Extraction and analysis of primary metabolites during Xanthomonas-Barley interaction

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

Intercellular host-associated bacteria shape the chemistry of the living eukaryotic environment. Pathogenic microorganisms like barley-associated *Xanthomonas* translucent (*Xt*) swiftly overtake the inner leaf tissue becoming the dominant community member during disease development. To define the metabolic signals associated with inner leaf colonization, we used untargeted metabolomics to characterize Xtu and Xtt primary metabolism signatures associated with mesophyll growth.

This protocol indicates how to do the infections, extract apoplastic fluids, perform GCMS, and the analysis.

GUIDELINES

Sugars degrade easily, therefore is good to use liquid nitrogen to quickly save the samples.

Also, there is high variation in the samples, therefore is good to have several samples and pool them to reduce the variation.

PROTOCOL integer ID:
92919

Keywords: primary metabolites, GCMS, Xanthomonas translucens, barley mesophyll, untargeted metabolomics

MATERIALS

Barley cv. Morex
Xtu, Xtt
Nutrient Agar
Methanol
Ribitiol

SAFETY WARNINGS

NA

Sample preparation

1 Sample preparation, you need Barley cv. Morex seeds, *Xanthomonas translucens* pv. *undulosa* strain UPB513, and *Xanthomonas translucens* pv. translucent strain UPB886.

The experiment requires samples for
Barley plants
The Barley cv. Morex was used for in-planta studies. All plants were sown at 20 °C 60 % humidity in Promix soil. Plants were grown for three weeks before inoculation. Plants should have at least two fully developed leaves.

Bacteria
Xanthomonas translucens pv. *undulosa (Xtu)* and *Xanthomonas translucens* pv. translucent *(Xtt)* were taken out of -80 °C glycerol stock and plated on Nutrient Agar media (3 g/L beef extract, 5 g/L peptone, 15 g/L agar) a week before the experiment and incubated at 28 °C. Three days before the experiment, a single bacteria colony was plated again on Nutrient Agar and incubated at 28 °C. On the day of inoculation, a bacterial loop was suspended in sterile water to achieve an absorbance of 0.1 at 600mn (10^8 CFU/mL).

Bacterial and mock inoculation

2 The youngest fully expanded leaf of the three-week-old plants was used for inoculations.

Fill a 1 mL needleless syringe (insulin-type syringae) with the inoculum. Press the syringe against the abaxial surface of the leaf and place a finger on the opposite side of the syringe to keep firm contact between the syringe and the leaf. Gently plunge the syringe, inoculating roughly 100-500 µL into the leaf. Most leaves will have a temporary transparency to the infiltrated area.

The leaves were syringe-infiltrated with either 1-2mL of the bacterial resuspension or mock water.

Aim to inoculate at least 30-40 leaves with each strain to have data to collect at each time point and to pool samples.

Apoplast collection

3 Leaves were collected after **6 h**, **12 h**, and **24 h post-inoculation**.

To eliminate any surface microorganisms, the leaves were cleaned using 75% ethanol. A pool of four leaves was cut at the base for fluid extractions and then weighed. A 10 ppm ribitol solution was used to vacuum infiltrate all the leaves. The ribitol was added to the for metabolites normalization.

After infiltration, the leaves were weighed again to determine the amount of water infiltrated; water density was assumed to be 1g/ml. We followed the protocol from Roman-Reyna and Rathjen 2017 for the mesophyll fluid extraction and test cytoplasmic contamination. We used 50 mL syringes to create the negative pressure.

CITATION

Roman-Reyna V, Rathjen JP (2017). Apoplastic Sugar Extraction and Quantification from Wheat Leaves Infected with Biotrophic Fungi..

LINK

https://doi.org/10.1007/978-1-4939-7249-4_11

At each time point, 2uL of Apoplastic fluid from each sample was plated in Nutrient Agar to count the colony-forming units and ensure that all samples had the same number of bacteria. After that, fluids were flash-frozen to avoid metabolite degradation.

For analysis, four pools were selected from each time point that has a similar number of bacteria.

Polar metabolites extraction

4 Polar extraction is based on the sugar and amino acids interaction with Methanol (MeOH) and Chloroform (ChlNa2).

For the polar extraction, ~500uL of apoplast was mixed with 1mL of (5:2:2 MeOH:ChlNa2:H2O). The sample was vortex and incubated for 5min at 28C in a 20 rpm, 28°C .

Samples were then centrifuged 2200 x g, 15°C 15min. The methanol phase was collected and 200uL were sent to the West Coast Metabolomics Center (UC Davis) for Untargeted Primary Metabolism by GC-TOF MS (GCTOF - C).

Untargeted Primary Metabolism with GC-TOF MS.

5 Samples were sent to the West Coast Metabolomics Center (UC Davis) for Untargeted Primary Metabolism by GC-TOF MS.

The following methods were shared by the Center.

For more details please see:

CITATION

Fiehn O, Wohlgemuth G, Scholz M, Kind T, Lee DY, Lu Y, Moon S, Nikolau B (2008). Quality control for plant metabolomics: reporting MSI-compliant studies..
LINK
https://doi.org/10.1111/j.1365-313X.2007.03387.x

5.1 Derivatization

Samples were extracted using 1mL of 3:3:2 ACN:IPA:H2O (v/v/v). Half of the sample was dried to completeness and then derivatized using 10 uL of 40 mg/mL of Methoxyamine in pyridine. They are shaken at 30C for 1.5 hours. Then 91 uL of MSTFA + FAMEs were added to each sample and were shaken at 37C for 0.5 hours to finish derivatization.

5.2 Data Acquisition

Samples are then vialed, capped, and injected into the instrument. They used a 7890A GC coupled with a LECO TOF.

The derivatized sample was injected (0.5uL, splitless method) onto a RESTEK RTX-5SIL MS column with an Intergra-Guard at 275°C with a helium flow of 1 mL/min. The Column details are Restek corporation Rtx-5Sil MS (30 m length x 0.25 mm internal diameter with 0.25 µm film made of 95% dimethyl/5%diphenylpolysiloxane).

The GC oven is set to hold at 50°C for 1 min then ramp to 20°C/min to 330°C and then hold for 5 min. The transfer line is set to 280°C while the EI ion source is set to 250°C. The Mass spec parameters collect data from 85m/z to 500m/z at an acquisition rate of 17 spectra/sec.

Mass spectrometry parameters are used as follows: a Leco Pegasus IV mass spectrometer is used with unit mass resolution at 17 spectra s-1 from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source.

5.3 Data Processing

ChromaTOF 2.32 software (Leco Corp) is used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram.

Apex masses are reported for use in the BinBase algorithm. Result *.txt files are exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database.

The BinBase algorithm (rtx5) used the settings: validity of chromatogram (<10 peaks with intensity >10^7 counts s-1), unbiased retention index marker detection (MS similarity>800, validity of intensity range for high m/z marker ions), retention index calculation by 5th order polynomial regression. Spectra are cut to 5% base peak abundance and matched to database entries from most to least abundant spectra using the following matching filters: retention index window ±2,000 units (equivalent to about

For the BinBase software please see:

CITATION

Skogerson K, Wohlgemuth G, Barupal DK, Fiehn O (2011). The volatile compound BinBase mass spectral database..

LINK

https://doi.org/10.1186/1471-2105-12-321

5.4 Raw Data Normalization

The data is given as peak heights for the quantification ion (mz value) at the specific retention index.

West Coast Metabolomics Center (UC Davis) provides a variant of a 'vector normalization' in which they calculate the sum of all peak heights for all identified metabolites for each sample. They called it "mTIC" in analogy to the term TIC used in mass spectrometry (for 'total ion chromatogram'), but with the notification "mTIC" to indicate that we only use genuine metabolites (identified compounds). Data is normalized to the total average mTIC.

Further analyses

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West Coast Metabolomics Center (UC Davis) provided the normalized data and annotated the peaks based on retention times and mass spectra compared to the MassBank of North America.

West Coast Metabolomics Center provided a table with a list of KEGG IDs, names, and peak intensities, which was used as input for MetaboAnalyst. The Raw/derived files (cdf extension) are available on https://www.ebi.ac.uk/metabolights project MTBLS7676.

Statistical analyses were conducted in MetaboAnalyst 5.0.

CITATION

Xia J, Wishart DS (2011). Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst..

LINK

https://doi.org/10.1038/nprot.2011.319

We selected the module "Statistical Analysis [One Factor]" for the analysis. With thedata type "Peak Intensities" the input data was the CSV table from theWest Coast Metabolomics Center.

For partial least squares discriminant analysis (PLSDA), the table was uploaded as a single file. For Fold-Change (FC) analysis data, the data was separated into six files based on time points (6, 12, 24) and Xt strains (UPB886, UPB513) before uploading.

PCA and FC analysis data underwent the same normalization steps. All data was imported into the Statistical Analysis module with the data type “Peak intensities”. The statistical filter used was Standard deviation and for sample normalization, we selected the option 'Normalization by reference feature'. The reference feature was Ribitol, which was spiked into the extraction buffer. Finally, to reduce data skewness we selected Log transformation (base 10).