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Jun-tao LIU

Yan-ling LIU

Zhi CHENG

Shi-jing CHEN

Wei-Hua HUANG
Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China;, whhuang@whu.edu.cn

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Electrochemical Monitoring of Cell Wall-Regulated Transient Extracellular Oxidative Burst from Single Plant Cells

LIU Jun-tao, LIU Yan-ling, CHENG Zhi, CHEN Shi-jing, HUANG Wei-hua*
(*Key Laboratory of Analytical Chemistry for Biology and Medicine* (*Ministry of Education*), *College of Chemistry and Molecular Sciences, Wuhan University, Wuhan* 430072, *China*)

Abstract: Oxidative burst by rapid, transient generation of reactive oxygen species (ROS) is a fundamental process for plant disease defense and signal transduction. ROS can be protective or toxic depending on their concentrations, and critical balance between ROS production and scavenging is regulated by complex and perfect mechanisms in plant cells. However, the process of rapid and transient ROS burst from plant cells and their regulatory mechanisms are far from being completely understood, owing to the methodological obstacles in real-time monitoring such a fast process. In this work, we showed the application of microelectrode electrochemistry with high spatiotemporal resolution to monitor the kinetics of ROS burst from single plant cell and to investigate the regulatory functions of cell wall. An agarose chip with numerous microwell arrays was fabricated to efficiently trap individual plant cells for electrochemical detection. The results revealed that the oxidative burst from single *Arabidopsis thaliana* cells is characterized by numerous transient spikes with fast kinetics. Further experiments indicated that intact plant cells released much less H_2O_2 molecules with faster kinetics to the extracellular environment compared with single protoplasts, while the ROS level could be recovered when the activity of cell wall peroxidase (POD) was inhibited. The results provide evidence that cell wall participates in regulating transient oxidative burst transient by a mechanism involving cell wall POD machinery at single cell level. We think that the results presented here would facilitate better understanding on ROS burst and their regulatory mechanisms.

Key words: oxidative burst; single plant cell; cell wall; microelectrochemical sensor; real-time detection
CLC Number: O657 **Document Code**: A

Reactive oxygen species (ROS) have been known to play important roles in regulation of plant developmental programs[1] and in activation of plant defensive system[2]. A rapid and transient production of ROS (phase I ROS burst) which usually lasts for a few minutes is found to elicit defense system, when plants successfully recognize the abiotic or biotic stress[3-4]. ROS can be protective or toxic depending on their concentrations[5-6], and critical balance between ROS production and metabolism is regulated by complex and perfect mechanisms in plant cells[7]. Although there have been some reports indicated that several enzymatic and non-enzymatic machineries on plasma membranes and cell walls are involved in ROS production[8-9] and scavenging[10], the regulatory mechanisms on ROS production and metabolism are far from being completely understood[4,11].

Various methods including chemiluminescene[12], fluorescence[13-14] and electrophoresis[15] have been developed for ROS analysis. However, deep insight into the rapid and transient ROS burst mechanism requires a method with high sensitivity and spatiotemporal resolution to realize real-time ROS burst monitoring. Electrochemical technique using microelectrodes has been widely used for real-time detection of secreted chemical messengers at single cells[16-17], and its application in investigation of oxidative stress from single animal cells has also been successfully demonstrated[18]. In previous work, by using platinum nanoparticles modified carbon fiber microdisk elec

Received: 2014-07-23, Revised: 2014-10-11 *Corresponding author, Tel: (86-27) 68752149, E-mail: whhuang@whu.edu.cn
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trodes (NPt/CFMDE), we have successfully detected the ROS burst characterized by a large number of "transient oxidative microburst" quantum events from single living protoplasts[19] in real-time. This research achievement enables us to further investigate the kinetic characteristics of ROS burst from plant cells and its regulatory mechanisms. Although protoplasts were always used as a model to study the ROS, absence of cell wall makes them lack many types of enzymatic and non-enzymatic machineries that are quite indispensable in ROS homeostasis[20-21]. Therefore, it is of great importance to detect extracellular ROS burst and to study its kinetics at both single protoplasts and intact cells in real-time for obtaining more comprehensive understanding of ROS regulatory mechanisms.

In this study, we aim to use microelectrode electrochemistry to acquire the quantitative and kinetic characteristics of quantal ROS microburst and to investigate the function of the cell wall in homeostasis during rapid and transient ROS burst. The isolated protoplasts were cultured in Km8P medium to regenerate cell walls. The results showed that the ROS level of intact cell was dramatically decreased as compared with protoplast, while it could be re-upregulated when plant cell wall peroxidase POD activity was inhibited. The results demonstrated that the high level of phase I extracellular oxidative burst generated from protoplast could be well regulated by cell wall peroxidase for ROS homeostasis.

1 Experimental

1.1 Materials and Reagents

5% Nafion, Catalase, 2,1,3-benzothiasiazol (BTH), Fluorescent Brightener 28 (FB28), 4-aminobenzoic acid (PABA) agarose, gelatin were purchased from Sigma (St. Louis, MO). Cellulase R-10 and macerozyme R-10 were purchased from Yalult (Japan). Hydrogen peroxide and tetraammineplatinum(II) chloride monohydrate were purchased from Shenshi Chemical Co Ltd (Wuhan, China), Km8P medium, 2-(2,4-dichloro-phenoxy)acetic acid (2,4-D) and kinetin (KT) for protoplasts culture were kindly supplied by Prof. Li Yang-Sheng in College of Life Sciences at Wuhan University. Carbon fiber was purchased from Goodfellow Cambridge Limited (Oxford, U.K.). All other chemicals unless specified were of reagent grade and used without further purification.

1.2 Fabrication of the NPt/CFMDE

The NPt/CFMDEs were fabricated as we previously described with some modifications[19]. Briefly, a CFMDE was firstly prepared with protruding length of 100 ~ 200 μm and diameter of 5 ~ 7 μm. The CFMDE was dipped into the 1% Nafion (diluted with ethanol) solution for 5 s, and then placed in air for 5 min, repeated the above process for 3 ~ 5 times. The surface of the CFMDE was platinized at a constant potential of -0.7 V in a 0.01 $mol \cdot L^{-1}$ tetraammineplatinum (II) chloride monohydrate ($Pt(NH_3)_4Cl_2 \cdot 2H_2O$) solution until an electric quantity of 2 μC was reached, where a platinum electrode was used as the counter electrode and an Ag/AgCl electrode as the reference electrode. Finally, the NPt/CFMDE was electrochemically activated by continuous potential cycling from -0.20 to 1.80 V (vs. Ag/AgCl) at a scan rate of 100 $mV \cdot s^{-1}$ for 400 s in PBS solution. When not in use, the electrode was stocked in PBS solution in the refrigerator at 4 ℃. The electrochemical and scanning electron microscope characterization were performed on an electrochemical workstation (CHI 660A, CH Instruments, Shanghai, China) and a scanning electron microscope (Quanta 200, Fei, Holland) respectively. To minimize difference of electrochemical behavior between different electrodes, only those electrodes with the same limiting diffusion currents were chosen for cell experiments.

1.3 Protoplasts Isolation and Cell Wall Regeneration

The process for separating protoplasts from Arabidopsis thaliana has been previously described in detail[22]. Briefly, several young leaves were cut from the plant, and then washed with water. In order to culture the protoplast successfully, the whole process must be performed in a sterile environment. After the dirt was washed off, the leaves were transferred into a petri dish and washed by sterile water for three times. Then they were soaked in 75% alcohol for 10 s and

washed with sterile water again. The clean leaves were cut into 10 mm × 1 mm pieces and then put into CPW9M medium (27.2 mg of KH_2PO_4, 101.0 mg of KNO_3, 1480.0 mg of $CaCl_2 \cdot 2H_2O$, 246.0 mg of $MgSO_4$, 0.16 mg of KI, 0.025 mg of $CuSO_4$ dissolved in 1 L ultrapure water containing 9% mannitol, pH 5.6) solution containing 1.5% cellulase and 0.5% macerozyme. The suspension was incubated in darkness for 4 h at 26 ℃ on the shaking bed at a speed of 45 $r \cdot min^{-1}$. Then, the suspension was filtered by 45 μm nylon sieve. The filtered suspension was kept still for 30 min and the bottom solution containing suspension isolated protoplasts were dispersed into PBS buffer to get a cell density of about 5×10^4 mL^{-1}. For cell wall regeneration, the solution containing enzyme was replaced by Km8P medium[23-24]. The protoplasts were cultured in liquid protoplast culture medium (Km8P, 0.05 $mg \cdot L^{-1}$ 2,4-D and 0.1 $mg \cdot L^{-1}$ KT) in a plastic petri dish at 26 ℃ in dark for two days. The regenerated cell walls were tested by FB28 at a final concentration of 10 $\mu mol \cdot L^{-1}$ under a ZEISS Observer Z1 microscopy (ZEISS, Germany).

1.4 Fabrication of the Agarose Microwell Array Chip

The agarose microwell array chip was reproduced using an elastic polydimethylsiloxane (PDMS) stamp which was fabricated using standard soft-lithography technology. A master mold for the PDMS stamp was obtained by patterning SU-8 2010 photoresist (Microchem, USA) on a silicon wafer (a spinning speed of 1000 $r \cdot min^{-1}$ for 20 μm thickness and 3500 $r \cdot min^{-1}$ for 10 μm thickness, respectively). After the silicon masters were exposed to trimethylchlorosilane for 1 min, the mixed PDMS (RTV615, GE Toshiba Silicones Co. Ltd) prepolymer (polymer/curing agent mass ratio of 10:1) was casted onto the master, degassed, and cured at 75 ℃ for 1 h, then peeled off the master mold and the PDMS stamp was obtained (Fig. 1C). Before protoplast or cell detection, 4% agarose soltion (dissolved by CPW9M at about 80 ℃) was transferred to a Petri dish, waiting for about 1 min, and the PDMS stamp was then put onto the agarose hydr ogel for 45 min at room temperature. The agarose chip containing microwell array was successfully reproduced after PDMS stamp was peeled off the agarose (Fig. 1D-F). Similarly, a gelatin chip was obtained by firstly mixing of 12.5% gelatin solution dissolved in CPW9M with 10% transglutaminase solution and then was reproduced by a PDMS stamp at 37 ℃ for 2 h for gelation in an incubator.

1.5 Real-time Electrochemical Monitoring of Oxidative Burst from Single Plant Cells

All amperometric measurements were conducted in a temperature-controlled chamber on an inverted microscope (Axiovert 200M, Zeiss, Göttingen, Germany) placed in a Faraday cage. A two-electrode configuration was employed where an Ag/AgCl electrode was used as the reference electrode. The tip of NPt/CFMDE was positioned precisely close to the surface of plant cells immobilized into the agarose microwell by a micromanipulator (TransferMan NK2, Eppendorf, Hamburg, Germany). In the opposite direction, a glass microcapillary filled with 10 μL 0.1 $mmol \cdot L^{-1}$ BTH connected to a manual piston pump (CellTram Oil, Eppendorf, Hamburg, Germany) was positioned by another micromanipulator to the place where the distance between the cell and the outlet of the microcapillary was about 50 μm to induce the oxidative burst. During the whole experiment, the temperature was kept at 25 ℃. The oxidative burst from single protoplasts was amperometrically recorded by a patch clamp amplifier (EPC10, Heka Elektronik, Germany) at a constant potential of 600 mV. Signals were sampled at 20 kHz, bessel filtered at 2.9 kHz, and for graphical display only, current traces were digitally filtered at 1 kHz. Raw amperometric data were collected using "Pulse" and then analyzed by Igor Pro (Igor Pro, Wave Metrics) and Minianalysis 6.0 software, only events larger than fivefold the RMS (root mean square) noise were taken as oxidative microburst events and used for analysis.

2 Results and Discussion

2.1 Immobilization of Plant Cells with Agarose Microwell Array Chip

The position of each cell must be fixed during

electrochemical detections. Protoplasts and plant cells, unlike mammalian cells which can be tightly adhered onto poly-L-lysine (PLL) or laminin precoated substrates, are not easily immobilized. In previous paper[19], we developed a method that used a 0.15% gelatin coated glass substrate to immobilize plant protoplasts, but some of the protoplasts still lost their positions when the puff of stimulant arrived. Here we developed a robust and versatile technique that uses a microwell array chip based on photolithography and softlithography for large-scale single cells trapping and immobilization.

A SU8 master on silicone wafer was firstly prepared by standard photolithography technique[25], and based on this master, the structures can be well reproduced. Various materials including PDMS, gelatin and agarose were used to fabricate the microwells chips and their trapping efficiencies were compared. The hydrophobic properties of PDMS led to a low single cell trapping efficiency. There were only a few protoplasts or plant cells dropped into the PDMS microwell even after treating PDMS chip with plasma for 1 min. Gelatin hydrogel is a hydrophilic and biocompatible material, but swelling of gelatin hydrogel in aqueous solution usually resulted in size changes of the microwells and ideal immobilization efficiency could not be easily achieved. Agarose gel is another hydrophilic and biocompatible material but with less swelling compared with gelatin, and the embedded structures is easily reproduced by using a PDMS stamp. Considering the average size of protoplasts (37.0 ± 5.4 μm, mean ± s.d.) and intact cell (21.0 ± 3.8 μm) after wall generation from *Arabidopsis thaliana*, two kinds of agarose chips with different sizes of microwells (40 μm in diameter and 20 μm in depth for protoplast; 25 μm in diameter and 10 μm in depth for intact plant cell, 40 μm for the distance between adjacent wells) were fabricated to trap individual cells. The single protoplasts and plant cells were well dispersed into the microwells, and slightly swelling of agarose made the protoplasts and cells tightly immobilized inside the microwells during the electrochemical detection.

2.2 Characteristics of Extracellular ROS Burst from Single *Arabidopsis Thaliana* Protoplasts

Plants can release ROS such as $O_2^{\cdot-}$, H_2O_2 and nitric oxide (NO) in the first few minutes to protect themselves once being attacked by pathogens[4]. Electrochemical monitoring on single *Arabidopsis thaliana* protoplasts evoked by BTH (Fig. 1A) induced numerous transient microburst events (Fig. 1B), which was similar to our previous observations from single oilseed rape protoplasts. At the given time (25 s from the first spikes detected), the average quantal events of 94 ± 11 (mean ± s.d., n =12) were detected. Catalase (0.2 mg·mL^{-1}) that can rapidly eliminate H_2O_2 (Fig. 1C) was used to identify the released species we detected. No amperometric spikes could be obtained (Fig. 1D) when 0.2 mg·mL^{-1} catalase was added into the protoplasts solution before electrochemical monitoring, which indicated that the released molecules evoked by BTH we detected at 0.6 V (vs. Ag/AgCl) were H_2O_2.

Microelectrode recordings at single cells can expose a number of quantitative and kinetic features[26-27], which are previously unknown for transient oxidative burst from plant cells. Statistics on some quantitative and kinetic parameters of the "quantal" bursts of H_2O_2 from the single *Arabidopsis thaliana* protoplasts are presented in Fig. 1E-H and Tab. 1. The maximum amplitude of the oxidation current (I_{max}) and the charge content (Q) represent a measure of the signal molecules content, whereas the rise-time of the spike (T_{rise}, 10% ~ 90%) and the width at half-maximum ($T_{1/2}$) represent the kinetics of single microburst events. Amperometric data yield the mean values of I_{max} = 60.75 ± 28.50 pA and Q = 144.65 ± 57.94 fC (averaged 2018 spikes from 12 protoplasts), corresponding to 4.51 ± 1.82×10^5 H_2O_2 molecules released per each quantal events. The average $T_{1/2}$ and Trise values were 1.11 ± 0.83 ms, 0.99 ± 0.66 ms, respectively, indicating the fast kinetic characteristics of the plasma membrane NADPH oxidase- mediated extracellular transient bursts[4, 28].

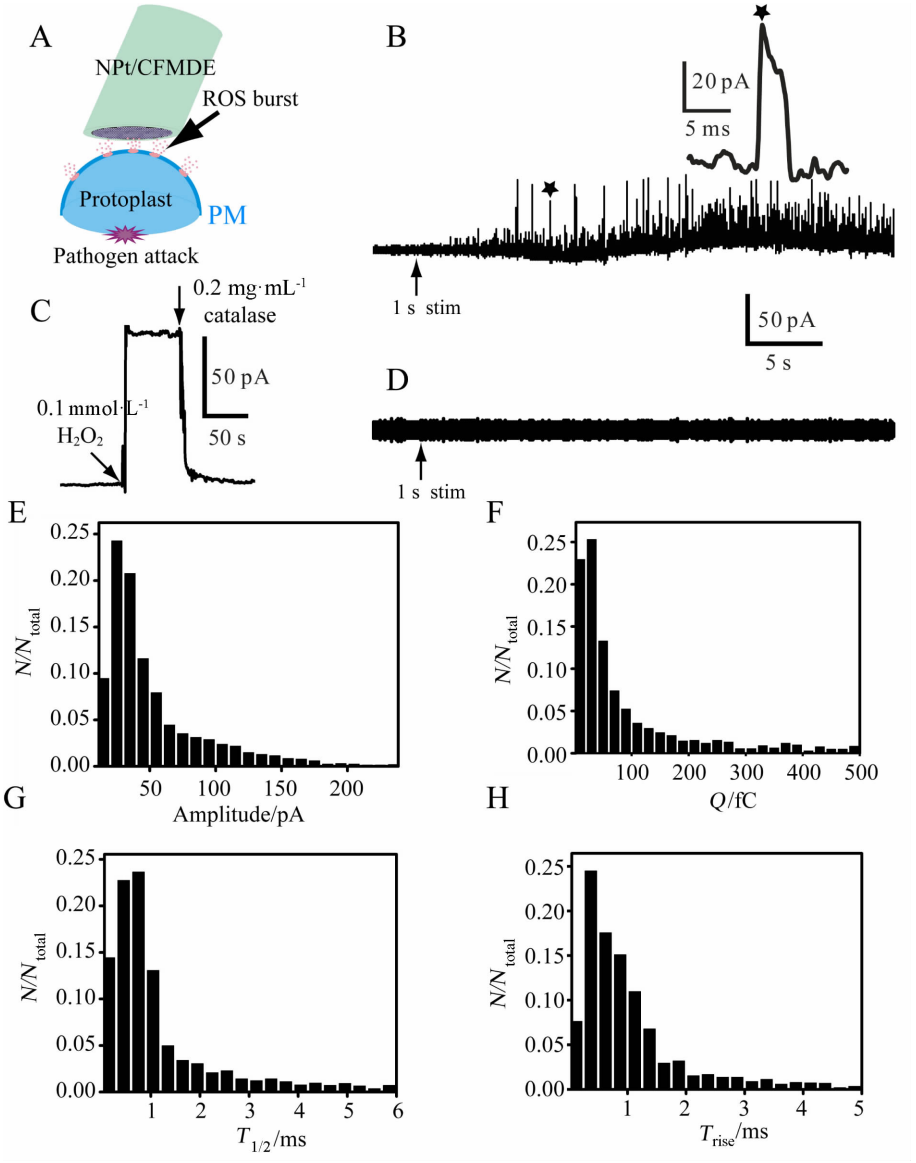

Fig. 1 Amperometric detection of ROS burst from single *Arabidopsis thaliana* protoplasts

A. Schematic diagram showing the detection principle; B. Amperometric signals showing a large number of transient oxidative microburst events from a living protoplast evoked by BTH; C. Amperometric trace showing the H_2O_2 was eliminated by adding catalase; D. No amperometric spikes were detected when catalase added into the protoplast solution; E-H. Histograms of maximum amplitude of the oxidation current (I_{max}), charge content (Q), the width at half-maximum peak currents ($T_{1/2}$) and rise-time of the spike (T_{rise}), respectively.

2.3 Real-Time Monitoring of Extracellular ROS Burst from Single Intact Plant Cells

Protoplasts without cell wall are usually used as the model to study the ROS in biology. However, plant cell walls play significant roles in ROS homeostasis, where various kinds of enzymatic or non-enzymatic machineries are involved in ROS production and scavenging[29]. Enzymolysis was employed for cell prepa ration, hence the cell walls are enzymatically hydrolyzed and only protoplasts could be obtained. Protoplasts are spherical with a clearly visible plasma membrane and populations of small green chloroplasts. In order to obtain single intact plant cells, the protoplasts were cultured in Km8P medium for cell wall regeneration. After culture for 48 hours, about 25% of the cultured protoplasts regenerated cell wall. Compared with protoplasts (37.0 ± 5.4 mm in diame-

ter), the intact plant cells have apparent different appearance with smaller size (21.0 ± 3.8 μm in diameter), less regular shape, and transparent bodies (the populations of small green chloroplasts can not be clearly observed), which facilitate us to distinguish them from protoplasts for electrochemical detection. Moreover, the structure of cellulose in cell walls can be stained by FB28 (Fig. 2B, C).

Fig. 2D depicts a representative amperometric trace from a single plant cell. Compared with protoplasts, the quantal microburst frequency was obviously decreased, and an average of 41 ± 10 events was recorded during the first 25 s from the first amperometric spike detected. Furthermore, the individual burst events were also greatly reduced. The average I_{max} and Q values for individual burst events were 16.93 ± 6.83 pA and 18.27 ± 7.78 fC (12 cells, 1164 spikes), corresponding to 28% and 13% of those from single protoplasts in the released H_2O_2 molecule concentration and amount, respectively (Fig. 2E, F and Tab. 1).

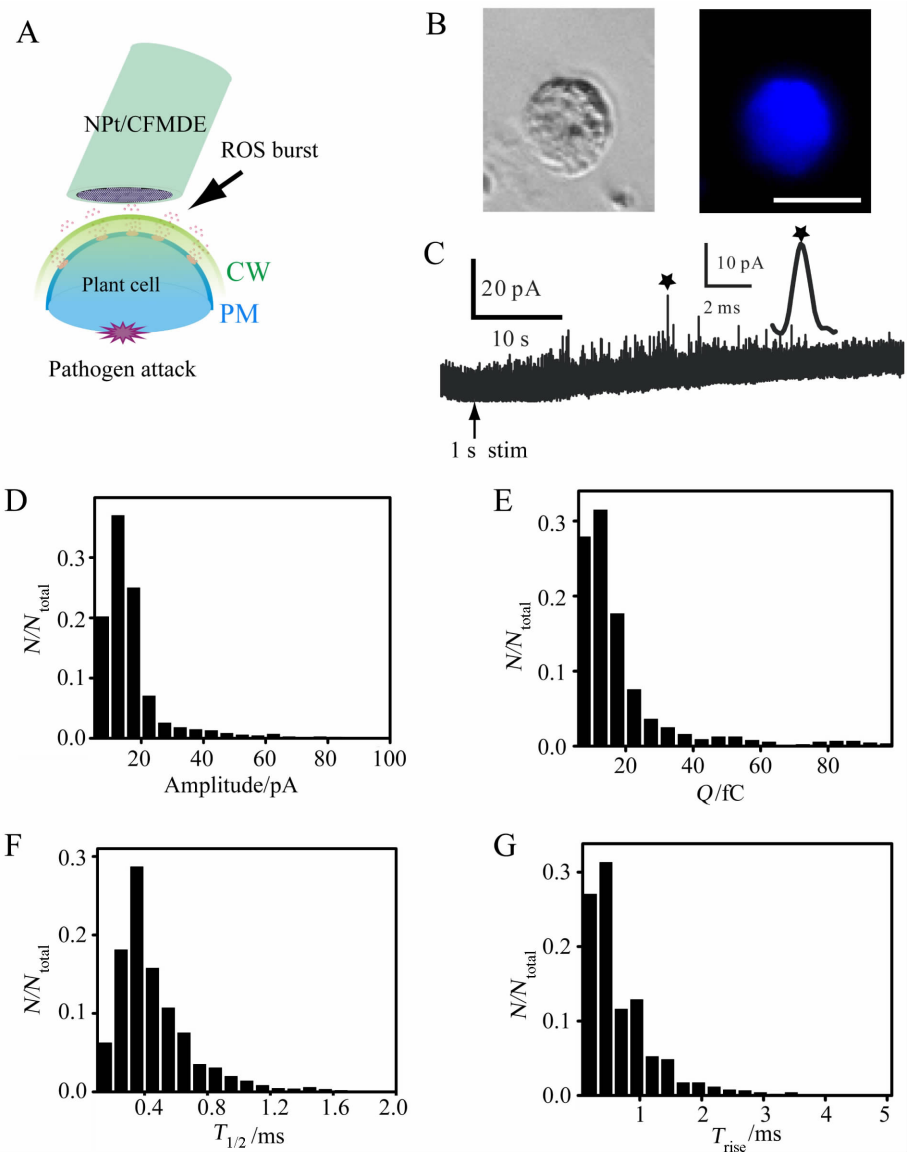

Fig. 2 Amperometric detection of ROS burst from single intact *Arabidopsis thaliana* cells after cell wall regeneration

A. Schematic diagram showing the detection principle; B,C. Bright-field and fluorescent microscope images of a single plant cell, scale bar represents 20 μm; D. Amperometric signals showing the downregulated transient oxidative microburst events from a living plant cell; E-H. Histograms of I_{max}, Q, $T_{1/2}$ and Trise of individual oxidative microburst events, respectively.

Taking account of the lower ROS bursts frequency after cell wall regeneration, much less total amount of H_2O_2 molecules were detected from single intact plant cells.

In plant tissues, H_2O_2 with a high concentration was also a toxic molecule for plant themselves. Cell wall forms an effective barrier to plasma membrane and our results indicated that the high level of ROS produced by protoplast could be well controlled by plant cell wall. This phenomenon was accordance with the previous biological results[29-30].

The ROS microburst events from intact cell showed a slightly faster kinetics than that from protoplasts. The average $T_{1/2}$ and Trise values were 0.46 ± 0.18 ms and 0.74 ± 0.42 ms, respectively (Fig. 2G, H). This is an interesting phenomenon. Amperometric peaks with larger $T_{1/2}$ and Trise should be expected if the cell walls act as only a physical barrier to ROS, since the plasma membrane NADPH oxidase-mediated ROS must diffuse more distances before arriving at the electrode surface for detection. However, in the case the electrode was placed touching the cell wall surface, the pulse widths we detected obviously decreased. The time parameters should be corresponding to the kinetics of ROS burst from cell wall instead of from plasma membrane, which indicated that the kinetics of ROS burst was also possibly regulated by cell wall. Further experiments are needed to explain this matter.

2.4 Cell Wall POD in Downregulation of the Extracellular ROS Burst

In plant cells, peroxidase (POD) mainly exists on the cell wall and plays dual roles on ROS metabolism by either eliminating high concentration of ROS or producing of ROS in specifically cases[28, 31]. Although its functions in regulation of oxidative burst are widely demonstrated at plant tissues, such investigation has not been performed at single cell levels. As mentioned above, plant cells displayed a dramatically decreased ROS burst after cell wall regeneration. Here we used PABA (10 μmol·L^{-1}), a POD inhibitor[32] to test the function of POD (the PABA was existed in the suspension cell solution during the experiment) in ROS homeostasis. We found that the I_{max} and Q val-

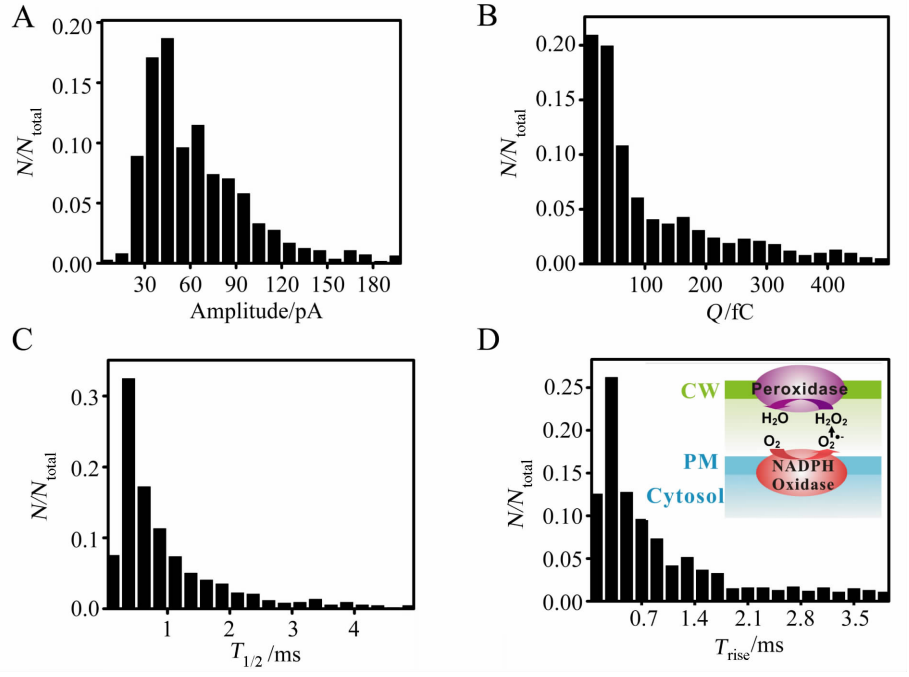

Fig. 3 Amperometric detection of ROS burst from single intact *Arabidopsis thaliana* cells after PABA added. Histograms of I_{max} (A),Q (B), $T_{1/2}$ (C) and T_{rise} (D) of individual oxidative microburst events from single *Arabidopsis thaliana* cells in which cell wall POD was inhibited by PABA, the possible mechanism of cell wall POD in scavenging H_2O_2 is shown in the inset of (D).

Tab. 1 Mean values (mean ± s.d.) of quantitative and kinetic parameters of amperometric spikes from the oxidative bursts on single *Arabidopsis thaliana* protoplasts and intact cells

Cell type	Frequency[a]	I_{max}/pA	Q/fC	$T_{1/2}$/ms	T_{rise}/ms
Protoplasts (2018 spikes from 12 protoplasts)	94 ± 11	60.75 ± 28.50	144.65 ± 57.94	1.11 ± 0.83	0.99 ± 0.66
Plant cells[b] (1164 spikes from 12 cells)	41 ± 10	16.93 ± 6.83	18.27 ± 7.78	0.46 ± 0.18	0.74 ± 0.42
Plant cells[c] (1084 spikes from 9 cells)	53 ± 8	49.25 ± 20.09	100.15 ± 47.97	0.97 ± 0.65	0.91 ± 0.62

a. number of amperometric spikes during 25 s from the first spikes detected; b. *Arabidopsis thaliana* cells after cell wall regeneration; c. POD inhibited *Arabidopsis thaliana* cells.

ues were obviously increased after adding PABA into the plant cell medium. The average of I_{max} and Q values for microburst events were 49.25 ± 20.09 pA and 101.5 ± 49.97 fC (9 cells, 1084 spikes, Fig. 3A, B and Tab. 1), corresponding to 2.9 fold and 5.5 fold enhancement compared with that from intact plant cells, and recovered to 81% and 69% of that from protoplasts, respectively (Tab. 1). The average values of $T_{1/2}$ and Trise were 0.97 ± 0.65 ms and 0.91 ± 0.62 ms, respectively (Fig. 3C, D). The results demonstrated that during the phase I ROS burst, one of the functions of cell wall POD is possibly to downregulate ROS level released to the extracellular environment by scavenging part of H_2O_2 produced on plasma membrane (Fig. 3D).

3 Conclusions

Electrochemical detection using highly sensitive microsensor provides an effective tool for monitoring fast kinetics of ROS burst in real-time and investigating their potential mechanisms at single cell levels. In this study, we used highly sensitive NPt/CFMDE to detect the kinetics of ROS burst and to investigate cell wall's regulatory functions from single plant cell. Agarose microwell array chip supplied a high throughput and large-scale approach to immobilize plant cells. Microelectrode recordings at single cells exposed a number of previously unknown quantitative and kinetic features on transient oxidative burst from plant cells. Our detection demonstrated that the intact plant cells after cell wall regeneration released much less H_2O_2 molecules with faster kinetics to the extracellular environment compared with single protoplasts, while the ROS level could be recovered when the activity of cell wall POD was inhibited. The results indicated the direct evidence that the cell wall participated in regulating transient ROS burst, and the high level ROS burst could be efficiently downregulated to the extracellular by a mechanism involving cell wall POD machinery. The regulated ROS bursts possibly have dual roles in preventing damages to plant cells by high ROS level and acting messenger molecules to elicit the activation of defense mechanisms. Our results presented here are in reasonable agreement with those obtained by traditional biological assay. In a word, we successfully demonstrated the application of electrochemical tools to obtain biological functions and mechanisms of ROS at individual plant cell levels, and would facilitate better understanding on ROS burst and their regulatory mechanisms.

Acknowledgements

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References:

[1] Gilroy S, Swanson S. Ros in plant development[J]. Physiologia Plantarum, 2010, 138(4): 384-392.

[2] Torres M A. Ros in biotic interactions[J]. Physiologia Plantarum, 2010, 138(4): 414-429.

[3] Orozco-Cardenas M L, Narvaez-Vasquez J, Ryan C A. Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate[J]. Plant Cell, 2001, 13(1): 179-191.

[4] Torres M A, Jones J D, Dangl J L. Reactive oxygen species signaling in response to pathogens[J]. Plant Physiology, 2006, 141(2): 373-378.

[5] Gemes K , Poor P, Horvath E, et al. Cross-talk between salicylic acid and nacl-generated reactive oxygen species and nitric oxide in tomato during acclimation to high salinity[J]. Physiologia Plantarum, 2011, 142(2): 179-192.

[6] Shetty N P, Jorgensen H J L, Jensen J D, et al. Roles of reactive oxygen species in interactions between plants and pathogens[J]. European Journal of Plant Pathology, 2008, 121(3): 267-280.

[7] Dixit R, Cyr R. Cell damage and reactive oxygen species production induced by fluorescence microscopy: Effect on mitosis and guidelines for non-invasive fluorescence microscopy[J]. Plant Journal, 2003, 36(2): 280-290.

[8] O'Brien J A, Daudi A, Finch P, et al. Peroxidase-dependent apoplastic oxidative burst in cultured arabidopsis cells functions in MAMP-elicited defense[J]. Plant Physiology, 2012, 158(4): 2013-2027.

[9] Bolwell G P, Davies D R, Gerrish C, et al. Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms[J]. Plant Physiology, 1998, 116(4): 1379-1385.

[10] Srivastava V, Schinkel H, Witzell J, et al. Downregulation of high-isoelectric-point extracellular superoxide dismutase mediates alterations in the metabolism of reactive oxygen species and developmental disturbances in hybrid aspen[J]. Plant Journal. 2007, 49(1): 135-148.

[11] Kotchoni S O, Gachomo E W. The reactive oxygen species network pathways: An essential prerequisite for perception of pathogen attack and the acquired disease resistance in plants[J]. Journal of Biosciences, 2006, 31(3):389-404.

[12] Yamashiro N, Uchida S, Satoh Y, et al. Determination of hydrogen peroxide in water by chemiluminescence detection, (i) flow injection type hydrogen peroxide detection system[J]. Journal of Nuclear Science and Technology, 2004, 41(9):890- 897.

[13] Li H M, Li O L, Wang X, et al. Simultaneous determination of superoxide and hydrogen peroxide in macrophage raw 264.7 cell extracts by microchip electrophoresis with laser-induced fluorescence detection[J]. Analytical Chemistry, 2009, 81(6): 2193-2198.

[14] Chen Z Z, Li Q L, Wang X, et al. Potent method for the simultaneous determination of glutathione and hydrogen peroxide in mitochondrial compartments of apoptotic cells with microchip electrophoresis-laser induced fluorescence[J]. Analytical Chemistry, 2010, 82(5): 2006-2012.

[15] Gong X C, Li Q L, Xu K H, et al. A new route for simple and rapid determination of hydrogen peroxide in raw264. 7 macrophages by microchip electrophoresis [J]. Electrophoresis, 2009, 30(11): 1983-1990.

[16] Wightman R M, Jankowski J A , Kennedy R T, et al. Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells[J]. Proceedings of the National Academy of Sciences, 1991, 88(23): 10754-10758.

[17] Schuhmann W, Schulte A. Single-cell microelectrochemistry[J]. Angewandte Chemie International Edition, 2007, 46(46): 8760-8777.

[18] Amatore C, Hu R, Guille M, et al. In situ electrochemical monitoring of reactive oxygen and nitrogen species released by single mg63 osteosarcoma cell submitted to a mechanical stress[J]. The Journal of Chemical Physics, 2010, 12(34): 10048-10054.

[19] Ai F, Chen H, Zhang S H, et al. Real-time monitoring of oxidative burst from single plant protoplastsusin microelectrochemical sensors modified by platinum nanoparticles[J]. Analytical Chemistry, 2009, 81(20): 8453-8458.

[20] Patykowski J. Role of hydrogen peroxide and apoplastic peroxidase in tomato-botrytis cinerea interaction [J]. Acta Physiologiae Plantarum, 2006, 28(6): 589-598.

[21] Minibayeva F, Kolesnikov O, Chasov A, et al. Wound-induced apoplastic peroxidase activities: Their roles in the production and detoxification of reactive oxygen species [J]. Plant Cell and Environment, 2009, 32(5): 497-508.

[22] Forsberg J, Landgren M, Glimelius, K. Fertile somatic hybrids between *brassica napus* and *Arabidopsis thaliana* [J]. Plant Science, 1994, 95(2): 213-223.

[23] Saka K, Katterman F R, Thomas J C. Cell regeneration and sustained division of protoplasts from cotton[J]. Plant Cell Reports, 1987, 6(6): 470-472.

[24] Thomas T D. Isolation, callus formation and plantlet regeneration from mesophyll protoplasts of tylophora indica (burm. F.) merrill: An important medicinal plant[J]. *In Vitro* Cellular & Developmental Biology-Plant, 2009, 45 (5): 591-598.

[25] Xia Y, Whitesides G M. Soft lithography[J]. Angewandte Chemie International Edition, 1998, 37(5): 550-575.

[26] Wightman R M. Probing cellular chemistry in biological

systems with microelectrodes[J]. Science, 2006, 311(5767): 1570-1574.

[27] Amatore C, Arbault S, Guille M, et al. Electrochemical monitoring of single cell secretion: Vesicular exocytosis and oxidative stress[J]. Chemical Reviews, 2008, 108(7): 2585-2621.

[28] Quan L J, Zhang B, Shi W W, et al. Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network[J]. Journal of Integrative Plant Biology, 2008, 50(1): 2-18.

[29] Morina F, JovanovicL, Mojovic M, et al. Zinc-induced oxidative stress in *verbascum thapsus* is caused by an accumulation of reactive oxygen species and quinhydrone in the cell wall[J]. Physiologia Plantarum, 2010, 140(3): 209-224.

[30] Luo Y, Liu Y B, Dong Y X, et al. Expression of a putative alfalfa helicase increases tolerance to abiotic stress in arabidopsis by enhancing the capacities for ros scavenging and osmotic adjustment[J]. Journal of Plant Physiology, 2009, 166(4): 385-394.

[31] Deepak S A, Ishii H, Park P. Acibenzolar-s-methyl primes cell wall strengthening genes and reactive oxygen species forming/scavenging enzymes in cucumber after fungal pathogen attack[J]. Physiological and Molecular Plant Pathology, 2006, 69(1): 52-61.

[32] Brisch E, Daggett M A, Suprenant K A. Cell cycle-dependent phosphorylation of the 77 kda echinoderm mi crotubule-associated protein (EMAP) *in vivo* and association with the p34(cdc2) kinase[J]. Journal of Cell Science, 1996, 109(12): 2885-2893.

超微电极实时监测植物细胞壁参与调控的单细胞活性氧爆发

刘俊桃,刘艳玲,程 治,陈时靖,黄卫华*

(武汉大学 化学与分子科学学院,生物医学分析化学教育部重点实验室, 湖北 武汉 430072)

摘要: 植物细胞活性氧爆发在植物的抗病以及信号转导中起着非常重要的作用,植物内活性氧产生及代谢受到复杂而精确的机制调控,从而维持正常的活性氧水平以发挥其生理功能. 然而,在单细胞水平开展活性氧爆发实时监测及其调控机制研究一直受到很大的挑战. 本文以碳纤维微盘电极(CFMDE)为基底电极,利用 Nafion 的模板效应, 采用电化学沉积法制得纳米铂颗粒修饰电极 (NPt/Nafion/ CFMDE); 同时采用基于聚二甲基硅氧烷(PDMS)的软光刻技术,制备了一种高效固定植物悬浮细胞的琼脂糖阵列微孔芯片. 使用 NPt/Nafion/CFMDE 实时监测了单个拟南芥原生质体活性氧爆发,并证明电化学监测活性氧的主要成分为过氧化氢. 在此基础上,采用浅层培养法培养原生质体再生植物细胞壁. 电化学监测结果表明,与单个原生质体相比,植物细胞在受到刺激时释放的过氧化氢量显著降低;然而当采用过氧化物酶抑制剂抑制植物细胞壁上过氧化物酶活性后,植物细胞释放过氧化氢量显著回升. 研究结果表明,细胞壁在活性氧爆发过程具有很好的调控功能,可望促进植物细胞活性氧爆发及其调控机制的研究.

关键词: 氧爆发; 单细胞; 细胞壁; 微电极; 实时监测