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Apoplastic redox metabolism: Synergistic phenolic oxidation and a novel oxidative burst ☆

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Abstract

The plant apoplast is an important mediator of communication between the cell cytoplasm and its surroundings. Plant cell suspensions offer a convenient model system to gain insight into apoplastic physiology. Here, we describe a novel phenomenon that took place when two naturally occurring phenolics were added together to either soybean or tobacco cell suspensions. Acetosyringone (AS) and/or hydroxyacetophenone (HAP), phenolics found in the extracellular/apoplast of tobacco cells, were added to soybean or tobacco cell suspensions undergoing an oxidative burst. Individually, AS appeared to be utilized as a typical peroxidase substrate to scavenge hydrogen peroxide, while HAP was utilized at a much lower rate. However, when added together the rate of utilization of both phenolics increased and surprisingly resulted in the production of hydrogen peroxide. We have further characterized this novel phenomenon in suspension cells. This study demonstrates that certain phenolics in plants can cause co-oxidation which, as in animals, could alter the structure and bioactivity of surrounding phenolics.
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Keywords: Co-oxidation; Phenolics; *Glycine max*; *Nicotiana tabacum*; *Pseudomonas syringae*; Oxidative burst; Hydrogen peroxide; Apoplast; Redox metabolism; Suspension cells

1. Introduction

The apoplast matrix that surrounds the plant cell is a complex and dynamic micro-environment. When outside stresses, such as environmental changes or pathogen attack occur, the micro-environment of the apoplast is likely to be the first line of defense. The cross-linked cell wall polysaccharides provide a backbone to the matrix within which various proteins, enzymes, metabolites, and inorganic ions are associated. Localized responses to bacterial or fungal attacks often result in structural alterations, such as lignification and papillae formation, involving multiple matrix components including callose, phenolics, and hydroxyproline-rich proteins [1,2], or physiological alterations of the apoplast environment, such as increased pH or production of reactive oxygen species.

Due to the complexity and inaccessibility of the apoplast, examining its rapidly changing stress-related chemistry in vivo remains a challenge. However, analysis of the extracellular/apoplast chemistry of cell suspensions, which mimic stress-related symptoms observed in planta, can provide insight. In our studies of extracellular phenolics during plant/bacterial interactions in suspension cells, we found that qualitative and quantitative changes in

Abrreviations: AS, acetosyringone; HAP, 4-hydroxyacetophenone; ROS, reactive oxygen species; HKbac, heat-killed bacteria

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phenolic composition were dependent on several factors with the predominant factor being the type of bacteria present [3,4]. When suspension cells were treated with a pathogen that caused an incompatible response and oxidative burst, the concentration of the extracellular phenolics decreased or disappeared as a result of oxidation. We also found that an extracellular phenolic produced by tobacco cells, AS, had a bioactive effect that accelerated the plant/bacterial interaction when added exogenously [5].

In this report, we demonstrate a novel phenomenon that brought new insight to our understanding of apoplastic redox metabolism. In an attempt to protect apoplastic phenolics in soybean suspensions from oxidation prior to their analysis, we added two commercially available plant phenolics, AS and HAP, to serve as antioxidants. Surprisingly, we discovered that the relatively high concentrations of these exogenous phenolics were (1) rapidly oxidized and (2) resulted in a large oxidative burst. We demonstrate that certain phenolics trigger a unique chemistry in the extracellular/apoplast environment of suspension cells that results in a prooxidant environment rather than an antioxidant environment as expected. In addition, we demonstrate that phenolic co-oxidation can occur in the extracellular/apoplast environment of suspension cells. Phenolic co-oxidation is currently an area of intense study and concern in the pharmaceutical industry [6,7] where the co-administration of certain phenolic drugs can cause modification of their structure and bioactivity.

The same principle of phenolic co-oxidation in animals could occur in the plant apoplast, where the introduction of certain phenolics, whether from the cell cytoplasm or invading micro-organisms, could lead to structural and bioactive modification of pre-existing apoplast phenolics. Because this could be insightful for understanding the complexity of apoplastic redox metabolism, we decided to investigate and characterize this phenomenon in suspension cells. The chemical mechanisms involved are also currently being investigated.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (P-8250), soybean peroxidase (P-1432), AS (3,5-dimethoxy-4-HAP, D134406), 4-HAP (278564), and all suspension culture ingredients were purchased from Sigma-Aldrich Chemicals Inc., St. Louis, MO, USA. The peroxidases were purchased and measured in units as described by Sigma; one unit (U) will oxidize 1 μmole of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) min^{-1}.

2.2. Plant material

Soybean (*Glycine max* L. Merr. cv Harosoy) suspension cells were originally isolated from hypocotyl callus and maintained in Gamborg's B-5 medium (Gibco, Grand Island, NY, USA) augmented with 1 mg L^{-1} 2,4-D, pH 5.0. Suspension cultures of tobacco (*Nicotiana tabacum* L. cultivar Hicks) were derived from pith and maintained on MS media, supplemented with 0.2 mg L^{-1} KH_2PO_4, 0.2 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin, pH 5.8. Soybean and tobacco cultures were routinely transferred, 10 into 80 mL of fresh media in 250 mL flasks, every 4 days and incubated on a rotary shaker at 150 rpm and 27 °C in the dark.

Suspension cells were washed and suspended in assay buffer, containing 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 175 mM mannitol and 0.5 mM MES, pH 6. Then 25 mL of the cell suspensions, 0.05 g mL^{-1}, were dispensed into 50 mL beakers on a rotary water bath shaker set at 27 °C and 180 rpm to keep cells suspended. Stock solutions, 20 mM, of AS and HAP, were made in assay buffer and were added directly to the cell suspensions using less than 125 uL per beaker to produce final phenolic concentrations ranging up to 100 μM. All experiments were preformed at least twice with two or more replicates per treatment.

2.3. Heat-killed bacteria (HKBac) preparation

Cultures of *Pseudomonas syringae* pv. *syringae* 61, isolate WT, were grown for 20 h in Kings B broth, centrifuged, washed, and suspended in deionized water. Based on optical density, the concentration of the suspension was adjusted with water so that addition of about 200 μL of the bacterial suspension to plant cell suspensions resulted in the desired final concentration of about 10^8 cfu mL^{-1}. The bacterial suspensions were then autoclaved for 15 min and frozen until used.

2.4. HPLC-UV quantification of phenolics

One-milliliter samples of cell suspensions were filtered through Miracloth and centrifuged at 12,000*g* for 5 min prior to HPLC analysis. AS and HAP were separated and quantified using a Onyx monolith C18 analytical column, 100 × 4.6 mm i.d., (Phenomenex, Torrence, CA, USA) with a Waters (Milford, MA) quaternary pump, autosampler, photodiode array detector, and Empower data acquisition on a Dell Pentium 4 computer. Aliquots, 150 μL, of samples were acidified with phosphoric acid (0.1%) and placed in the autosampler using a 10 μL injection volume. An isocratic mobile phase of 30% methanol in 0.01% aqueous phosphoric acid, 2 mL min^{-1}, separated the phenolics within 4 min. Quantification using peak height was preformed using the UVmax wavelength for each peak, AS, 300 nm, HAP, 276 nm, and calibration with standards.

2.5. FOX2 (ferrous oxidation in xylenol orange) assay for hydrogen peroxide

In this spectrophotometric method, ferrous ions are oxidized by hydrogen peroxide to ferric ions, which bind