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Biochemical and Biophysical Research Communications 328 (2005) 130–136
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Involvement of acetosyringone in plant–pathogen recognition☆

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Received 22 December 2004
Available online 6 January 2005

Abstract

In this study, acetosyringone was identified as one of the major extracellular phenolics in tobacco suspension cells and was shown to have bioactive properties that influence early events in plant–bacterial pathogenesis. In our model system, tobacco cell suspensions treated with bacterial isolate *Pseudomonas syringae* WT (HR+) undergo a resistant interaction characterized by a burst in oxygen uptake several hours after inoculation. When the extracellular concentration of acetosyringone in tobacco cell suspensions was supplemented with exogenous acetosyringone, the burst in oxygen uptake occurred as much as 1.5 h earlier. The exogenous acetosyringone had no effect on tobacco suspensions undergoing susceptible interactions with *Pseudomonas tabaci* or a non-resistant interaction with a near-isogenic mutant derivative of isolate *P. syringae* WT (HR+). Resistant interactions with isolate *P. syringae* WT (HR+) also produce an oxidative burst which oxidizes the extracellular acetosyringone. This study demonstrates that acetosyringone, and likely other extracellular phenolics, may have bioactive characteristics that can influence plant–bacterial pathogenesis. Published by Elsevier Inc.

Keywords: Oxidative burst; Oxygen uptake; Reactive oxygen; Hydrogen peroxide; *Pseudomonas syringae*; *Pseudomonas tabaci*; *Nicotiana tabacum*; Phenolics; Apoplast

We have been investigating extracellular phenolics that build up in plant cell suspensions and repress the oxidative burst triggered by bacterial pathogens to which the plant is resistant [1]. We report here the identity of one of the major phenolics in tobacco cell suspensions to be acetosyringone. Acetosyringone is generally considered to be a wound-induced metabolite in plants, which more than 10 years ago was shown to trigger virulence genes in the wound pathogen *Agrobacterium tumefaciens* [2–4]. Since then, studies of the bioactive influence that acetosyringone and other extracellular phenolics may have on plant pathogenesis have been scarce. In human and animal physiology, a new awareness of the wide range of regulatory effects that plant phenolics often possess has led to a renaissance in the search and study of bioactive phenolics [5–8]. Ironically in plants, the source of these compounds, little attention has been given to their potential bioactive role in influencing physiological events.

We report for the first time the bioactive effect of acetosyringone on early physiological events that take place during resistant plant–bacterial interactions. Tobacco plants that are resistant to the bean pathogen *Pseudomonas syringae* undergo rapid hypersensitive cell death in the leaves at the site of infection, which

☆ *Abbreviations:* CFU, colony-forming units; HR, hypersensitive response; RLU, relative light units.

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0006-291X/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.bbrc.2004.12.153

prevents spread of the pathogen. We have studied this interaction using tobacco cell suspensions [9–12]. When these cell suspensions are inoculated with *P. syringae*, a burst in oxygen uptake occurs within 4–6 h followed by hypersensitive cell death after 12–15 h. The burst in oxygen uptake is unique to resistant interactions and is associated with recognition of the pathogen by the plant. By monitoring this resistant recognition response we found that increased levels of acetosyringone could reduce the response time of tobacco cells to *P. syringae*. Acetosyringone had no apparent effect on the susceptible interaction of tobacco with *Pseudomonas tabaci* or on interactions with a near-isogenic transposon mutant of *P. syringae*, *isolate* B7 (HR−), which cannot produce a resistant response in plants or cells. These results indicate that extracellular phenolics can significantly influence host interactions with pathogens and potentially other microbes, and warrant further examination of their bioactive properties.

Materials and methods

Chemicals. Horseradish peroxidase (P-8250) and all other chemicals were purchased from Sigma–Aldrich, St. Louis, MO, unless otherwise noted.

Tobacco cell suspensions. Suspension cells of tobacco (*Nicotiana tabacum* L. cv. Hicks line T4) were maintained and prepared as previously described [13]. Two-day-old tobacco cells were washed and suspended at a cell density of 0.05 g/ml in assay buffer (0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 175 mM mannitol, and 0.5 mM Mes, pH 6). The cell suspensions, 25 ml, in 50-ml beakers, were equilibrated for 0.5 h prior to treatment in a rotary water bath shaker at 27 °C and 180 rpm. Bacterial or chemical treatments were added directly to the suspensions. All experiments were performed at least twice with two replicates per treatment.

Bacterial isolates. Pseudomonas syringae isolate WT (HR+) causes a resistant hypersensitive reaction with rapid cell death visible after 15–24 h when introduced into tobacco leaves or suspension cells. *P. syringae* isolate B7 (HR−) is a mutant of isolate *P. syringae* WT (HR+), containing a single Tn5 insertion, that does not induce a hypersensitive response [9]. *P. tabaci* isolate 11528 is a tobacco pathogen causing susceptible disease symptoms after 3–4 days in leaves. Cultures of *Pseudomonas* isolates were maintained on Kings B [14] agar as previously described [13]. Medium for isolate B7 was supplemented with nalidixic acid (25 μg/ml) and streptomycin (40 μg/ml). Bacterial cultures were grown for 20 h in Kings B broth, centrifuged, and washed, and suspended in sterilized deionized water. Based on optical density, the concentration of the bacterial inoculum was adjusted with sterilized deionized water so that addition of about 200 μl to tobacco cell suspensions would result in a final concentration of 5×10^7 CFU/ml of bacteria, unless otherwise noted. Bacterial concentrations in tobacco cell suspensions were verified periodically by dilution-plating.

UV absorbance. Samples (1.0 ml) of cell suspensions were removed periodically and filtered through Miracloth, and the filtrate was centrifuged at 12,000*g* for 5 min to remove fine particles. Absorbance readings and scans, from 200 to 400 nm, were performed with a Beckman Model DU-650 spectrophotometer.

Extracellular antioxidant assay. The extracellular antioxidant capacity was estimated using a chemiluminescent assay that determined the quantity of H_2O_2 consumed by samples. The assay was performed using a modification of the luminol-dependent-chemiluminescent assay described previously [1,12]. Samples (0.4 ml) of treated or untreated suspension cells were dispensed into tubes and placed into an EG&G Berthold Autolumat 953 luminometer (Bad Wildbad, Germany). Two stock solutions were prepared: (A) 0.5 mM H_2O_2 in the same assay buffer used for cell suspensions; and (B) horseradish peroxidase, 28.8 U/ml, and 1.71 mM luminol in 1 M $NaPO_4$, pH 7. The luminometer first added stock solution A (50 μl) followed 4.5 s later by stock solution B (50 μl). The final concentrations were 50 μM H_2O_2, 1.44 U/ml peroxidase, and 171 μM luminol. Chemiluminescence was measured as relative light units (RLU) every 0.1 s for 20 s; the maximum measurement is proportional to the H_2O_2 concentration. Standard curves were prepared with dilutions of H_2O_2 in assay buffer. Under these assay conditions, the extracellular antioxidant in each sample had sufficient time to react with the added H_2O_2; the remaining H_2O_2 reacted with luminol. The decrease in RLU in suspension samples compared to buffer controls corresponds to the H_2O_2 consumed by extracellular antioxidant in each sample and provides an estimate of the extracellular antioxidant concentration of each sample.

Oxygen uptake assay. Oxygen electrodes (Microelectrodes, Londonderry, NH, USA), and data acquisition hardware and software (National Instruments, Austin, TX, USA) were used as previously described [10]. Oxygen electrodes were calibrated to 100% in aerated buffer (~284 μM oxygen) in a water bath shaker maintained at 27 °C. The multi-electrode apparatus enabled simultaneous monitoring of oxygen uptake rates in 16 beakers of tobacco cell suspensions with continuous shaking and aeration. The basic concept underlying this technique is that changes in the rate of oxygen uptake by the plant and bacterial cells will result in a change in the steady state oxygen concentration of the suspension. Based on calibration tests, at any given oxygen concentration of the assay buffer, the rate of oxygen flux from the air is known and should equal the oxygen uptake flux by the tobacco and/or bacterial cells [10,13].

HPLC–UV quantification of acetosyringone. One-milliliter samples of tobacco cell suspensions were filtered through Miracloth and centrifuged at 12,000*g* for 5 min prior to HPLC analysis. When not analyzed immediately, samples were flushed with N_2 and stored overnight at −20 °C. Phenolics were separated by C_{18} RP-HPLC using a Waters (Milford, MA) quaternary pump, autosampler, photodiode array detector, and Empower data acquisition on a Dell Pentium 4 computer. A 250 × 4.6 mm i.d., 5 μm Luna C18(2) analytical column (Phenomenex, Torrence, CA) was used with a binary mobile phase gradient of methanol in 0.01% aqueous phosphoric acid as previously described [15]. Aliquots, 100 μl, of samples were acidified with phosphoric acid (0.1%) and placed in the autosampler using a 30 μl injection volume. External standards of acetosyringone were used for quantification based on absorbance at 300 nm.

HPLC–MS identification of acetosyringone. Atmospheric pressure ionization mass spectrometry analysis was performed on a Quattro LC benchtop triple quadrupole mass spectrometer (Micromass, Manchester, UK) using the electrospray ionization interface in the negative mode (ES^-) as previously described [15]. Mass spectrometric data were acquired in the full scan mode over the *m/z* 50–400 range. Sensitivity of the mass spectrometer was optimized using an acetosyringone standard. A Waters 2690 HPLC system using the same column and gradient as described for HPLC–UV analysis was utilized for separation of the phenolics. Samples of tobacco suspensions for mass spectroscopy were prepared as for HPLC–UV followed by acidification with phosphoric acid (0.1%) and extraction with ethyl acetate. Dried samples were dissolved in methanol:water, (1:1, v/v) plus 0.1% formic acid, and 20 μl injected per run with a Waters autosampler.

Nuclear magnetic resonance spectroscopy. Phenolics isolated by HPLC–UV were dissolved in 0.8 ml CD_3OD, and ^1H NMR spectra were acquired deuterium locked at 25 °C using a Bruker QE 300 MHz NMR spectrometer. Chemical shift values were assigned relative to the frequencies of residual non-deuterated water and methanol externally referenced to tetramethylsilane (TMS).