New Phytologist Supporting Information

Article title: Jasmonates induce Arabidopsis bioactivities selectively inhibiting the growth of breast cancer cells through CDC6 and mTOR

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SUMMARY

Phytochemicals are often used *in vitro* and *in vivo* in cancer research. The plant hormones jasmonates (JAs) control the synthesis of specialized metabolites through complex regulatory networks. JAs possess selective cytotoxicity in mixed populations of cancer and normal cells. Here, direct incubation of leaf explants from the non-medicinal plant *Arabidopsis thaliana* with human breast cancer cells, selectively suppresses cancer cell growth. High -throughput LC-MS identified Arabidopsis metabolites. Proteins and transcript levels of cell cycle regulators were examined in breast cancer cells.

A synergistic effect by methyljasmonate (MeJA) and by compounds upregulated in the metabolome of MeJA treated Arabidopsis leaves, on the breast cancer cell cycle, is associated with CDC6, CDK2, CYCD1 and CYCD3, indicating that key cell cycle components mediates cell viability reduction. Bioactives such as indoles and quinolines and OPDA, in synergy, could act as anticancer compounds.

Our work suggests a universal role for MeJA-treatment of Arabidopsis in altering the DNA replication regulator CDC6, supporting conservation, across kingdoms, of cell cycle regulation, through the crosstalk between the target of rapamycin, mTOR, and JAs.

This study has important implications to identify metabolites with anti-cancer bioactivities in plants with no known medicinal pedigree and it will have applications in developing disease treatments.

Methods (part)

Metabolite profiling by liquid chromatography-mass spectrometry (LC-MS/MS)

Metabolite profiling was performed using a QToF (Quadrupole Time of Flight) 6520 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled to a 1200 series Rapid Resolution HPLC system.

Data extraction and processing

The raw data files (Agilent *.d) of leaf disc-containing samples were processed with Mass Profiler (Version B.08.00, Agilent, Palo Alto, CA, USA) to extract features of interest (FOIs) using the built-in molecular feature extraction algorithm. Differentially expressed features were identified by 3-way ANOVA ($p<0.05$) using the Benjamini-Hochberg multiple comparison correction. This list was used for cluster and heat map generation. To lead the discovery of JA-regulated specialized metabolites with potential in inhibiting human breast cancer cell growth, we run linear regression models on the normalized (zero mean and unit variance) log2 transformed abundances of each metabolite (total of 1757) with subsequent tests of a priori defined treatment contrasts (Hothorn *et al.*, 2008). These tests served as filtering conditions and metabolites that met them, were aggregated into corresponding sets (Table 1). All tests were performed with at a significance level of P=0.05.

For further analysis, including medium only and medium plus T-47D cells, the raw data files were aligned and subjected to recursive molecular feature extraction using ProFinder (Version B.10.00, Agilent, Palo Alto, CA, USA). The resulting set of compounds were exported to MassProfiler Professional (Agilent) and analysed to identify plant specific and MeJA-induced features.

Where available MS/MS spectra of FOIs were extracted from raw data files using MassHunter Qualitative Analysis software (version B07.00) and compared with MS/MS data from Metlin and MassBank to provide putative identifications. The identification of JA and OPDA was further confirmed by comparison of retention time and spectra with standards. The stereoisomers of these compounds were not resolved by the chromatographic method used. Predicted MS/MS spectra were generated with the MetFrag tool (https://msbi.ipb-halle.de/MetFrag) (Ruttkies *et al.*, 2016).

Methods S1 (part)

Metabolite profiling by liquid chromatography-mass spectrometry (LC-MS/MS)

Human breast cancer cells, co-incubated with excised leaf disks from *A. thaliana* using the experimental setup of the bioassay described, and combining 16 wells (100 µl volume per

well) to a total volume of 1.6 ml, were removed by centrifugation (15.000 g, 5 min) and the supernatant was snap frozen in liquid nitrogen.

Samples were thawed on ice and 300 µl of each was extracted for 60 min at -20 ºC in 900 µl acetonitrile (ACN) containing 1% acetic acid and 0.6 µl umbelliferone (3.6 mg/ml stock), used as the internal standard for sample recovery quality control. After centrifugation (15 min at 15.000 g, 4 ºC), the supernatant was transferred to a fresh tube and dried using a vacuum concentrator (SpeedVac, ThermoFisher Scientific) at 4 ºC for ~2h. The dry pellet was subsequently reconstituted in 150 µl of 80% methanol, mixed and centrifuged (15 min at 15.000 g, 4 ºC). The supernatant was filtered through a 0.2 µm PVDF syringe filter (Chromacol, ThermoFisher Scientific) into a glass autosampler vial.

Metabolite profiling was performed using a QToF (Quadrupole Time of Flight) 6520 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled to a 1200 series Rapid Resolution HPLC system. Five microlitres of sample extract was loaded onto a Zorbax StableBond C18 1.8 µm (particle size), 2.1 x 100 mm (diameter by length) reverse-phase analytical column (Agilent Technologies). For positive ion mode, mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. For negative ion mode, mobile phase A comprised 5% acetonitrile with 1 mM ammonium fluoride in water and mobile phase B was 95% acetonitrile with 1 mM ammonium fluoride in water. The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 30 min – 100% B; 30 min 30 sec – 0% B; 34 min – 0% B, 2 min recalibration post-time. The flow rate was 0.25 mL min^{-1} and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionization were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 L min^{-1} and a nebulizer pressure of 35 psig. The capillary voltage was 3250 V in both positive and negative ion mode. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the autoMS/MS function starting with a survey scan with a mass range of 50 – 3000 m/z at 4 scans s^{-1}, followed by the MS/MS scan with a mass range of 25 – 3200 m/z at 3 scans s^{-1} with a sloped collision energy of 3.5 V/ 100 Da with an offset of 5 V.

Data extraction and processing

The raw data files (Agilent *.d) of leaf disc-containing samples were processed with Mass Profiler (Version B.08.00, Agilent, Palo Alto, CA, USA) to extract features of interest (FOIs) using the built-in molecular feature extraction algorithm. This process identifies compounds as co-eluting adducts (H^+, Na^+, K^+, NH_4^+) using a 15 ppm mass tolerance and a 0.3 min retention time tolerance. The retention times of features were aligned across samples. This analysis was carried out for samples analysed in positive and negative ion mode and the resulting combined 5359 features (including predicted neutral mass, molecular formula, retention time and abundance) were exported as text files to MassProfiler Professional (Agilent) for statistical analysis. Log_2 abundance values were percentile (value 75) normalised and baselined to the median of all samples.

Features of interest were exported to MassHunter ID Browser (version B 08.00, Agilent) for generation of predicted molecular formulae based on accurate mass and isotope abundance and searching of an in-house Arabidopsis compound database constructed from AraCyc (https://www.plantcyc.org/databases/aracyc/16.0). Further annotation of FOIs was carried out by accurate mass searches in MassBank (http://www.massbank.jp/), Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), MZCloud (https://www.mzcloud.org/), Human Metabolome Database (http://www.hmdb.ca/), LipidBank (http://lipidbank.jp/) and FooDb (http://foodb.ca/).