Methods

Extraction and quantification of CKs using High-Performance Liquid Chromatography - (High Resolution Accurate Mass) - Tandem Mass Spectrometry (HPLC-(HRAM)MS/MS)

Prior to extraction, all samples were spiked with the isotope-labeled CK internal standards (OlChemIm, Olomouc, Czech Republic). Cytokinins were extracted from root tissues with Bieleski #2 extraction solvent (methanol:formic acid:water [15:1:4, v/v/v]), and purified by solid phase extraction (SPE) using MCX reverse phase (RP) cartridges. Purified samples were separated on C18 HPLC column and analyzed in positive ionization mode on high resolution-accurate mass QExactive Orbitrap mass spectrometer (FisherScientific, Ottawa, ON, Canada) coupled to a Dionex UltiMate 3000 HPLC (FisherScientific, Ottawa, ON, Canada). CKs were identified based on their analyte-specific retention times and parallel reaction monitoring (PRM) channels. CKs were quantified by isotope dilution analysis based on the direct comparison of the endogenous analyte peak area to that of the recovered internal (Xcalibur 4.1 software, Thermo Scientific, Waltham, MA, US).

The samples were scanned for the presence of 28 CKs: *cis*-zeatin (cZ), *cis*-zeatin riboside (cZR), *cis*-zeatin-9-glucoside (cZ9G), *cis*-zeatin nucleotide (cZNT), *cis*-zeatin *O*-glucoside (cZOG), *cis*-zeatin riboside-*O*-glucoside (cZROG), dihydrozeatin (DZ), dihydrozeatin nucleotide (DZNT), dihydrozeatin-*O*-glucoside (DZOG), dihydrozeatin riboside (DZR), dihydrozeatin riboside-*O*-glucoside (DZROG), dihydrozeatin-9-*N*-glucoside (DZ9G), isopentenyladenine (iP), isopentenyladenine nucleotide (iPNT), isopentenyladenine-7-glucoside (iP7G), isopentenyladenine-9-glucoside (iP9G), isopentenyladenosine (iPR), 2-methylthio-isopentenyladenine (2MeSiP), 2-methylthio-isopentenyladenosine (2MeSiPR), 2-methylthio-

zeatin (2MeSZ), 2-methylthio-zeatin riboside (2MeSZR), *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), *trans*-zeatin-7-glucoside (tZ7G), *trans*-zeatin-9-glucoside (tZ9G), *trans*-zeatin nucleotide (tZNT), *trans*-zeatin *O*-glucoside (tZOG), and *trans*-zeatin riboside-*O*-glucoside (tZROG).

Extraction and quantification of acidic hormones (ABA, IAA, JA) using High Performance Liquid Chromatography - Electrospray Ionisation - Tandem Mass Spectrometry (HPLC-(ESI)-MS/MS)

Prior to extraction, all samples were spiked with the labeled ABA ($^{2}H_4$ABA; 60 ng; NRC-PBI, Saskatchewan, SK, Canada) and IAA ($^{13}C_6$IAA; 10 ng; OlChemIm, Olomouc, Czech Republic) internal standards. Extraction of the three acidic phytohormones followed the protocols of Simura et al [1] with slight modifications. Briefly, root samples were extracted with 50% acetonitrile and purified using HLB RP, SPE cartridges. For the HPLC–MS/MS analysis, purified phytohormone samples were dissolved in 300 µL starting conditions solvent (acetonitrile:acetic acid:water [5:0.08:94.92, v/v/v]) and transferred to insert-equipped vials.

Levels of ABA, IAA and JA were measured using high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-(ESI)-MS/MS; QTrap5500; ABI Sciex Concord Ontario, Canada, coupled with Agilent 1100 series HPLC; Agilent, Mississauga, ON, Canada). An aliquot of 40 µl was injected on a Kinetex C18 column (2.1 × 50 mm, 2.6 µm; Phenomenex, Torrance, U.S.A.). Phytohormones were eluted using component A: dd-water, and component B: acetonitrile, both with 0.08% acetic acid, at a flow rate of 0.28 mL min^{-1}. The initial conditions of 5% B remained constant for 0.5 min, then changed linearly to 100% B over 3.5 min. This ratio was held constant for 2 min before immediately returning to

starting conditions and re-equilibrating for 4 min. Acidic phytohormones were identified in negative ionization mode, based on their analyte specific retention times and multiple reaction monitoring (MRM) channels using previously described MS settings [2].

Levels of ABA and IAA were quantified by isotope dilution analysis based on the direct comparison of the endogenous analyte peak area to that of the recovered internal (Analyst 1.6.2 software, AB SCIEX, Concord, ON, Canada). Levels of JA were quantified based on the recovery of $^{2}H_{4}$ABA. The content of acidic phytohormones in canola root samples was standardized to 1 g of plant fresh weight (FW).

Extraction and quantification of primary metabolites (free amino acids, sugars and sugar phosphates, organic acids) and secondary metabolites (glucosinolates) using High Performance Liquid Chromatography – High-Resolution Accurate Mass – Full Scan Mass Spectrometry (HPLC-(HRAM)-MS)

Samples for extraction of primary and secondary metabolites were spiked with 10ng of two labeled aromatic CKs ($^{13}C_{5}$-oT and $^{2}H_{7}$-BAR) and extracted with ice-cold methanol (methanol-water [8:2, v/v]) following procedures of Chen et al [3] with modifications. Each filtered extract was split in half and each 500 µL transferred to a new 2 mL tube. Divided extracts were evaporated to dryness at ambient temperature in a speed vac concentrator. Sample residues designed for the analysis of primary metabolites were redissolved in 500 µl of 90% acetonitrile (acetonitrile: water, v/v) and samples for the analysis of glucosinolates were redissolved in 500 µL of 5% acetonitrile with 0.1% formic acid (acetonitrile: formic acid: water, v/v/v). Additionally, sample mixtures composed of 10uL of each sample extract were prepared

separately for primary and secondary metabolite analysis and used to generate MS/MS for compound identification. All samples were filtered using 0.2 µm PVDF spin filter with 2 mL receiver tubes (InnoSep Spin, Canadian Life Sciences, Peterborough, Canada) and transferred to insert-equipped 2 mL HPLC vials. A volume of 25 µL of each sample was injected into a Dionex UltiMate 3000 HPLC coupled to a QExactive Orbitrap mass spectrometer.

Primary metabolites were resolved with an InfinityLab Poroshell 120 HILIC-Z column (2.1 × 100 mm, 2.7 µm; Agilent, Santa Clara, CA, US), using a flow rate of 0.2 mL min^{-1} with a mobile phase of 10 mM ammonium bicarbonate in water (A) and 10 mM ammonium bicarbonate in 95% acetonitrile (B). The following gradient was used to elute the analytes: mobile phase 100% B decreased to 90% over 2.5 min and to 50% over the next 5 min and returned to 100% over 0.5 min for 12 min of column re-equilibration.

Glucosinolates were resolved with a Kinetex C18 column (2.1 × 50 mm, 2.6 µm) using a flow rate of 0.3 mL min^{-1} with a mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The following gradient was used to elute the analytes: mobile phase B was held at 0% for 1.25 min before increasing to 50% over 2.75 min and to 100% over the next 0.5 min. Solvent B was then held at 100% for 2 min before returning to 0% over 0.5 min for 4 min of column re-equilibration.

The Orbitrap QExactive was operated with a heated electrospray ionization (HESI) probe in positive and negative mode [4]. Each sample was analyzed using a mass range of *m/z* 70–900, and data were acquired at 70,000 resolution, automatic gain control (AGC) target of $1e^6$, and maximum injection time (IT) of 100 ms. Additionally, the top 10 data-dependent acquisition

experiments were performed for the mixed sample from each group (HILIC and C18) to obtain compound MS/MS spectra [3].

Processing of all full scan and ddMS2 data was conducted using the Xcalibur 4.1 software. Metabolites were identified by accurate mass, comparison of retention times to authentic standards or by accurate mass and comparison of fragmentation patterns to MS/MS databases (METLIN, PubChem). Metabolite relative concentration was normalized based on the average recoveries of $^{13}C_5$-oT and $^{2}H_7$-BAR as the internal standards.

The raw and pre-processed data for metabolomics using clubroot-resistant and susceptible canola roots is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench [5], where it has been assigned Study ID ST002330. The data can be accessed directly via its Project DOI: http://dx.doi.org/10.21228/M8MD91.

References

1 J. Šimura, I. Antoniadi, J. Široká, D. Tarkowská, M. Strnad, K. Ljung and O. Novák, Plant Hormonomics: Multiple phytohormone profiling by targeted metabolomics, *Plant Physiol.*, 2018, **177**, 476–489.

2 S. C. Farrow and R. N. Emery, Concurrent profiling of indole-3-acetic acid, abscisic acid, and cytokinins and structurally related purines by high-performance-liquid-chromatography tandem electrospray mass spectrometry, *Plant Methods*, 2012, **8**, 42.

3 C. Chen, C. Li, Y. Wang, J. Renaud, G. Tian, S. Kambhampati, B. Saatian, V. Nguyen, A. Hannoufa, F. Marsolais, Z.-C. Yuan, K. Yu, R. S. Austin, J. Liu, S. E. Kohalmi, K. Wu, S. Huang and Y. Cui, Cytosolic acetyl-CoA promotes histone acetylation predominantly at H3K27 in Arabidopsis, *Nature Plants*, 2017, **3**, 814–824.

4 A. Kisiala, S. Kambhampati, N. L. Stock, M. Aoki and R. J. N. Emery, Quantification of Cytokinins Using High-Resolution Accurate-Mass Orbitrap Mass Spectrometry and Parallel Reaction Monitoring (PRM), *Anal. Chem.*, 2019, **91**, 15049–15056.

5 Sud, M., Fahy, E., Cotter, D., Azam, K., Vadivelu, I., Burant, C., Edison, A., Fiehn, O., Higashi, R., Nair, K. S., Sumner, S., & Subramaniam, S. (2016). Metabolomics Workbench: An international repository for metabolomics data and metadata, metabolite standards, protocols, tutorials and training, and analysis tools. *Nucleic Acids Res.*, **44**, D463–D470.