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Paper-based electroanalytical devices for *in situ* determination of salicylic acid in living tomato leaves

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ABSTRACT

Detection of phytohormones *in situ* has gained significant attention due to their critical roles in regulating developmental processes and signaling for defenses in plants at low concentration. As one type of plant hormones, salicylic acid has recently been found to be one of pivotal signal molecules for physiological behaviors of plants. Here we report the application of paper-based electroanalytical devices for sensitively *in situ* detection of salicylic acid in tomato leaves with the sample volume of several microliters. Specifically, disposable working electrodes were fabricated by coating carbon tape with the mixture of multiwall carbon nanotubes and nafion. We observed that the treatment of the modified carbon tape electrodes with oxygen plasma could significantly improve electrochemical responses of salicylic acid. The tomato leaves had a punched hole of 1.5 mm diameter to release salicylic acid with minor influence on continuous growth of tomatoes. By incorporating the tomato leaf with the paper-based analytical device, we were able to perform *in situ* determination of salicylic acid based on its electrocatalytic oxidation. Our experimental results demonstrated that the amounts of salicylic acid differed statistically in normal, phytoene desaturase (PDS) gene silent and diseased (infected by *Botrytis cinerea*) tomato leaves. By quantifying salicylic acid at the level of several nanograms *in situ*, the simple paper-based electroanalytical devices could potentially facilitate the study of defense mechanism of plants under biotic and abiotic stresses. This study might also provide a sensitive method with spatiotemporal resolution for mapping of chemicals released from living organisms.

1. Introduction

Plants are constantly attacked by biotic stresses, such as pathogenic bacteria, and abiotic stresses, including drought and cold. In order to protect themselves, plants have evolved complicated and effective defense mechanisms, such as innate immune responses and systemic acquired resistance (Boller and He, 2009; Chisholm et al., 2006). Activation of such defense pathways requires small signaling molecules, namely, phytohormones (Bari and Jones, 2009; Zhang and Zhou, 2010). Such plant hormones are essential to regulate multiple physiological processes of plants, including growth, development, reproduction and response to various stresses. Plant yields could be incresed by hormone regulation through controlling crop size and shape, enhancing crop stress tolerance, and increasing grain productivity (McSteen and Zhao, 2008; Peleg and Blumwald, 2011; Santner et al., 2009).

As one of important phytohormones, salicylic acid has been demonstrated to be involved in many physiological processes of plants, including growth and development (Vicente and Plasencia, 2011). One of the most well-known roles of salicylic acid is signaling for defense in plant immune responses (An and Mou, 2011; Hayat et al., 2012; Loake and Grant, 2007). Significant progress has been made during the past decade to understand mechanisms in the network of salicylic acid-mediated defense signaling (An and Mou, 2011). It has been validated that pathogen infection can lead to accumulation of salicylic acid in infected leaves, leading to enhanced expression of pathogenesis related

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genes (An and Mou, 2011; Hayat et al., 2012). Moreover, both local and systematic acquired resistances depend on signaling pathway mediated by salicylic acid, which could always crosstalk with other phytohormones, such as jasmonic acid and auxin, etc. (An and Mou, 2011; Bari and Jones, 2009; Zhang and Zhou, 2010). More importantly, these responses are regulated by tissue specific or localized biosynthesis and transport of phytohormones in plants and their mapping in the plant body is critical for understanding these processes (Brunoud et al., 2012). Therefore, *in situ* and real time analysis of salicylic acid especially in shoot tissues, such as leaves, would be essential for investigating salicylic acid-mediating defense mechanisms. A convinient method has yet to be developed to measure the salicylic acid levels in shoots in a timely fashion.

Conventionally, the quantification of salicylic acid in plants can be carried out with high performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC–MS), etc. (Aboul-Soud et al., 2004; Giannarelli et al., 2010). In these approaches, the samples harvested from plants normally need to be ground in liquid nitrogen and then filtered for extraction of salicylic acid followed by drying with flowing nitrogen. The required sample amount, the intricate procedure and the expensive instruments make it impossible to rapidly detect salicylic acid *in situ*. Alternatively, salicylic acid could also be electrochemically detected in multi-component samples based on its electroactivity in its form of free acid but not in its conjugates. For example, salicylic acid has been quantified with bare carbon paste electrodes and modified electrodes in the bulk solution (Gualandi et al., 2011; Torriero et al., 2004; Wang et al., 2010a,b). However, *in situ* detection of salicylic acid in live plants has never been demonstrated so far.

Pioneered by Whitesides' research group, a number of paper-based analytical devices have been developed as low cost platforms based on coupling of modern techniques, such as photolithography and screen printing, etc. (Cheng et al., 2010; Ellerbee et al., 2009; Martinez et al., 2008). Recent investigations have shown great potentials of paper-based analytical devices in biological and medical applications, such as cell electroporation and blood tests (Gong et al., 2010; Songjaroen et al., 2012; Vella et al., 2012; Yang et al., 2012). A specific advantage of paper-based analytical devices is that the required volume of sample solution could be as low as several micro litres. For example, Whitesides' research group demonstrated that enzyme-linked immunosorbent assay could be carried out using the array on patterned paper with the sample volume of only 3 μL (Cheng et al., 2010). Since Whitesides' and Henry's research groups have already demonstrated integration of electrochemical detections in paper-based analytical devices (Dungchai et al., 2009; Nie et al., 2010; Santhiago et al., 2013), it is believed that those devices could open more opportunities for *in situ* analysis of biomaterials that requires low sample volumes. In addition, previous works have demonstrated that the utilization of carbon-based nanomaterials could significantly enhance analytical performance of biosensors (Shi et al., 2013). For example, the applications of carbon nanotube (McLamore et al., 2011; Shi et al., 2011a,b) and graphene (Shi et al., 2012) have addressed the problem of low-sensitivity of biosensors in basic physiology/pathophysiology studies.

In this work, we applied paper-based analytical devices for *in situ* electrochemical detection of salicylic acid in live tomato leaves. Interestingly, we demonstrated that electrochemical responses of salicylic acid could be significantly improved after treatment of MWCNTs/Nafion modified carbon tape electrodes with oxygen plasma. By coupling the modified carbon tape electrode in paper-based electroanalytical device, the amount of salicylic acid could be differentiated *in situ* in normal, gene-silenced and diseased tomato leaves. Our strategy suggested that paper-based electroanalytical devices could potentially become effective platforms for measurement of some chemicals in live organisms.

2. Materials and methods

2.1. Chemicals and materials

Salicylic acid at the analytical grade was purchased from Sigma (St Louis, MO, USA). The dispersion of MWCNTs (length, 10–20 μm, outside diameter, >50 nm, inside diameter, 5–15 nm) in water with the weight ratio of 2% was purchased from Nanjing Xianfeng Nanomaterials Co., Ltd. (Nanjing, Jiangsu, China). Nafion with the concentration of 5% was obtained from Dupont (Wilmington, DE, USA). Other chemicals were of analytical grade unless otherwise mentioned. The qualitative filter paper (Whatman No. 1) was purchased from Whatman International Ltd. (Maidstone, UK). The Indium tin oxide (ITO) conductive glass (355.6 mm wide, 406.4 mm long, 1.1 mm thick, STN, 10 Ω) was purchased from Nanbo Display Technology Co. LTD. (Shenzhen, China). The conductive double-sided carbon adhesive tape (8 mm wide, 0.16 mm thick and 20 m long) was purchased from SPI Supplies (West Chester, PA, USA).

2.2. Sample preparation

The stock solution of salicylic acid (0.1 M) was dissolved in ethanol and diluted freshly by phosphate buffered solution with the pH value of 7.0 and stored at 4 °C before use. The tomato seedlings were grown in a plant growth room at 20–24 °C. Four-week-old tomato seedlings were inoculated with fungal pathogen *Botrytis cinerea* (*B. cinerea*) through foliar spraying with spore suspension of strain B05.10 at the concentration of 2×10^5 spores/mL until the whole plants were uniformly covered with tiny droplets. Plants sprayed with water were used as mock-inoculated controls. Then the inoculated and control seedlings were kept in above 90% relative humidity. The knock-down of phytoene desaturase (PDS) was obtained using tobacco rattle virus (TRV) derived virus-induced gene silencing (VIGS) vector, which could result in photobleaching phenotype (Romero et al., 2011; Velasquez et al., 2009).

Tomato leaves with normal sizes were collected to extract salicylic acid for HPLC–MS/MS determination according to previous protocols (Li et al., 2011). Briefly, tomato leaves were ground in liquid nitrogen and resuspended with propanol/water/HCl (2:1:0.002 in volume ratio) in a vial followed by oscillation. Dichloromethane was added for the first extraction and centrifugation. The subnatant was collected and the residue was mixed with dichloromethane for second extraction and centrifugation. Both subnatants were mixed and then dried. Methanol was added to solve the sample by sonication. The methanol solution consisting of samples was passed through the activated C18 column and eluted with methanol. The elution solution with the volume of 2 mL was collected in 4 mL chromatography bottle and dried with vacuum at 35 °C. The residue was solved with 0.5 mL methanol and then transferred to a 2 mL chromatography bottle followed by drying with vacuum. Finally the residue was solved in 400 μL mixture of methanol/0.05% formic acids solution with the ratio of 1:1. The sample solution consisting of salicylic acid was analyzed with HPLC–MS/MS.

2.3. Paper-based electroanalytical devices

A piece of carbon tape (12 mm long, 7 mm wide) was attached on the conductive surface of ITO glass (20 mm long, 7 mm wide)