Metabolic effect of drought stress during the grain filling growth stage in wheat measured by Isotopic Ratio Outlier Analysis (IROA)

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Abstract

Metabolomic approaches have been documented to have great value in phenotyping and diagnostic analyses in plants[1]. The IROA® protocol[2,3] was applied to determine the biochemical response of wheat metabolomes to water-stress during the grain filling growth stage. SS8641, a high-yield soft-red winter wheat, was grown under well-watered and drought conditions. In this IROA phenotypic analysis, controlled greenhouse-grown leaves containing carbon at natural abundance were compared to Standard wheat leaves that were grown to contain universally-distributed ~97% ^{13}C; namely, a targeted analysis using a biologically-relevant Internal Standard. The IROA patterns allowed the identification of the isotopically labeled peaks and their ^{12}C isotopomers, and the removal of artifacts, noise and extraneous peaks. By pooling experimental and Standard samples, variances introduced during sample-preparation and analysis were controlled.

Introduction

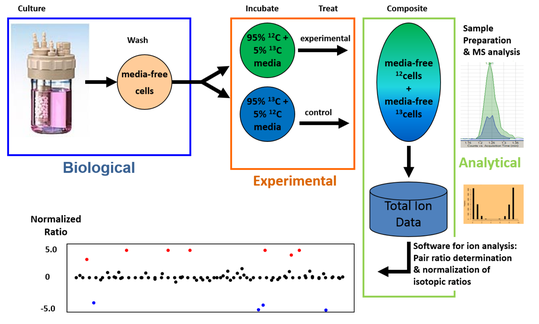


Figure 1. The IROA "Basic" global process overview.

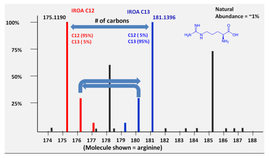


Figure 2. The IROA peaks. In the case of arginine, the ^{12}C M^{+} located at 175.1190 and its ^{13}C mate at 181.1396 clearly indicate a 6 carbon molecule. The corresponding M^{+1} and M^{-1} peaks are plus or minus the mass of a neutron. Natural abundance peaks do not have IROA patterns.

IROA Basic Protocol

The greatest challenge of most metabolic profiling experiments is the ability to differentiate peaks of biological origin from artifactual peaks, and to accurately identify and quantitate the peaks of interest. The IROA "Basic" global labeling and bioinformatics protocol (Figure 1), utilizes isotopically-defined media in which all nutrients are labeled with either 5%^{13}C, "^{12}C IROA media" (experimental), or 95%^{13}C, "^{13}C IROA media" (control), so that all biological compounds carry a distinct molecular signature and molecules can be distinguished from each sample set, as they have differing masses. Control and experimental samples can be analyzed as a single composite sample by LC-MS. Algorithms pair all identified biological peaks, and unlabeled natural abundance artifacts may be identified and discarded. All biological compounds will have two paired peaks; the peak from the ^{12}C-media is mirrored by a second peak from the ^{13}C-media (see Figure 2). The distance between the monoisotopic peaks readily identifies the number of carbons in the compound. The corresponding M^{+1} and M^{-1} peaks (and M^{+2} and M^{-2} etc. peaks) which are a mass difference of 1.00335 amu (mass difference between a ^{12}C and ^{13}C isotope), gives the IROA peaks a characteristic U-shape "smile" pattern. Accurate mass together with the knowledge of the number of carbons in a molecule greatly facilitates metabolite identification.

IROA Phenotypic Protocol

Where it is not possible to label the biological sample, the IROA "Phenotypic" Protocol (Figure 3) is applied. Here the sample is collected at natural abundance and mixed with a fully predefined "Standard" that has been isotopically labeled using IROA ^{13}C media. An ideal Standard would be one that represented the entire metabolome of the sample under study. All peaks of the IROA-labeled Standard may be easily identified according to their characteristic M-1 peak. The natural abundance metabolite peaks from experimental samples may be readily identified as their exact mass and position are established relative to their counterparts in the Standard. Compounds present in a Standards can be well characterized, produced in sufficient quantities, stored and used to compare samples across multiple experiments. Artifacts have no match in the Standard and can be discarded. Whereas in a IROA basic dataset the ratio of the peak areas represents the relative deviation of the metabolic pool sizes brought about by the experimental condition, in an IROA Phenotypic experiment the overall pattern of deviations from the Standard will define phenotype by difference from the Standard.

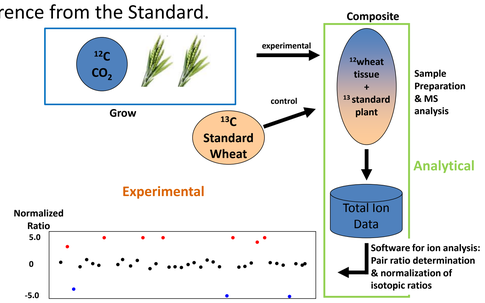
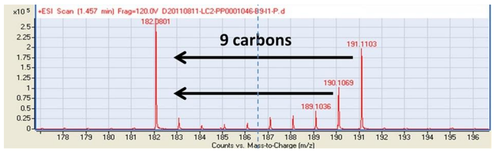


Figure 3. IROA Phenotypic Protocol. Wheat leaves are pooled with an isotopically-labeled wheat Standard (97% ^{13}C-enriched, IsoLife), processed, and analyzed by mass spectrometry. Software algorithms are used to sort the peaks, locate the M-1/^{13}C ratio of the labeled metabolites and match the experimental M natural abundance peaks. The ratio of the paired peak areas are measured and normalized and outliers determined. The IROA Phenotypic experiment is considered a complex targeted analysis.

Theory & Discussion

Using the IROA Phenotypic Protocol, both control Standard and experimental samples are analyzed as a pooled sample so that variability and ion suppression are accounted for, and fewer samples are required to be analyzed. Since the Standard is labeled, and even though the experimental samples do not carry any isotopic labeling, their exact mass and position are established relative to the Standard and noise and artifacts can be removed using software algorithms allowing for a very dramatic reduction in data size. The number of carbons are calculated for each IROA peak and this information together with accurate mass for either of the monoisotopic peaks makes it possible to unambiguously determine the empirical formula for masses below 400.

In this experiment a total of 724 IROA peaks were identified over 72 samples analyzed in both positive and negative mode; 8 time points, 3 conditions - well-watered and two different water-stress conditions, using biological triplicates.



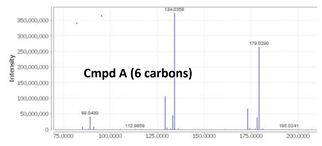
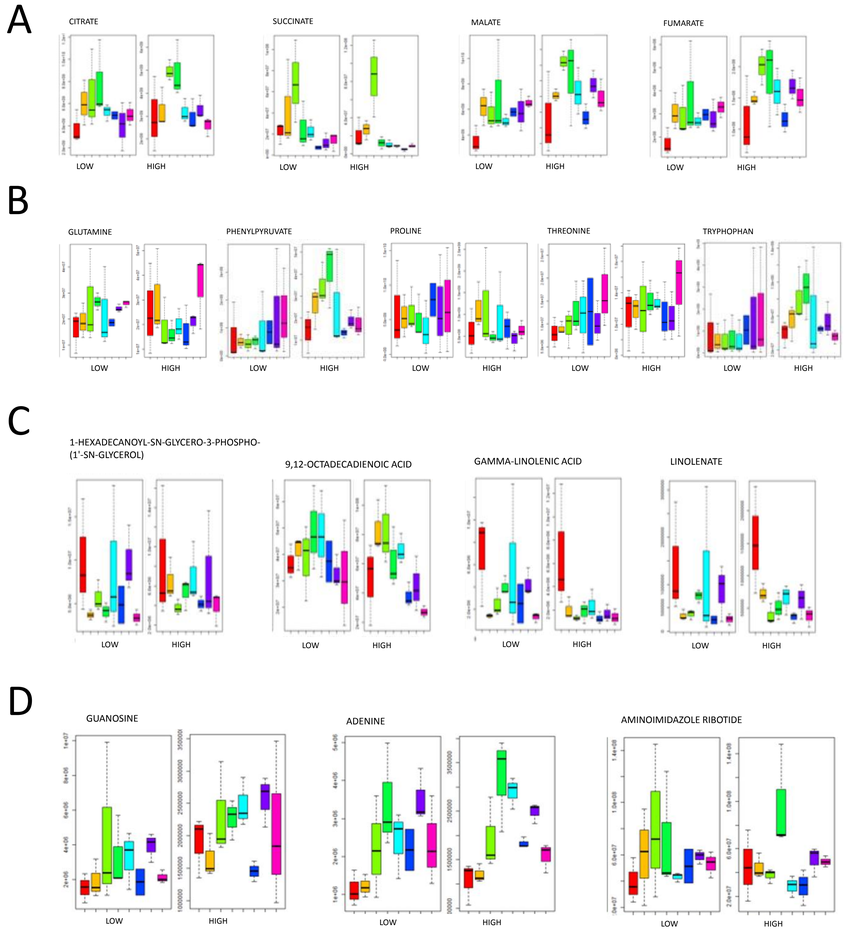


Figure 4. Representative IROA peaks. These peaks are examples of IROA phenotypic peaks.

Results

This study describes the use of an analytical and bioinformatic metabolomics technology, "IROA Phenotypic Protocol" (Figure 3), applied to the understanding of controlled greenhouse-grown wheat profiles under well-watered or one of two water-stress conditions. The IROA-labeled Standard used was wheat leaves derived from 7 week old *Triticum aestivum* seedlings grown using $^{13}CO_2$ as the only carbon source. 724 IROA peaks were found using ClusterFinder™ software. The ^{13}C IROA peaks in the labeled IROA control Standard were used to identify and quantitate their associated natural abundance peaks in the greenhouse-grown plants, allowing for a complex targeted analysis. The resulting dataset was analyzed as discussed in the Methods.



Methods

Experiment design: Seeds of wheat variety SS8641, developed by Jerry Johnson, University of Georgia were grown at day:night temperature of 20:15°C, relative humidity of 40±2%, 16h daylight until full anthesis period. A control group was maintained well-watered at Field Capacity while two groups (W1, W2) were subjected to water-stress (25%, 50% FC, respectively). Leaves were harvested 2 weeks post-anthesis every 3hrs during a 24h period, flash frozen in LN2, lyophilized and ground. For each sample (24 samples in triplicate, total of 72 samples), 10 mg dry weight was mixed with an equal quantity of ground wheat leaf grown under CO2 maintained at 97.5% ^{13}C and 2.5% ^{12}C (IROA control Standard). The combined wheat leaf samples were held at -80°C until preparation.

Sample Preparation: An aliquot of mixed sample in a small round-bottomed vial, containing approximately 270 mg 0.7 mm zirconia beads (Biospec.com), two 3 mm borosilicate glass beads (Kimble Chase), was suspended in 800 µl of MAA (MeOH:ACN:acetone, 1/1/1). The vial was capped and homogenized on a bead beater for 10 minutes at maximum speed. After homogenization the vials were centrifuged for 10 min at 4°C at 4,750 RPM. 500 ul of the supernatant was recovered, filtered, and dried under nitrogen. The dried sample was re-dissolved in 50 µl of distilled water for analysis.

LC-MS: Samples were stored in freezer and at 4 °C on the instrument autosampler. All data were acquired on the Thermo Scientific Q Exactive using external mass calibration. Sample analyses were conducted in both positive and negative ESI modes as separate LC/MS/MS runs. Full-scan MS were collected at a mass resolution of 70,000 FWHM from m/z 70 – 1000. Data-dependent MS/MS scans were acquired at 17,500 FWHM.

Data processing and analysis: The dataset was analyzed by the IROA ClusterFinder software (K). The 724 peaks identified tentatively by ClusterFinder were compared against libraries of compounds for their IROA characteristics; ^{12}C base peak, ^{12}C M+1, ^{13}C base peak, ^{13}C M-1, and intervening peaks. The metabolic phenotype of each experimental sample consists of the relative concentration of a number of metabolites. In the IROA method, all compound measurements are made relative to a ^{13}C Standard; in this case, an isotopically-labeled wheat Standard (97% ^{13}C-enriched, IsoLife). Therefore, these measurements represent the deviation of each metabolite relative to the isotopically-labeled Standard.

References

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3. Stupp GS, Clendinen CS, Ajredini R, Szewc MA, Garrett T, Menger RF, Yost RA, Beecher C, Edison AS. "Isotopic Ratio Outlier Analysis Global Metabolomics of Caenorhabditis elegans." *Analytical Chemistry* 2013 *85*(24), 11858-11865. DOI: 10.1021/ac4025413.

Summary

This IROA Phenotypic study was performed in wheat to understand key growth processes that characterize drought that could be used to mediate the selection of advantageous genotypes. The IROA results demonstrated clearly that during water-stress conditions (25% Field Capacity, LOW) compared with well-watered at Field Capacity (HIGH) disruption was seen in many pathways including the Krebs cycle, and amino acid, lipid and nucleotide metabolism (see Fig 5 A, B, C, D, respectively). The significantly enhanced quantitation of having a complex internal IROA Standard present in every experimental sample is a major benefit of the IROA protocol. All experimental biological peaks will have a ^{13}C-labeled pair and therefore artifacts will have no match in the Standard and may be removed from consideration and prevented from becoming false positives. The application of the IROA Standard as a "recovery"-type standard, i.e. put in prior to sample prep and carried throughout the rest of the sample preparation, is a means of further enhancing data quality and reducing sample-to-sample variation (correcting both in-sample and between sample variations).