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Priming agents of plant defence stimulate the accumulation of *mono*- and *di*-acylated quinic acids in cultured tobacco cells

Msizi I. Mhlongo [a], Lizelle A. Piater [a], Paul A. Steenkamp [a, b], Ntakadzeni E. Madala [a], Ian A. Dubery [a, *]

[a] Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park, 2006, South Africa
[b] CSIR – Biosciences, Pretoria 0001, South Africa

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ABSTRACT

Plants rely solely on innate immunity for defence against pathogen attack. The specific determinants of different stresses are not recalled, but plants are able to launch a strong defence response subsequent to being pre-sensitized. In the current study, microbe-associated molecular pattern molecules, namely lipopolysaccharides, flagellin-22 and chitosan, and two chemical-based resistance inducers, acibenzolar-S-methyl and isonitrosoacetophenone, were used to trigger a primed state in *Nicotiana tabacum* cells. With the aid of UHPLC-qTOF-MS/MS in combination with multivariate data models, the primed response triggered by these agents was studied using a metabolite fingerprinting approach. These structurally and functionally diverse priming agents were all found to trigger the accumulation of a group of chlorogenic acids, including *mono*-acylated and di-acylated caffeoylquinic acids (3-CQA, 5-CQA, 3,4-diCQA and 4,5-diCQA). A new role for chlorogenic acids as dynamic role players in priming of plants is proposed.

Introduction

Plants are under continuous attack from different biotic stressors. Since current methods of plant pathogen control relies largely on environmentally unfriendly and detrimental conventional pesticides, there is a need for alternative strategies that rely on the plants' innate immune system. The activation of plant resistance mechanisms involving inducible defense mechanisms as a complementary crop protection approach is increasingly being adopted and gaining much interest [1,2]. By definition, plant priming or conditioning is a process whereby a plant is pre-exposed to an eliciting or inducing agent (both natural and synthetic), leading to a physiologically altered state that allows it to respond in a more pronounced manner to secondary stresses [3,4]. Investigations into the molecular and biochemical basis of priming have indicated modifications to chromatin and epi-genetic changes, accumulation of dormant mitogen-activated protein kinases (MPK3/6) and signal amplification, as well as changes in metabolism as serving as a memory for priming in systemic plant immunity [5,6].

Plants produce numerous biochemical compounds, originating from different secondary metabolic pathways such as the phenylpropanoid (PP) pathway [7,8], as part of their defence arsenal that enable them to resist biotic stresses [9,10]. PPs involved in plant resistance include cinnamates, coumarins, benzoates and flavonoids, and act as anti-herbivore-, anti-microbial-, cell wall-strengthening- or signalling molecules [11–14]. Chlorogenic acids (CGAs) are also phenolic compounds and metabolic intermediates produced by the shikimate- and PP pathways [8,15]. CGAs are formed as ester molecules by the combination of cinnamic acid derivatives (caffeic-, ferulic- or *p*-coumaric acid) and (−)-quinic acid (QA). These molecules are widely distributed in the plant kingdom [16], and possess biological functions including antibacterial, anti-viral, anti-fungal and anti-oxidant properties that enable their function in plant defence and resistance [17–20]. This bioactivity is not only limited to plant defence but includes biomedical properties [21,22].

In the current study, *Nicotiana tabacum* cell suspensions were treated with different priming agents (isonitroacetophenone, acibenzolar-S-methyl, chitosan, flagellin peptide and lipopolysaccharide), and the metabolite perturbations due to the treatments were monitored with the aid of UHPLC-MS/MS fingerprinting. Metabolites of which the levels were affected by these inducers were highlighted with the aid multivariate data statistical analysis and two *mono*-caffeoylquinic acids (CQAs) and two *di*-acylated

* Corresponding author.
E-mail address: idubery@uj.ac.za (I.A. Dubery).

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CQAs were identified and shown to be statistically significantly induced.

Materials and methods

Cell treatment and metabolite extraction

N. tabacum cells (20 mL) were cultured [23] and treated, 3 days after sub-culturing, with 1 mM isonitroacetophenone (INAP), 300 μM acibenzolar-S-methyl (ASM), 100 μg/ml chitosan (CHT), 200 nM flagellin (FLG22), 100 μg/ml lipopolysaccharide (LPS) and no inducer (control cells) at room temperature with continuous shaking at 80 rpm for 24 h. CHT, FLG22 and LPS were prepared as previously reported [24] and dissolved in culture medium to minimise physiological changes. For maximum reproducibility, the experimental design included three biological replicates for each inducer and the control. Following elicitation, cells were harvested by filtration and further washed with 20 mL culture medium. Two grams of cells was weighed and homogenised in 20 mL 100% methanol (1:10 m/v) using a probe sonicator (Sonopuls, Bandelin, Germany) set at 55% power for 15 s, repeated twice. The homogenates were centrifuged at 5000 rpm for 20 min and the supernatants transferred into 50 mL Falcon tubes. Using a rotary evaporator the supernatants were evaporated to approximately 1 mL at 55 °C followed by drying to completeness with a speed vacuum centrifuge at 55 °C. The remaining pellets were re-constituted in 400 μL 50% (v/v) UHPLC-grade methanol in milliQ water, and filtered through a 0.22 μm nylon filter into glass vials fitted with 500 μL inserts. The filtered extracts were stored at −20 °C until analysed.

Ultra-high performance liquid chromatography-quadropole time-of-flight mass spectrometry (UHPLC-qTOF-MS)

Chromatographic analyses of the extracts were performed on an UHPLC-high definition quadrupole time-of-flight MS instrument (UHPLC-qTOF SYNAPT G1 HD-MS system, Waters, Manchester, UK) using an Acquity HSS T3 column (1.7 μm, 2.2 mm × 150 mm; Waters, Manchester, UK) [25]. A binary solvent system consisting of eluent A and B, where eluent A was 0.1% formic acid in water and eluent B acetonitrile (Romil Chemistry, UK), was used. A gradient method at constant flow rate of 0.4 mL/min was used for analyte separation, and the conditions were: 5% B over 0.0–2.0 min, 5–12% B over 2.0–2.10 min, 12–65% B over 2.10–10.50 min, 65–95% over 10.50–11.00 min, held constant at 95% over 11.00–12.00 min, 95-5% over 12.00–13.00 min, and the column was washed with 5% B over 13.00–15.00 min. The photodiode array (PDA) detector scanning range was set from 200 to 500 nm, with 1.2 nm resolution and a sampling rate of 20 points sec^{-1}. The MS detector was set to collect both negative (ESI−) and positive (ESI+) ions, and the condition were as follows: capillary voltage of 2.5 kV, sample cone voltage of 30 V, MCP detector voltage of 1600 V, source temperature of 120 °C, desolvation temperature of 400 °C, cone gas flow of 50 L/h, desolvation gas flow of 800 L/h, *m/z* range of 100–1000, scan time-of 0.15 s, interscan delay of 0.02 s, mode set as centroid, lockmass set as leucine enkephalin (556.3 μg/mL), lockmass flow rate of 0.4 mL/min, and mass accuracy window of 0.5 Da.

In-source collision-induced dissociation (CID) was used to generate simulated MS^1 fragmentation of chlorogenic acid. The general MS settings described above were used but the cone voltage raised to 60 V to produce the *m/z* fragment of 191 Da. The scan method was adapted to select the *m/z* 191 fragment and to fragment the molecule further to produce simulated MS^2 and MS^3 fragments. For simulated MS^3 experiments the trap collision energy was individually adjusted for each compound to generate a fragment ion mass spectrum. The mass scan range was also changed to 50–650 Da to allow the detection of low molecular weight fragment ions.

Data analyses

MassLynx™ raw data (.raw) was converted using the DataBridge software (Waters, MA, USA) to NetCDF files. These were exported to the XCMS online statistical package (https://xcmsonline.scripps.edu), an automated, web-based metabolomics data processing software that identifies biomarker features of which the relative intensity varies between sample groups, for multivariate data analyses. The software further calculates the *p*-values as well as fold-changes of the metabolites (variables) across different samples of varying biological background. The method parameters were chosen for UHPLC/UHD-qTOF specificities and were as follows: (i) Feature detection set as centWave method, minimum peak width = 5, maximum peak width = 20, (ii) Retention time correction set as Obiwarp method, Profstep = 1, alignment set as *mz* width = 0.015, minfraction = 0.5, bw = 5, and statistics set as statistical test = Unpaired parametric *t*-test (Welch *t*-test), paired *t*-test and post-hoc analysis with the threshold *p*-value = 0.01 and fold-change = 1.5. Upon completion of the XCMS analyses, principal component analysis (PCA) score plots were generated (Supporting information, Fig. S1) to detect major differences between controls and treated samples. Two metabolites responsible for the separation were identified from the PCA and cloud plots and found to have *m/z* values of 354.30 and 516.45. These two ions were further investigated using tandem MS/MS with the aid of an in-source collision-induced dissociation (ISCID) approach to reveal their identity. Based on the quasi-molecular ion peaks $[M-H]^-$ and the resulting fragments, the elemental composition and identity of these molecules were identified using the Dictionary of Natural Products (DNP) and other online databases such as Chemspider. Due to unavailability of authentic standards, apple cider was used as a source of CGAs. Cider contains all regional isomers of CQAs except for the diCQAs. The latter were only characterized based on their UV spectra and MS fragmentation patterns compared to already published data [16].

Results and discussion

To evaluate the influence of these MAMP- and chemical inducers, UHPLC-qTOF-MS in combination with PCA was used to identify metabolites of which the levels are affected [25]. As seen from the PCA score plots (Supporting information, Fig. S1), treated samples were found to cluster separately from the control samples, suggesting different metabolite distribution patterns. Potential bio-markers responsible for the separation as seen from the PCA and cloud plots and identified from the loading plots were further evaluated with regard to the fold-change and significant *p*-values. Amongst these metabolites, peaks with pseudo-molecular ions *m/z* 353.08 and *m/z* 515.01 were found to be up-regulated due to the priming agents. Based on the fragmentation patterns of these ions and the calculated/predicted molecular formulae, they were identified as CGAs [16,26]. All data presented in this study used the recommended IUPAC numbering system and the structures of the positively identified CGAs are shown in Scheme 1.

To substantiate our identification, fragmentation of the MS/MS-based studies were investigated and compared to the hierarchical diagnostic fragmentation key proposed by Clifford et al. [16]. MS/MS data was monitored with the single ion monitoring (SIM) mode to single out peaks with desired *m/z* (Fig. 1). Such peaks were further investigated by generating the corresponding mass spectra (Fig. S2 and Table 1). During MS analyses CGAs fragments generate