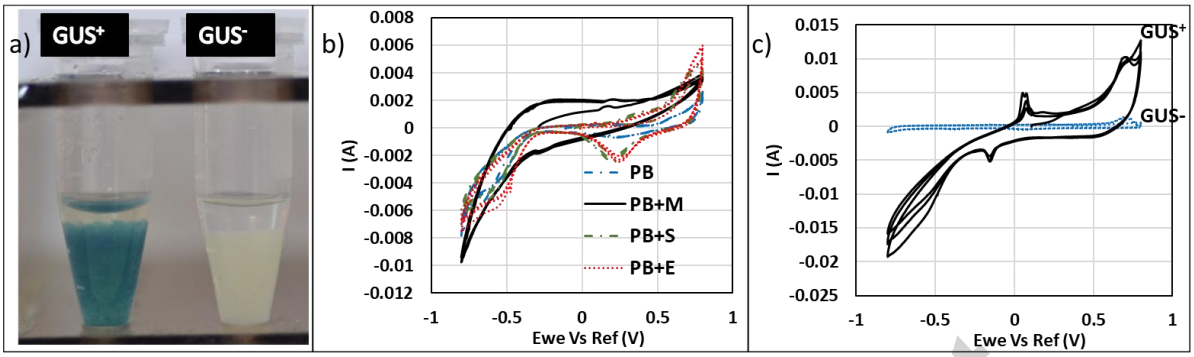

Fig 2. a) X-gluc staining of transgenic (GUS+) and wild type (GUS-) Msk8 cells, b) Cyclic voltammetry (cycles 1,2,3) from -0.8 to 0.8V with a scan rate of 100mV/s in the presence of 0.1M Phosphate Buffer (PB), PB+media (M), PB+0.1M substrate (S), PB+0.1M commercially available GUS enzyme (E). Where, I/ current (Ampere) and Ewe/ working electrode potential (Volt) maintained Vs reference electrode (Ref), c) Cyclic voltammetry (cycle 3) from -0.8 to 0.8V of GUS- and GUS+ Msk8 cells in suspension with PhG substrate.

We studied the current response of the three-electrode electrochemical chip containing GUS$^+$ cells suspended in the solutions of different PhG concentrations at 0.7 V for a duration of 0-3600 s (Figure 3a). After the PhG was added at 500 s there was a steady rise in the current signal that correlated with substrate concentration. Without the addition of substrate, the chronoamperogram showed a negligible increase in the current. A calibration graph was plotted by calculating the current signal (ΔI, which was defined as the current density at 500 s subtracted from the current density at the 3600 s) for various substrate concentrations (Figure 3b, c). The curve typical of Michaelis-Menten kinetics(Michaelis and Menton, 1913) was observed. The current reached saturation after about 3000 s. In initial experiments in the Msk8 tomato cells that constitutively express GUS, PhG substrate was detected with an adequate sensitivity of 0.078 mA/mM-cm^2 and lower LOD of 0.1 mM. The sensor had a linear range from 0.1 mM to 1 mM, the highest concentration tested. The data from the calibration experiment were also plotted as a Lineweaver-Burk plot (Figure 3d); the V_{max} and

K_m were 0.34 µA and 0.43 mM, respectively. The K_m of the GUS enzyme calculated using Lineweaver-Burk plot is in the range of the theoretical value as reported by Fishman et al. for the GUS enzyme obtained from calf liver (0.148 mM)(Fishman and Bernfeld, 1955). For the GUS enzyme of *E. coli* origin, the K_m is in the range of 0.018 to 3.05 mM for PhG(Kleber, 1991). The enzyme concentration (0.09mM) can be calculated by the Dixon's(Bisswanger, 2008) method using the calibration chart (Figure.2 b).These experiments established the feasibility of utilizing electrochemical sensing to detect constitutive enzyme expression in cells.

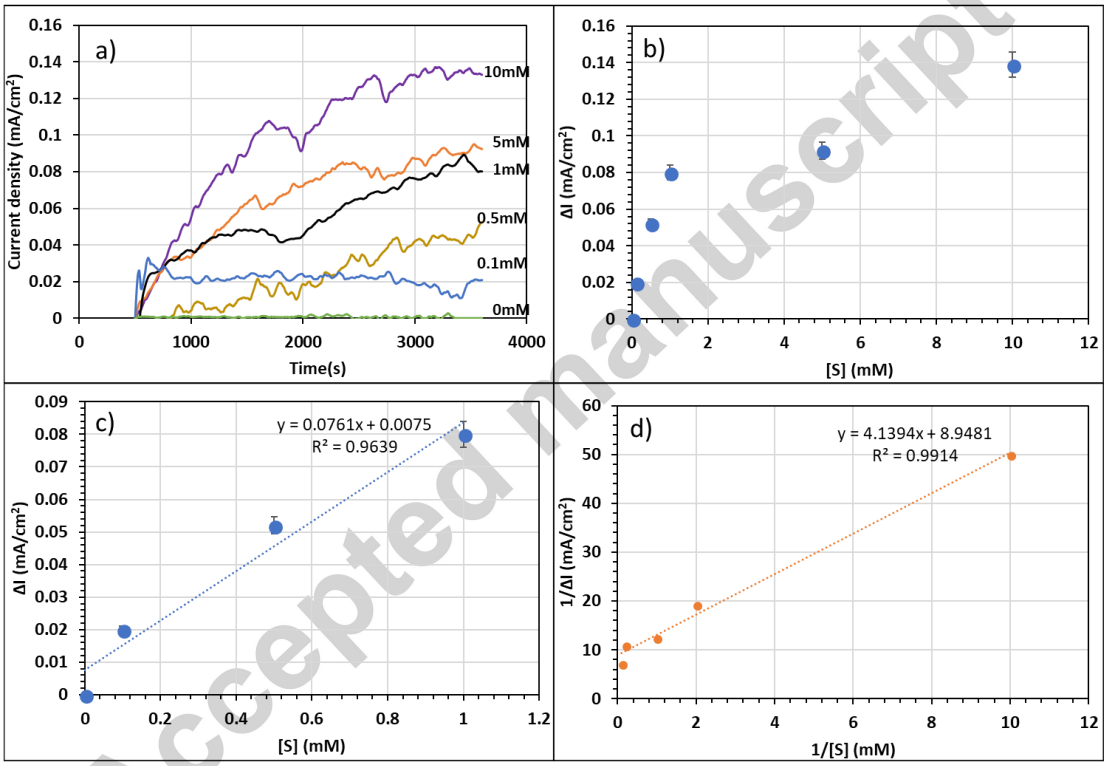

Fig 3. a) Chronoamperometry at 0.7 V of 30 µl of Msk8 GUS$^+$ cells with substrate concentrations ranging from 0.5 to 10 mM. b) Plot of ΔI vs. concentration of pNPG, where ΔI was calculated from the difference in the current density at 500 s from the current density at 3600 s from data shown in panel a. c) Linear range of the chronoamperometry based on the data shown in panel is from 0 to 1 mM pNPG. d) Lineweaver-Burk plot used to calculate enzyme kinetic parameters K_m and V_{max}.

3.1.2 Whole *Nicotiana tabacum* based biosensing

We next demonstrated successful measurement of an electrochemical response in real time on a plant expressing GUS by attaching the sensing chip directly to the leaves. PhG

solution was injected at the abaxial side of the leaf using a needleless syringe. The solution diffused throughout the intracellular matrix of the leaf. Blank measurements were performed by injecting PB. The chip was then mounted at the site of substrate injection using polydimethylsiloxane (PDMS) for support and a clip for plant chip attachment (Figure 4a, b).

A real-time electrochemical response was examined in the wild-type (GUS^-) and GUS^+ transgenic tobacco plant (constitutively over-expressing the *GUS* gene). We performed two sets of experiments. First, we evaluated GUS^+ and GUS^- plants in the absence of the substrate to test if there is any electroactive plant biochemical that interferes with the current signal at 0.7 V. The chronoamperogram showed a very negligible current response (Figure 4c), demonstrating the absence of a strong electroactive species that could interfere with the true current signal. Second, we measured the electrochemical response in GUS^- and GUS^+ after injecting the substrate. For the GUS^- plant we saw an increase in the current after injection of the substrate. The signal reached steady state soon and dropped after 1300s. This could again be due to a small amount of the free phenolphthalein present in the substrate as discussed in the section describing the cell-based experiment (Figure 2c).

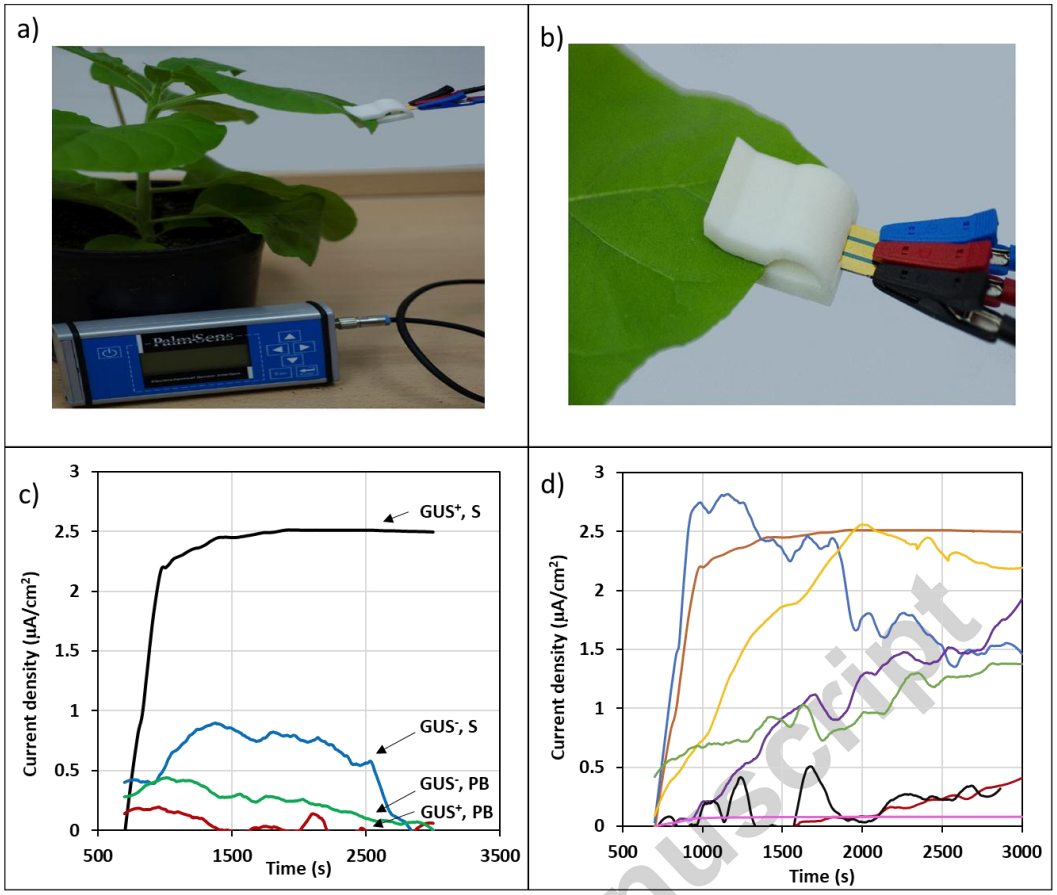

Fig 4. a) Chip is connected to a portable potentiostat and to the leaves using 3D printed chip holder containing the chip, b) 3D printed white polymer chip holder containing the chip in contact with the abaxial side of the leaf c) Chronoamperometry at 0.7 V vs. Ag/AgCl of GUS$^-$ and GUS$^+$ N. tabacum plants with substrate PhG (S) and without substrate (PB), d) Chronoamperometric measurements on GUS$^+$ plant with PhG at different leaf and different positions of the leaf.

Upon injection of substrate into the GUS$^+$ plant, the significant increase of the Faradaic current due to the oxidation of the product generated by the enzyme in the transgenic plant was significant when compared to the two control experiments, GUS$^+$ plants without PhG and GUS$^-$ plants with PhG (Figure 4c). The same experiments when repeated with different chips gave similar trends (Figure 4d). However, since we cannot control the level of GUS expression or the extent of diffusion of the substrate adjacent to the sensor, we observed different levels of increment in the current signal. One of the major challenges that we face is the issue of fluctuating substrate diffusion in the leaf, which requires further investigation and calibration. The manual injection method we used for the substrate is a major issue in such a

measurement, which we did not take into consideration in our preliminary chip design. We could use a flexible electrode integrated with a micro-electro-mechanical systems (MEMS) as the second-generation biosensor, which can perform automated substrate injection and detection simultaneously. Future directions for this research will involve optimization of substrate concentration and calibration of the MEMS integrated device to acquire the desired response. To the best of our knowledge our work is the first to report an *in vivo* electrochemical scheme for detection of the expression of an enzyme directly from a plant.

3.2 Biosensing after heat shock induced expression of biosensing enzyme

A more advanced scheme demonstrated here is the profiling of the biochemical changes in the cells and plants due to external heat stimulus. For this purpose, the cells were genetically engineered to express the reporter gene *uidA* driven by a heat shock promoter. A temperature rise induces the expression of the GUS enzyme in the cells.

3.2.1 Plant cell based biosensing

In the second part of our work we explored the feasibility of bio-electrochemical detection of the heat-induced expression of the GUS enzyme in cultured cells and plants. The BY2 cell line stably transformed with pBINPLUS AtHsp18.2::GUS vector (i.e expressing a GUS enzyme whose expression is induced only after heat shock) showed strong X-Gluc staining 48 hours after heat shock, whereas non-heat shocked BY2 cells were negative (Figure 5a).

We also validated the biological measurement by means of the chronoamperometry of the cells subjected to heat shock or not in the absence and presence of substrate PhG (Figure 5b). There was not a significant increase in the current in either heat-shocked cells treated with PhG or heat-shocked cells without PhG. In the heat-shocked cells treated with GUS substrate an increase in current indicated that the oxidation of phenolphthalein correlated with substrate

PhG concentration. The ΔI (subtracting current density at 300 s from current density at 3600 s) versus concentration of PhG was then plotted as a calibration curve (Figure 5c). The calibration showed a linear increase of current for the concentration range of 0.2 mM to 2 mM PhG. The sensitivity of 0.029 mA/mM-cm^2 was found in the sensors performance.

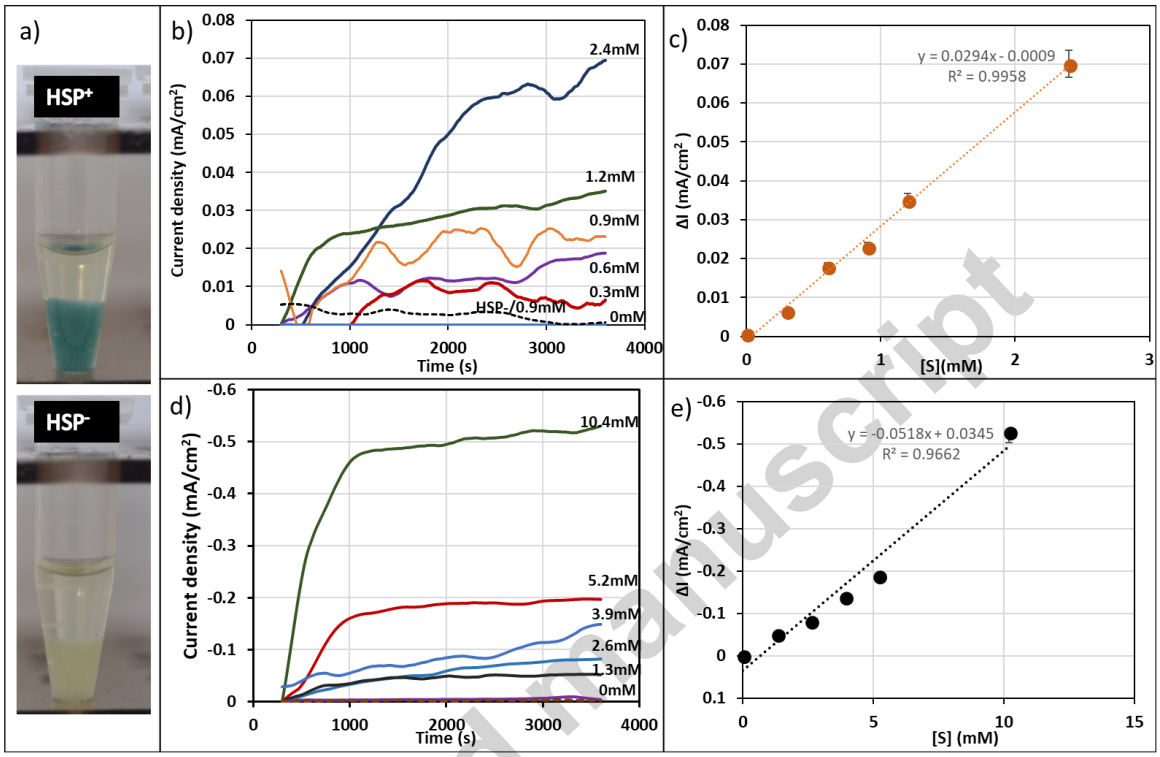

Fig 5. a) X-gluc staining of heat shock induced (HSP+) and without heat shock (HSP-) BY2 cells, b) Chronoamperometry of 30 µl of heat-shock-induce and non-heat-shocked (HSP-) BY2 cells at -0.4 V in the presence of different concentration of PhG, c) ΔI vs. C concentration of PhG, where ΔI was calculated from the difference in the current density at 300 s from the current density at 3600 s from data shown in panel b, d) Chronoamperometry of heat shock induced (HSP+) and and without heat shock induction (HSP-) BY2 cells at -0.4V in the presence of different concentration of pNPG, e) Calibration chart ΔI vs. C concentration of pNPG (ΔI calculated from the different in the current density at 300 s from the current density at 3600 s).

Another electroactive product that can be utilized for electrochemical detection of GUS expression is p-nitrophenol (pNP). One of the reasons for our choice of pNPG as the substrate is because it produces pNP as the product of enzyme reaction. pNP has low redox potential (-0.4 V) on the Au electrode and hence the current signal is not affected by the background signal of water hydrolysis, which is detected at around 1.23 V. The phenolphthalein is

oxidized at 0.7 V and thus the signal from water splitting may result in unacceptable background.

The inset in Figure S2a) shows the mechanism of the reduction reaction of pNP into 4-hydroxyaminophenol on the electrode surface. This was studied using CV as shown in Figure S2b). The CV of pNPG and GUS together showed no oxidation peak in the first cycle because the pNP in its native form is not oxidizable. However, there is a peak at -0.4 V due to reduction of pNP produced by the reaction of GUS and pNPG. Neither enzyme alone nor substrate alone gave a redox peak, demonstrating their lack of electroactivity.

We also performed the chronoamperometry of heat-treated and untreated cells in the presence and absence of pNPG at -0.4 V versus Ag/AgCl. The chronoamperometry showed no a significant increase in the current in either heat-shocked cells treated with PhG or heat-shocked cells without PhG (Figure 5d). In heat-shocked cells treated with increasing concentrations of pNPG, we observed a linear increase in the current response indicating the reduction of the pNP. This difference in the current density at 300 s and the current density at 3600 s was plotted versus the pNPG concentration as a calibration curve (Figure 5e). A linear increase in the current was observed over the concentration range from 1.3 to 10.4 mM pNPG. Based on the calibration curve obtained from the chronoamperogram the sensitivity was 0.051 mA/mM-cm^2 and the LOD was 0.6 mM. The sensor showed better sensitivity for the heat-induced production of GUS enzyme when compared to the constitutively expressed enzyme. The sensitivity of the experiments with the PhG substrate was found to be lower than that of pNPG substrate. The increased sensitivity for pNPG is likely due to the higher electron transfer rate between the product formed with PhG and the electrode.

3.2.2 Whole *Nicotiana tabacum* based biosensing

After successfully showing the sensing of the biochemical changes in plant cells due to heat we exploited our approach to understand the biochemical response to heat in whole plants. Similar to BY2 cells, *N. tabacum* plants were stably transformed with pBINPLUS AtHsp18.2::GUS. Leaf disks of a transgenic plant showed X-Gluc staining only after heat shock although the staining pattern was not uniform, due to the diffusion of the substrate into the tissue (Figure 6a).

We then used the direct biochip interface to sense the GUS enzyme expression in transgenic tobacco plants after heat shock. In the control experiment in plants grown without heat treatment, application of PhG resulted in a slight current decrease that stabilized to a constant value. The heat-treated plant with PhG showed a significant current increase due to the oxidation of the product generated by the reaction between the heat-shock-induced GUS enzyme in the leaf and the injected PhG substrate (Figure 6b). This was successfully verified by analysis of different leaves of the plant (Figure 6c). Again, a different level of signal but with similar increasing trend was observed. This confirms with the non-uniform the enzyme expression as seen in Figure 6a) as well as error due to manual injection. For the heat-treated plants not injected with substrate a gradual decrease in the signal was observed typical of the linear dependence in the $\sqrt{t}$ as per the Cotrell equation (Figure 6d).

We have demonstrated that bio-electrochemical sensing is a straight-forward methodology. The electrodes can be made on a specially manufactured substrate using lithography or 3D printing, matching the pore patterns on the leaves or inserted into the plant. Substrates of the electrode can be flexible, disposable, and even biodegradable. The sensors can be grafted onto the plants in the field. In this way the growth and maintenance of the plant-sensor cyborg will be separated from the actual plant and will not be considered as a genetically modified organism. Plants can also be engineered to express the substrate, hence eliminating the need for external substrate injection. The reporter gene can be driven by

specific promoters detecting almost any desired factor (biotic or abiotic) that affects the plant. This system was applied in microbes(Belkin, 2003), indicating its feasibility in plants, with existing plant promoters, both endogenous and heterologous.

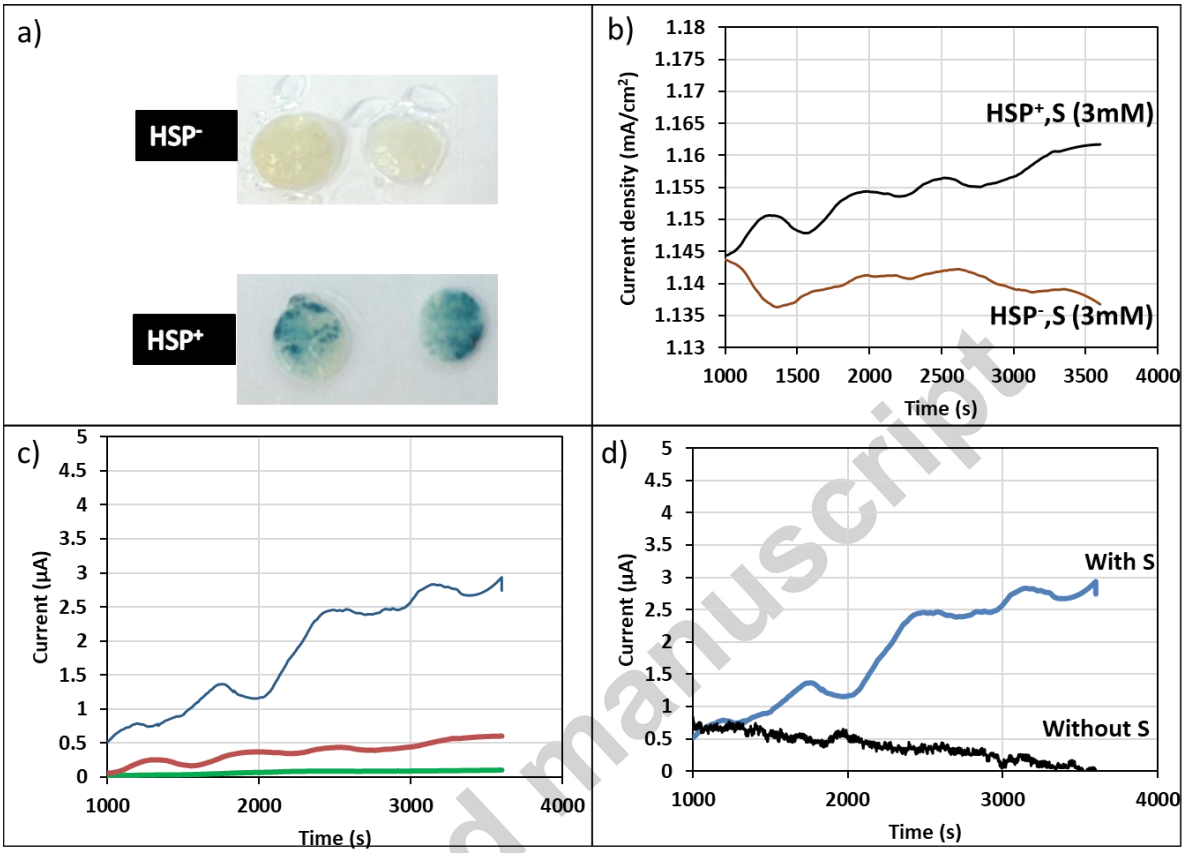

Fig 6. a) X-gluc staining of stable transgenic N. tabacum without heat shock (HSP-) and and after heatshock (HSP+) leaf disks, b) Chronoamperometry of heat shocked N. tabacum plant (HSP+, S) and non-heat-shocked plant (HSP-, S) at 0.7 V in the presence of 3 mM substrate PhG (S), Chronoamperometric measurements on HSP+ plant c) three different leafs with PhG, d) With PhG (blue) without PhG (black).

Conclusion

We demonstrated direct sensing of GUS enzyme produced in the plant cells and plant itself using a three-electrode electrochemical chip. Sensing was done in the cells and plants, constitutively and well as induced expression of GUS. We obtained a sensitivity of 0.076 mA/mMcm^2 and a limit of detection of 0.1mM. We explored two substrates, pNPG and PhG

with the cells, as a proof of concept that the signal produced is specific to the enzyme substrate reaction and not due to the extraneous reactions like water splitting and hydrogen evolution. The pNPG substrate was chosen because after reacting with the GUS enzyme it produces a product electroactive at -0.4 V. This electric potential is low that there is no interference due to the hydrolysis of water (1.23 V). We obtained a significantly higher sensitivity (0.029 mA/mMcm^2) for the heat shock induced cells than the constitutively GUS producing cells. The pNPG (0.051 mA/mMcm^2) substrate demonstrated better sensitivity than the PhG substrate. The heat induced GUS expression in the plants were also detected by our direct electrochemistry platform. The implementation of our device could allow sensing of external variables such as temperature, water content, nutrients, and pesticides and internal variables such as plant hormones that serve as signal transmitters (e.g., jasmonic acid or methyl jasmonate) and monitor the communication between plants. This technology can interact with the internet of things collecting data over a very large number of plants, over a large area for a long time and at very low cost. The current method suffers the error due to manual injection of substrate which can be further improved by using automated actuation using MEMS. Future of this technology is in more precise agriculture by monitoring all stages of planting, growing, harvesting, storage, distribution and food-quality forecasting.

Acknowledgments

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant no. 607417 (CATSENSE). This research was also supported by the Israel Science Foundation (grant No. 1616/17). We would also like to acknowledge the Boris Mints Institute for Strategic Policy Solutions to Global Challenges, the Department of Public Policy and the Manna Centre for Food Security,

Tel Aviv University for their generous support under the program "Plant based heat stress whole-cell-bio-sensor" (grant no. 590351) 2017.

References

Afzal, A., Duiker, S.W., Watson, J.E., Luthe, D., 2017. Trans. ASABE 60, 1063–1074. https://doi.org/10.13031/trans.12083

Alexandratos, N., Bruinsma, J., 2012. 20, 375. https://doi.org/10.1016/S0264-8377(03)00047-4

Bais, H.P., Park, S.-W., Weir, T.L., Callaway, R.M., Vivanco, J.M., 2004. Trends Plant Sci. 9, 26–32. https://doi.org/10.1016/J.TPLANTS.2003.11.008

Belkin, S., 2003. Curr. Opin. Microbiol. 6, 206–212. https://doi.org/10.1016/S1369-5274(03)00059-6

Bisswanger, H., 2008. Enzyme kinetics: principles and methods, Wiley Encyclopedia of Chemical Biology.

Caplan, A., Herrera-Estrella, L., Inzé, D., Haute, E. Van, Montagu, M. Van, Schell, J., Zambryski, P., 1983. Introduction of Genetic Material into Plant Cells. Science (80-.). 222, 815–821. https://doi.org/10.1126/SCIENCE.222.4625.815

Coppedè, N., Janni, M., Bettelli, M., Maida, C.L., Gentile, F., Villani, M., Ruotolo, R., Iannotta, S., Marmiroli, N., Marmiroli, M., Zappettini, A., 2017. Sci. Rep. 7, 16195. https://doi.org/10.1038/s41598-017-16217-4

Deng, X., 1996. Plant Cell Am. Soc. Plant Physiol. 8, 873–886. https://doi.org/10.1105/tpc.8.5.873

Felix, G., Grosskopf, D.G., Regenass, M., Boller, T., 1991. Biochemistry 88, 8831–8834. https://doi.org/10.1073/pnas.88.19.8831

Fishman, W., Bernfeld, P., 1955. Glucuronidases. Methods Enzymol.

Gmur, S., Vogt, D., Zabowski, D., Moskal, L.M., 2012. Sensors 12, 10639–10658. https://doi.org/10.3390/s120810639

Jefferson, R.A., Burgess, S.M., Hirsht, D., 1986. Biochemistry 83, 8447–8451.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987a. EMBO J. 6, 3901–7.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987b. EMBO J. 6, 3901–7. https://doi.org/10.1073/pnas.1411926112

Kim, G., Kim, G., Ahn, C.-K., Yoo, Y., Cho, B.-K., 2013. Sensors 13, 2986–2996. https://doi.org/10.3390/s130302986

Kleber, H.-P., 1991. D. Schomburg and M. Salzmann (Editors), Enzyme Handbook Volume 1, Class 4: Lyases. 810 S., Loose-Leaf-Binder. Berlin — Heidelberg — New York — London — Paris — Tokyo — Hong Kong — Barcelona 1990. Springer-Verlag. DM 248,00. ISBN: 3-540-52579-3. J. Basic Microbiol. 31, 320–320. https://doi.org/10.1002/jobm.3620310416

Koncz, C., Olsson, O., Langridge, W.H., Schell, J., Szalay, A.A., 1987. Proc. Natl. Acad. Sci. United States Am. 84, 131–135. https://doi.org/10.1073/pnas.84.1.131

Kuriyama, S., Arnold, M.A., Rechnitz, G.A., 1983. J. Memb. Sci. 12, 269–278. https://doi.org/10.1016/S0376-7388(00)80191-X

Kuriyama, S., Rechnitz, G.A., 1981. Anal. Chim. Acta 131, 91–96. https://doi.org/10.1016/S0003-2670(01)93537-8

López, O., Rach, M., Migallon, H., Malumbres, M., Bonastre, A., Serrano, J., 2012. Sensors 12, 15801–15819. https://doi.org/10.3390/s121115801

Manak, M.S., Paul, A.L., Sehnke, P.C., Ferl, R.J., 2002. Life Support. 8, 83–91.

Michaelis, L., Menton, M.I., 1913. Kinetics of invertase action. Biochem. Z. 49, 333–369.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Narasimhulu, S., Deng, X., Sarria, R., Cell, S.G.-T.P., 1996, U., 1996. Am Soc Plant Biol.

Navarro, P., Fern?ndez, C., Weiss, J., Egea-Cortines, M., 2012. Sensors 12, 15356–15375. https://doi.org/10.3390/s121115356

Oliphant, A.J., Wynne, R.H., Zipper, C.E., Ford, W.M., Donovan, P.F., Li, J., 2017. Biol. Invasions 19, 179–195. https://doi.org/10.1007/s10530-016-1271-6

Perez-Ruiz, M., Carballido, J., Agüera, J., Rodríguez-Lizana, A., 2013. Sensors 13, 3313–3330. https://doi.org/10.3390/s130303313

Popovtzer, R., Neufeld, T., Ron, E.Z., Rishpon, J., Shacham-Diamand, Y., 2006. Sensors Actuators B 119, 664–672. https://doi.org/10.1016/j.snb.2006.01.037

Sidwell, J.S., Rechnitz, G.A., 1985. Biotechnol. Lett. 7, 419–422. https://doi.org/10.1007/BF01166215

Smit, N., Rechnitz, G.A., 1984. Biotechnol. Lett. 6, 209–214. https://doi.org/10.1007/BF00140038

Strange, T.L., Petolino, J.F., 2012. Transgenic Plants. Cold Spring Harb. 847. https://doi.org/10.1007/978-1-61779-558-9

Takahashi, T., Komeda, Y., 1989. MGG Mol. Gen. Genet. 219, 365–372. https://doi.org/10.1007/BF00259608

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.-P., Pereira, A., Stiekema, W.J., 1995. Transgenic Res. 4, 288–290. https://doi.org/10.1007/BF01969123

van Leeuwen, W., Vermeer, J.E.M., Gadella, T.W.J., Munnik, T., 2007. Plant J. 52, 1014–1026. https://doi.org/10.1111/j.1365-313X.2007.03292.x

Vernick, S., Freeman, A., Rishpon, J., Niv, Y., Vilkin, A., Shacham-Diamand, Y., 2011. J. Electrochem. Soc. 158, P1. https://doi.org/10.1149/1.3507268

Wilczek, A., Szyp?owska, A., Skierucha, W., Cie?la, J., Pichler, V., Janik, G., 2012. Sensors 12, 10890–10905. https://doi.org/10.3390/s120810890

Highlights:

- Bio-electrochemical sensing of GUS enzyme in plant cell culture.
- Profiling GUS enzyme in plants using integrated Chip-on-Plant system.
- Chip-on-Plant functional sensor for detecting heat-stress in plants.