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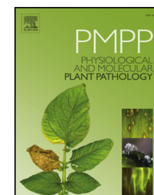

The dynamics of apoplast phenolics across the apoplast/symplast barrier in tobacco leaves following bacterial inoculation

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A B S T R A C T

One of the first detectable responses of plant leaves to bacterial infection is a change in the composition of its apoplast phenolics. In tobacco, two prominent phenols that are induced are acetosyringone (ACE) and acetovanillone (AV). Recently, we found another response that occurs in pathogenic interactions, which involves substantial increases in chlorogenic acid (CGA) in the apoplast about 10 h after inoculation. Since chlorogenic acids are known to be stored in the cell vacuole, we decided to monitor phenolic changes in both the symplast and apoplast during plant-bacterial interactions. This was carried out with *Pseudomonas fluorescens* (PF, saprophyte), *P. syringae* pv. *syringae* (PSS, HR-causing pathogen), and *P.s.* pv. *tabaci* (PTAB, compatible pathogen). Whole-tissue analysis demonstrated that the level of CGA in control tissue, ~650–700 μg/g fresh wt, was more than 100x greater than in the apoplast, ~5–6 μg/g fresh wt. The level of ACE in healthy whole tissue was very low, ~2 μg/g fresh wt, and AV was not detected. After inoculation with bacteria, the whole tissue levels of ACE and AV increased within 3 h. This increase was concurrent with increases of ACE and AV in the apoplast, which accounted for a significant portion of the whole tissue increase, suggesting the apoplast may have been the intended target for ACE and AV. Apoplast levels of CGA in the control and saprophytic interactions did not change appreciably. In the incompatible PSS interaction, the levels of CGA were similar to the control for the first 10 h, after which they increased dramatically just prior to tissue disintegration. Interestingly, only in the compatible interaction, PTAB, CGA levels increased slowly over the first 10 h, followed by a dramatic increase by 15 h. However, after several hours the CGA levels decreased, at which time previous studies have shown PTAB begins to multiply in the tissue. Using a technique of sequential washings of the apoplast, we found that the rate of dilution of CGAs compared to ACE and AV was clearly different, suggesting that different reservoirs for these phenols may exist within the apoplast. Finally, we suggest the CGA concentration in the apoplast wash provides a measurable parameter for the loss of integrity of the A/S barrier during plant/bacterial interactions. In the incompatible interaction with PSS this coincided with the loss of tissue integrity, while in the compatible interaction with PTAB there was a mild loss of integrity soon after inoculation and a stronger but transient loss about 10 h later.

1. Introduction

Bacterial pathogens that invade plant leaves generally do so through the leaf stomata and colonize the surface of cells that line the air space within. The apoplast of plant cells is the matrix outside the plant cell membrane which forms a continuum with neighboring cell matrixes. The apoplast of plant cells lining the air space within the leaf are in direct contact with the invading bacteria. The symplast is the intracellular protoplasm which also forms a continuum with neighboring cells, via plasmadesmata. The symplast of cells in contact with bacterium can respond to the invading pathogens by detection of microbial-associated molecular patterns (MAMPs), such as bacterial flagellin, triggering an initial array of plant defense mechanisms [18]. These mechanisms include the production of phenolic secondary metabolites as well as the production of reactive oxygen species, which appear in the apoplast resulting in a highly oxidative and toxic environment for the bacteria [12,21].

In a previous study with tobacco leaves, we found that the relative concentrations of apoplast phenolics respond to bacteria within hours of inoculation [5]. Three types of interactions were investigated;

Abbreviations: AV, acetovanillone; ACE, acetosyringone; AWF, apoplast wash fluid; A/S barrier, apoplast/symplast barrier; CGA, chlorogenic acid; PF, *Pseudomonas fluorescens*; PSS, *P. syringae* pv. *syringae*; PTAB, *P.s.* pv. *tabaci*; 5-O-CQA, 5-O-caffeoylquinic acid

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saprophytic, compatible (susceptible), and incompatible (resistant). Each of these interactions had a unique effect on two types of phenolic responses in the apoplast. The first response started within 2 h after inoculation and involved the induction of several phenolics, two of the major ones being acetovanillone (AV) and acetosyringone (ACE). The timing and concentration of these phenolics varied with the type of interaction [3,5]. This differential induction phenomenon was similar to that found in cell suspension cultures of potato and tobacco that were treated with bacteria [1,8,9].

The second response of phenolic changes in the apoplast occurred only with the pathogenic interactions and involved chlorogenic acid isomers (CGA), which are preformed metabolites stored in the cell vacuole [2,5,10,11,14]. About 10 h after inoculation, the CGA level in the apoplast abruptly increased 10 to 100 times, within a few hours. The rapidity and magnitude of the CGA increase suggested a possible leakage from the symplast due to a loss of integrity of the A/S barrier.

The objective of this study was to semi-quantify and characterize the changes of CGA, ACE and AV in both the symplast and apoplast to help determine their ultimate effects on plant/bacterial interactions. These metabolites have unique bioactive and redox characteristics [4,6,7] that can significantly affect the early stages of the interaction when susceptibility or resistance is determined. During the study, we found that a process of sequential elution of the phenolics from the apoplast may give insights as to the source, timing, and site of accumulation of these phenolics within the apoplast.

2. Materials and methods

2.1. Chemicals

All chemical standards were obtained from Sigma-Aldrich Chemicals, Inc (St. Louis, MO, USA): ACE (3′,5′-dimethoxy-4′-hydroxyacetophenone, ACE), AV (3′-methoxy-4′-hydroxyacetophenone, AV), chlorogenic acid (5-O-caffeoylquinic acid, 5-O-CQA), neochlorogenic acid (trans-3-O-caffeoylquinic acid, 3-O-CQA), and cryptochlorogenic acid (4-O-caffeoylquinic acid, 4-O-CQA). Nomenclature is based on 1977 IUPAC rules [16].

2.2. Preparation of bacteria

Pseudomonas syringae pv *syringae* (PSS), *P. s.* pv. *tabaci* (PTAB), and *P. fluorescens* (PF) were maintained and prepared as previously described in detail [5]. PSS produces a resistant hypersensitive response (HR) on tobacco with tissue collapse after 15–24 h, while PTAB produces a susceptible response developing symptoms after 24–48 h; PF is considered a saprophyte causing no visible symptoms. For experiments, bacteria were grown for 18–20 h in King's B broth (augmented with nalidixic acid for PSS) in dark, at 190 rpm, 30 °C. The cultures were washed with deionized water and suspended in deionized water to various concentrations based on optical density; 0.1 ODU $_{600nm}$ was about 10^8 cfu ml^{-1}.

2.3. Preparation of plant material

Tobacco plants (*Nicotiana tabacum*) *cv* Hicks were grown under greenhouse conditions as described previously [5]. Preliminary studies with PF had shown that leaf age could the influence relative composition of the response phenolics, therefore only a limited number of similar aged leaves were used. Leaves were numbered from the top down; leaves 4 and 5 were generally the first fully expanded leaves and used for experiments. The center panels were inoculated with bacteria as described previously [2]. This was carried out by pricking the leaf panel in a few spots with a needle and then gently pressing a plastic needleless syringe barrel, filled with inoculum, against the leaf. One treatment per leaf was used and each leaf half was treated as a replicate. Two to three plants were used per experiment and all experiments were repeated at least three times with 2–4 replicates of each bacterial treatment.

2.4. Extraction of phenolics

2.4.1. Whole-tissue phenolics

As described in detail previously [2], a leaf disc, 11 mm diameter ~0.03 g, was homogenized with 1 ml acidified water (0.2% phosphoric acid in deionized water). The cell debris was removed by centrifugation and the supernatant was analyzed by UPLC/UV/MS as described in sec. 2.4.5. The concentration of each phenolic was determined by comparison to standards. Quantification of the whole-tissue samples was based on a 1 ml sample size representing 0.03 g fresh wt of tissue.

2.4.2. Apoplast phenolics

The apoplast phenolics from treated leaves were obtained by water infiltration followed by centrifugation as described previously [5]. Leaf panels were cut from the leaf and weighed immediately. Each sample was processed separately which involved vacuum infiltration in sidearm flasks with deionized water followed by centrifugation to obtain the apoplastic wash fluid (AWF). Aliquots, about 200 µl, were placed in a tapered 0.350 ml glass vials with 2 µl of 10% phosphoric acid to acidify the sample and help stabilize it until analysis by UPLC/UV/MS as described Section 2.4.5. The concentration (µM) of each phenolic in the AWF was estimated by comparison with standards. The total sample volume was based on the weight change between the pre- and post-infiltration tissue. The fresh wt of the sample was the pre-infiltration weight.

2.4.3. Comparison of whole-tissue and AWF phenolics from the same leaf panel

Leaf panels that had been uniformly inoculated with bacteria or water were removed from the plant and used to prepare 3 different samples: 1) a tissue leaf disc was removed and treated as in Section 2.4.1 for whole tissue analysis, 2) the remaining panel tissue was weighed and infiltrated with water as described in Section 2.4.2 for AWF analysis, 3) from the remaining tissue, another leaf disc was removed for whole-tissue analysis and designated tissue minus AWF.

2.5. Analysis of phenolics by UPLC/UV/MS

As previously described in detail [2], phenolic analysis was conducted using an ultra performance liquid chromatography (UPLC) system (Waters Acquity UPLC, Milford, MA, USA) equipped with a refrigerated autosampler, a photodiode array detector (PDA), and ESI interface and single-quadrupole MS detector (Thermo Fisher Scientific, Waltham, MA, USA). Samples, 4 µl, were separated on an Acquity BEH C18 (2.1 × 50 mm, 1.7 µm) column. The PDA measured absorbance from 210 to 400 nm. Peaks were identified by their PDA retention times and spectra, and quantified by the peak height at the wavelength of maximum absorbance using Waters Empower software. Molecular weight or fragment masses (determined by mass spectrometer) were used to verify comparison of sample peaks with standards. Standard compounds (Section 2.1) were injected and analyzed using the same conditions to prepare standard curves.

2.6. Conductivity

The conductivity of apoplast washes was measured with a flow thru micro-conductivity electrode (MI 16–900; Microelectrodes Inc., Bedford, NH USA) connected to an Orion 150 A + conductivity meter (Thermo Fisher Scientific, Grand Island, NY, USA). The apoplast sample, 50 µl, was passed through the electrode and the conductivity read as µSiemens.

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