A facile colorimetric assay for determination of salicylic acid in tobacco leaves using titanium dioxide nanoparticles†

Po-Jen Tseng,a Chiung-Yi Wang,a Tzu-Yun Huang,a Yuan-Yu Chuang,a Shih-Feng Fu† and Yang-Wei Lin*a

A facile, colorimetric method for salicylic acid (SA) detection in tobacco leaves was developed using titanium dioxide nanoparticles (TiO2 NPs). The sensing strategy is based on the reaction of TiO2 NPs with SA at pH 5.5, which results in an intramolecular ligand to metal charge transfer transition between salicylate and Ti(n) on the surface of TiO2 NPs, and causes the TiO2 NP solution to turn yellow. The TiO2 NP probe exhibits high selectivity for SA over seven structural chemicals (4-hydroxybenzoic acid, 3'-hydroxybenzoic acid, benzoic acid, acetylsalicylic acid, phenol, methyl salicylate, and jasmonic acid). Moreover, the difference in the absorbance of the TiO2 NP solution is proportional to the concentration of SA over the range from 0.02 to 1.0 mM ($R^2 = 0.992$). By using the TiO2 NP probe in 5 mM sodium acetate (pH 5.5) solutions, the limit of detection for SA was 15.4 µM at a signal-to-noise (S/N) ratio of 3. Furthermore, the practicality of the TiO2 NP probe was validated for the determination of SA in tobacco leaves by demonstrating its advantages including simplicity and selectivity.

Introduction

Salicylic acid (SA; 2-hydroxybenzoic acid) has been shown to play an important role in plants for heat production, flowering, and germination processes.1–4 Moreover, it has been found to be a key compound for pathogen resistance and the associated signal transduction.5–8 Its accumulation can induce immune responses such as age-related resistance and systemic acquired resistance (SAR), and gene expression associated with these responses.9–11 It can also contribute to the hypersensitive response (HR), which is the action of programmed cell death induced by signals to restrict pathogen spreading.12–15 Therefore, it is necessary to quantify SA routinely in the field of plant immunity.16

To date, quantitative detection of SA in plant tissues has been achieved using liquid chromatography.17–19 The technique involves extraction of SA into organic solvents, followed by chromatographic separation and detection via spectroscopy. More recently, the concentration of SA has been further determined by mass spectroscopy (MS).20–24 In some research, the use of an enzyme-linked immunosorbent assay method has been reported for the quantitative analysis of SA in plant extracts.25,26 These methods are highly accurate, quantitative and can be adapted for high throughput analysis of many samples; however, the extraction and purification of SA are laboratory intensive.27 They are also destructive and cannot provide information on the spatial distribution of SA within plant tissues. Recently, Huang et al. demonstrated the SA biosensor Acinetobacter sp. ADPWH_lux.28,29 This strain which contains a chromosomal integration of a salicylate inducible luxCDABE operon provides the substrate and catalyst for SA luminescence. Measurement of SA from tobacco mosaic virus (TMV)-infected tobacco leaves using the biosensor and MS found similar results, demonstrating that this strain is adequate for the determination of SA in plants in vivo. However, the reaction time should be 2 h prior to imaging and this biosensor is laboratory intensive.

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† Electronic supplementary information (ESI) available: Fig. S1: diffuse reflectance spectra of (a) TiO2 NPs and (b) TiO2 NPs with SA (1.0 mM). Fig. S2: TEM images of (a) 24 µM TiO2 NPs and (b) 24 µM TiO2 NPs in the presence of SA (1.0 mM). Fig. S3: Raman spectrum of 240 µM TiO2 NPs in the presence of SA (1.0 mM). Fig. S4: SALDI-MS spectra of (a) 1.0 mM SA and (b) 24 µM TiO2 NPs in the presence of SA (1.0 mM). Fig. S5: zeta potential and hydrodynamic diameter of 24 µM TiO2 NPs in the presence of different concentrations of SA (0–10 mM). Fig. S6: effect of the TiO2 NP concentration (12–120 µM) on the values for (A – $A_0$)/$A_0$, where A and $A_0$ represent the absorbance at 420 nm of TiO2 NPs in the presence and absence of SA (1.0 mM), respectively (n = 3). Fig. S7: effect of the (A) buffer system, (B) pH, and (C) concentration of sodium acetate buffer solution on the absorbance difference (A – $A_0$), where A and $A_0$ represent the absorbance at 420 nm of TiO2 NPs in the presence and absence of SA (1.0 mM), respectively (n = 3). Fig. S8: UV-Vis spectra of various concentrations of SA (0.04 to 1.0 mM) in the absence of TiO2 NPs. Fig. S9: analysis of tobacco leaf extracts (aliquots spiked with SA [0.04–0.9 mM]) using 24 µM TiO2 NPs (n = 3). See DOI: 10.1039/c3ay42209g.
Herein, we report a facile, colorimetric detection method for SA in tobacco leaves using a nanoparticle-based sensor. In recent years, the study of metal oxide particles with well-defined nanostructures has become one of the most active research areas. Titanium dioxide nanoparticles (TiO2 NPs) are representative materials that have received considerable attention for use in dye-sensitized photovoltaic cells and photocatalysis.\textsuperscript{20, 21} In addition, the surface charge-transfer complexes between TiO2 NPs and enediols have been reported.\textsuperscript{22, 23} As mentioned in the study, upon photoexcitation, an electron is considered to be transferred from the HOMO of the enediols to the conduction band of the TiO2 through the LUMO of the enediols. Besides the intrinsic interest of these systems for studying electron-transfer processes, these enediols also act as surface photosensitizers, enabling the TiO2 to absorb and respond to the visible light region.\textsuperscript{14, 24}–\textsuperscript{26} SA has an enediol-type structure; the possibility of its quantitative detection of the TiO2 NP sensor was then validated for the detection of SA in tobacco leaf extracts. Finally, tobacco leaves were immersed in a solution of TiO2 NPs to determine the spatial distribution of SA levels with and without the appearance of the HR elicited by UV irradiation in an NN genotype tobacco. These results indicate that the TiO2 NP sensor is potentially useful for the non-destructive visualization of changes in the SA content in plant tissues.

**Experimental section**

**Materials**

Titanium(IV) isopropoxide, SA, acetylsalicylic acid (AA), sodium hydroxide, sodium acetate, jasmonic acid (JA), acetic acid, and acetonitrile were obtained from Sigma (St. Louis, MO, USA), and 4-hydroxybenzoic acid (4-HBA), 3-hydroxybenzoic acid (3-HBA), benzoic acid (BA), methyl salicylate (MeS), nitric acid, ethyl acetate, cyclohexane, boric acid and sodium phosphate were obtained from Acros (Morris Plains, NJ, USA). Phenol (ph) was purchased from Tokyo Chemical Industry (Tokyo, Japan), and trichloroacetic acid and methanol were obtained from Fluka (Ronkonkoma, NY, USA).

**Apparatus**

UV-Vis absorption spectra of the TiO2 NPs in the absence and presence of SA were recorded using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT, USA). UV-Vis diffuse reflectance spectra of the samples were measured by using an Evolution 220 UV-Vis spectrophotometer (Thermo Scientific Inc., NY, USA). A JEOL-1200EX II transmission electron microscopy system (JEOL, Tokyo, Japan) was used to measure the size and shape of the TiO2 NPs in the absence and presence of SA. The Raman spectrum of the TiO2 NPs in the presence of SA was recorded using a DXR Raman Microscope (Thermo Scientific Inc., NY, USA). The Microflex matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MS) experiment was performed in the negative ion mode (MALDI-TOF-MS, Bruker Daltonics, Bremen, Germany). The hydrodynamic diameters and zeta potentials of the TiO2 NPs in the presence of SA were measured using a Zetasizer Nano ZS90 apparatus (Malvern, UK). A high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) was used to drive electrophoresis. The CE system was coupled to a variable wavelength UV detector (SAPPHIRE 600, ECOM, Praha, Czech Republic). Data acquisition and processing were accomplished using a PC equipped with a Peak-ABC Chromatography Data Handling System (Shanghai Qianpu Software Company Ltd., Shanghai, China).

**Preparation of TiO2 NPs**

The TiO2 NPs were prepared via a sol–gel reaction according to a previously described procedure.\textsuperscript{36} Titanium isopropoxide (10 mL) was added to 0.1 M nitric acid (60 mL) with vigorous stirring at 80 °C, and a white precipitate formed instantaneously. After that, the slurry was heated at 80 °C and stirred vigorously for 8 h, leading to the formation of a sol and then a colloidal solution. The concentration of the as-prepared TiO2 NPs was estimated to be 240 μM (2 × 10\textsuperscript{17} particles per mL) by assuming that the titanium isopropoxide reacted completely to form TiO2 NPs.\textsuperscript{36}

**General procedure for colorimetric analysis**

A stock solution of SA (10 mM) prepared in deionized (D.I.) water was diluted to 0–1.0 mM, and each dilution was added to a 5 mM sodium acetate buffer solution (pH 5.5) containing 24 μM TiO2 NPs to give a final volume of 1000 μL. For selective determination of SA, seven structural analogues (4-HBA, 3-HBA, BA, AA, ph, MeS, and JA (1.0 mM each)) were each added to a 5 mM sodium acetate buffer (pH 5.5) solution containing 24 μM TiO2 NPs to give a final volume of 1000 μL. After equilibration at ambient temperature for 5 min, the mixtures were transferred separately into 96-well microtiter plate and their UV-Vis spectra were recorded. Determinations were performed in triplicate for three preparations of the samples.

**Determination of SA by capillary electrophoresis**

The concentration of SA by capillary electrophoresis (CE) with UV detection was determined according to a previously described study with slight modifications.\textsuperscript{27} Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with a 75 μm I.D. and 365 μm O.D. were used for the determination of SA. Before use, new capillaries were flushed with 0.5 mol l\textsuperscript{−1} NaOH for 3 h, rinsed extensively with water, and finally conditioned with the separation buffer solution for 30 min. The capillary length was 45 cm, and the detection window was located 10 cm from the outlet side. When not in use, the capillaries were stored in water to prevent buffer crystallization. 20 mmol l\textsuperscript{−1} phosphate/boric acid solution at pH 9 in the presence of 2% methanol and 2% acetonitrile was chosen as the running buffer. Each sample was injected at the elevated anode end (30 cm above the cathode) into the capillary using hydrodynamic injection over 10 s. The potential applied for the separation was +16 kV. UV detection was performed at 205 nm. Between runs, the capillary was rinsed consecutively with water and the running buffer.
Extraction of SA from tobacco leaves

The procedure used for SA extraction was based on a previously described method with slight modification.\textsuperscript{27} Tobacco leaf samples (0.5 g) were ground using a mortar and pestle. Samples were then transferred to a 1.5 mL Eppendorf tube wherein an aliquot (1 mL) of 99% methanol was added. This extraction mixture was mixed by vortexing for 5 min and then subsequently centrifuged (12 000 g) for 10 min. The supernatant was collected in a 1.5 mL Eppendorf tube, and the pellet was resuspended in 99% methanol (0.5 mL) and resubjected to sonication and centrifugation. The supernatants were combined and centrifuged again, and then the solvents were evaporated using a SpeedVac concentrator at a high drying speed. Trichloroacetic acid (10%, 500 \textmu L) was then added to the residue, and the solution was mixed using a vortex. Partitioning with 500 \textmu L ethyl acetate : cyclohexane (1 : 1, v/v) resulted in the separation of an upper organic solvent phase containing the free SA and a lower aqueous phase containing other compounds. This partitioning was carried out twice. The combined upper layers containing the free SA were evaporated to dryness using a SpeedVac concentrator at a medium drying speed. 5 mM sodium acetate solution (pH 5.5) was then added to the residue, and the solutions (leaf extracts) were mixed using a vortex.

Procedure for SA determination in tobacco leaf extracts and tobacco leaves

Tobacco leaf extracts were collected using the above method. After filtration through a 0.2 \textmu m membrane, aliquots of the leaf extracts (100 \textmu L) were spiked with a standard solution of SA at the desired concentrations. The spiked samples were then diluted to 1000 \textmu L using a 5 mM, pH 5.5 sodium acetate solution containing 24 \textmu M TiO\textsubscript{2} NPs. After equilibration at ambient temperature for 5 min, the mixtures were transferred separately into 96-well microtiter plates and their UV-Vis spectra were recorded using a microplate reader. Determinations were performed in triplicate for three preparations of the samples.

Tobacco (\textit{Nicotiana tabacum} L.) cultivar Xanthi nc (NN genotype) was grown in a glasshouse and used after approximately six weeks. For determination of SA (\textit{in situ}) in the tobacco leaves, four samples were prepared: tobacco leaves in the absence of SA (a) before and (b) after immersion in a TiO\textsubscript{2} NP solution for 10 min; (c) tobacco leaves in the presence of SA after immersion in a TiO\textsubscript{2} NP solution for 10 min, where the SA was introduced into the extracellular space of the leaves via infiltration through the lower epidermis using a syringe with no fitted needle; and (d) tobacco leaves in the presence of endogenous SA after immersion in a TiO\textsubscript{2} NP solution for 10 min, where the endogenous SA was induced \textit{via} UV irradiation at 254 nm for 20 min.

Results and discussion

Sensing strategy

It is known that surface modification of TiO\textsubscript{2} NPs with SA leads to the formation of a surface charge transfer complex. The SA acts as a surface photosensitizer, enabling the TiO\textsubscript{2} to absorb and respond to visible light (Scheme 1).\textsuperscript{34–36} As shown in the inset of Fig. 1, white TiO\textsubscript{2} NPs turn yellow upon immersion in a colourless solution of SA. The yellow colour is a clear indication of the formation of Ti(IV)–SA complexes.\textsuperscript{32} Fig. 1 presents the UV-Vis spectra of TiO\textsubscript{2} NPs in the presence and absence of SA. Spectra (a) and (b) represent the absorption of the TiO\textsubscript{2} NP and SA solutions, respectively. As can be seen, modification of the TiO\textsubscript{2} NPs with SA (1.0 mM) leads to an extension of the absorption of the solution into the visible region (spectrum (c)). This absorption increasing in the visible region is attributed to the formation of an intramolecular ligand to metal charge transfer transition between salicylate and Ti(IV) on the surface of TiO\textsubscript{2} NPs.\textsuperscript{32} Diffuse reflectance spectra of TiO\textsubscript{2} NPs and TiO\textsubscript{2} NPs with SA are also shown in Fig. S1.† According to the plots, the absorption edges of TiO\textsubscript{2} and TiO\textsubscript{2} with SA occur at about 311 nm and 420 nm, respectively, due to the excitation of electrons from the valence gap to the conductive gap. According to the plots in the inset of Fig. S1,† the band-gap energies are estimated to be 3.98 eV and 2.95 eV for TiO\textsubscript{2} NPs and TiO\textsubscript{2} NPs with SA. Therefore, the wavelength at 420 nm can be used for the quantitative SA concentration by using TiO\textsubscript{2} NPs. The TEM images in Fig. S2† show that the TiO\textsubscript{2} NPs in the presence of SA (1.0 mM) have size similar to those in the absence of SA (average 7.1 ± 0.8 nm). However, the SA was found to have an impact on

Scheme 1 Illustration of the colorimetric sensing of SA using TiO\textsubscript{2} NPs.

Fig. 1 UV-Vis spectra and photographic images of 5 mM, pH 5.5 sodium acetate buffer solutions of (a) 24 \textmu M TiO\textsubscript{2} NPs, (b) 1.0 mM SA, and (c) 24 \textmu M TiO\textsubscript{2} NPs in the presence of SA (1.0 mM).
the aggregation behavior of the TiO2 NPs, suggesting that SA adsorsbs onto the surfaces of the TiO2 NPs.25

To confirm the adsorption of SA on the surfaces of the TiO2 NPs, Raman, MS and dynamic light scattering analyses were performed for the TiO2 NPs in the presence of SA after a series of centrifugation/washing steps to ensure the removal of any unbound species. Fig. S3† presents the Raman spectrum obtained for the TiO2 NPs in the presence of SA (1.0 mM). The bands at 294, 397, 510, and 627 cm⁻¹ correspond to vibrations of the TiO2 NPs; the bands in the range from 1150 to 1600 cm⁻¹ correspond to the Raman resonances for adsorbed SA. The ring stretching mode is observed at 1462 cm⁻¹ together with C-O stretching vibrations (coupled with ring stretching modes) at 1228, 1306, and 1592 cm⁻¹. In addition, a band appears at 1137 cm⁻¹ that is associated with an in-plane CH band. Fig. S4† presents the SALDI-MS spectra of SA and the TiO2 NPs in the presence of SA (1.0 mM). The signals at m/z 136.96 and 182.94 are assigned to [salicylate–H]⁻ and Ti[iv]–SA complex ions, respectively. Considering the mechanism shown in Scheme 1, it is possible that Ti[iv]–SA complex exists on the surfaces of the TiO2 NPs. A Zetasizer Nano ZS90 apparatus was employed to confirm the adsorption of SA on the surfaces of the TiO2 NPs that were exposed to SA (0–10 mM) at pH 5.5. It can be seen in Fig. S5† that the hydrodynamic diameter of the TiO2 NP increased, and the zeta potential of the TiO2 NPs became less positive with an increase in the SA concentration. These results further confirmed the possible presence of SA on the surfaces of the TiO2 NPs. The possible reaction mechanism is presented in Scheme 1. The enediol group of the SA coordinated to Ti4⁺ ions (Ti atoms) on the TiO2 NP surface, leading to the formation of Ti[iv]–SA coordination compounds. Upon photoexcitation, an electron is considered to be transferred from the HOMO of the SA to the conduction band of the TiO2 through the LUMO of the SA, enabling the TiO2 to absorb and respond to the visible light region. Thus, we can determine the concentrations of SA by monitoring the increase in the visible absorbance of the TiO2 NPs.

**Assay optimization**

Additional assay parameters were then evaluated to further optimize the experimental protocol. Different concentrations of TiO2 NPs ranging from 12 to 120 µM were tested, and it was found that the intensity of the yellow colour of the TiO2 NP solution in the presence of SA increased with an increase in the TiO2 NP concentration (inset of Fig. S6†). The effect of the concentration of TiO2 NPs on the values of (A – A0)/A0, where A and A0 represent that the absorbance at 420 nm of TiO2 NPs in the presence and absence of SA (1.0 mM), respectively, is shown in Fig. S6.† The value of (A – A0)/A0 for the TiO2 NPs decreased with an increase in the concentration of TiO2 NPs, probably due to the inner filter effects of the TiO2 NPs at high concentration levels. Therefore, a concentration of 24 µM was chosen as the optimal TiO2 NP concentration in the present study.

To test the effect of the buffer system, different buffer systems were evaluated, including sodium acetate, sodium phosphate, and tris(hydroxymethyl)aminomethane–hydrochloric acid (Tris–HCl). As can be seen in Fig. S7A† the maximum difference in the absorbance for SA detection was obtained when a sodium acetate buffer was used. As is known, phosphate ions, which are adsorbed spontaneously onto TiO2 NPs, were used to modify the surface charges of metal oxides.28 As a result, the minimum difference in the absorbance for SA detection was achieved. Therefore, the sodium acetate buffer system was selected for further study. The influence of pH was then investigated over the range from 4.5 to 8.5. For SA detection, the difference in the absorbance of the TiO2 NP solution increased as the pH value of the sodium acetate buffer increased up to 5.5, above which it then decreased slightly (Fig. S7B†), probably due to the instability of the TiO2 NPs at higher pH. Therefore, a sodium acetate buffer at pH 5.5 was used for all further experiments. The influence of the sodium acetate buffer concentration in the range from 5 to 100 mM on the system was also tested (Fig. S7C†). The difference in the absorbance of the TiO2 NP solution decreased with an increase in the concentration of the sodium acetate buffer solution, probably due to the aggregation of the TiO2 NPs at higher buffer concentration. Therefore, a sodium acetate concentration of 5 mM was selected as the optimal value in the follow study.

**Validation of the assay**

To investigate the selectivity of the TiO2 NPs for SA (1.0 mM), seven structural analogs (4-HBA, 3-HBA, BA, AA, ph, MeS, and JA) (1.0 mM each) were added to the TiO2 NPs (one additional substance at a time). At pH 5.5, SA induced a significant increase in the absorbance at 420 nm (i.e., formation of Ti[iv]–SA complexes) (Fig. 2A). However, another one chemical led to a change in the absorption of the TiO2 NP solution: a colour change in the solution was observed for AA as a result of its hydrolysis. However, the use of 0.5% potassium fluoride as the inhibitor of AA hydrolysis was found to be effective.29 Next, to further test the practicality of using the TiO2 NPs as an SA sensor, analyses were conducted with mixtures containing SA (1.0 mM) and all of the seven structural analogues (4-HBA, 3-HBA, BA, AA, ph, MeS, and JA) (1.0 mM each). The results indicated that none of the structural chemicals caused any interference, and the difference in the values of the absorbance at 420 nm induced by SA in the absence and presence of the other substances was always less than 0.08 a.u. (Fig. 2B).

Under optimal conditions, the sensitivity of TiO2 NPs toward SA was then investigated. The absorbance of the Ti[iv]–SA complexes increased with an increase in the concentration of SA, and a linear relationship was obtained for the plot of the absorbance difference as a function of the concentration of SA over the range from 0.02 to 1.0 mM (R² = 0.992) (Fig. 3). Moreover, the limit of detection of the TiO2 NPs for SA (S/N = 3) was determined to be 15.4 µM. The UV-Vis spectra of various concentrations of SA in the absence of TiO2 NPs are also shown in Fig. S8.† There are no obvious absorbance differences in the visible region. In comparison to other optical methods, the new assay for SA is relatively rapid (5 min), and simple (no need to prepare and purify biosensor bacteria).16,25,26,28,29
Determination of SA in tobacco leaf extracts and tobacco leaves in situ

With the expectation that the TiO2 NP-based sensor would have great potential for use in the analysis of SA in plant samples, a standard addition method was applied to determine the concentration of SA in a tobacco leaf extract matrix. Notably, a linear correlation was found to exist between the absorbance difference and the concentration of SA spiked in the leaf extract matrix over the range from 0.04 to 0.90 mM ($R^2 = 0.997$) (Fig. S9†). The spike recoveries for these measurements were 102.2%–104.6%. To confirm that measurements of SA obtained using TiO2 NPs are as accurate as conventional methods, the spiked SA concentration (0.5 mM) in leaf extracts was measured using CE with UV detection. The two methods gave almost identical results: 0.52 ± 0.055 mM and 0.55 ± 0.014 mM for the TiO2 NP probe and CE/UV methods, respectively ($n = 3$). Using a $F$-test (the $F$ value was 19 at a 95% confidence level), the $F$ values calculated at the 95% confidence level was 15.4, revealing that no significant differences existed between the precision of the new assay and the CE/UV method. Then, using a $t$-test (the $t$-test value was 2.776 at a 95% confidence level), the $t$ values calculated at the 95% confidence level is 0.09. These results showed that the data obtained from the two methods were not significantly different. Our results further suggest that the TiO2 NP probe has the potential to be a quantitative in situ assay of SA in tobacco leaves.

Before attempting to use TiO2 NPs to detect the endogenous SA in leaves, it was necessary to determine whether or not the TiO2 NPs would remain capable of reporting the presence of SA following infiltration into plant leaves. Thus, SA was introduced into the extracellular space of leaves via infiltration through the lower epidermis using a syringe with no fitted needle. The leaves infiltrated with SA were then immersed in a buffer solution containing 24 μM TiO2 NPs, and a colour change was readily detectable (photographic image c in Fig. 4). On the other hand, only a slight colour change (photographic image b in

Fig. 2 (A) Selectivity of 24 μM TiO2 NPs toward SA ($n = 3$). The concentration of SA was 1.0 mM, and the concentration of each of the other interference was 1.0 mM. Inset: photographic images of TiO2 NP solutions in the presence of SA and other possible interfering compounds. (B) Tolerance of 24 μM TiO2 NPs toward SA in the presence of other possible interfering compounds ($n = 3$). The concentration of SA was 1.0 mM, and the concentration of each of the other substances was 1.0 mM.

Fig. 3 UV-Vis spectra and linear responses (inset) of the absorbance difference plotted with respect to the concentration of SA with TiO2 NPs ($n = 3$). Photographs of 24 μM TiO2 NPs with respect to the concentration of SA (0.02 to 1.0 mM).

Fig. 4 (A) Photographic images and (B) statistical results (yellow to green ratio%) for tobacco leaves in the absence of SA (a) before and (b) after immersion in a 24 μM TiO2 NP solution; (c) tobacco leaves in the presence of SA after immersion in a 24 μM TiO2 NP solution (SA was introduced into the extracellular space of the leaves by infiltration through the lower epidermis using a syringe with no fitted needle); and (d) tobacco leaves in the presence of endogenous SA after immersion in a 24 μM TiO2 NP solution. The endogenous SA was induced via UV irradiation at 254 nm for 20 min ($n = 3$).
Table 1 Various sensing systems for the determination of SA

<table>
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<th>Method</th>
<th>Sensing system (probe)</th>
<th>Analytical ranges</th>
<th>LOD</th>
<th>Reaction time</th>
<th>In situ detection</th>
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<td>Luminescence</td>
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<td></td>
<td>Acinetobacter sp. ADPWH_lux</td>
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<td>0.1 μM</td>
<td>2 h at 37 °C</td>
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<td>28 and 29</td>
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<td>Chromatography/MS</td>
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<td></td>
<td>HPLC-MS/MS</td>
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<td>21 and 22</td>
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<tr>
<td></td>
<td>HPLC-MS/MS</td>
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<td>10 min</td>
<td>Impossible</td>
<td>23</td>
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<tr>
<td></td>
<td>GC-MS/MS</td>
<td>72.5–109 μM</td>
<td>72.5 μM</td>
<td>10 min</td>
<td>Impossible</td>
<td>24</td>
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<tr>
<td>ELISA (absorbance)</td>
<td>Mouse monoclonal antibody</td>
<td>0.4–400 μM</td>
<td>0.4 μM</td>
<td>1.5 h at 37 °C</td>
<td>Impossible</td>
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<td>Colorimetric</td>
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<td>15.4 μM</td>
<td>5 min at 25 °C</td>
<td>Possible</td>
<td>This study</td>
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</table>

* Not provided.

Fig. 4) was observed when leaves without infiltrated SA were immersed into the TiO₂ NP solution. Next the in situ detection of the endogenous SA in tobacco leaves after UV irradiation (254 nm) for 20 min was demonstrated using this approach; the tobacco leaves turned yellow upon immersion in a TiO₂ NP solution (photographic image d in Fig. 4). Notably, the yellow to green ratio in the presence of SA was higher than that in the absence of SA (P < 0.05) (Fig. 4B). This was also carried out on the same extract using the CE with UV detection. The amounts of SA found by the TiO₂ NP and CE/UV methods were 4.14 μg mL⁻¹ (SA/leaf extract) and 4.97 μg mL⁻¹, respectively. The two methods gave almost similar results. Therefore, it was concluded that TiO₂ NP probe has the potential to be used as a nanosensor for the non-destructive, in situ qualitative assessment and visualization of changes in SA accumulation in plant tissues. This novel probe possesses three attractive features when compared with other reported methods (Table 1): (1) expensive enzymes/substrates (ELISA), sophisticated instruments (Chromatography-MS) and complex molecular engineering (Acinetobacter sp. ADPWH_lux) are not required; (2) fast-reaction time, an intramolecular ligand to metal charge transfer transition between salicylate and TiO₂ NPs, is rapid (5 min); (3) non-destructive detection—the analysis of tobacco leaves in situ is possible without performing tedious sample pretreatment.16,20–26,28,29

Conclusions

A facile, colorimetric method for the detection of SA in tobacco leaves using a sensor based on TiO₂ nanoparticles was described. Under optimum conditions, the TiO₂ NP probe exhibits high selectivity for SA. Notably, the absorbance difference of the TiO₂ NP solution is proportional to the concentration of SA over the range from 0.02 to 1.0 mM (R² = 0.992) with a limit of detection of 15.4 μM at a signal-to-noise (S/N) ratio of 3. The practicality of the TiO₂ NP sensor was validated through the detection of SA in tobacco leaf extracts. Moreover, when tobacco leaves were immersed in a solution of TiO₂ NPs, the changes in the SA accumulation with and without UV light irradiation in an NN genotype tobacco were determined. Therefore, this new TiO₂ NP nanosensor has potential application for non-destructive visualization of the changes in SA accumulation in plant tissues.

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Notes and references