Differential Metabolomic Profiling of Wheat Cultivars by IROA (Isotopic Ratio Outlier Analysis)

Chris Beecher[1], Francis Beecher[2], Felice A de Jong[1]

[1]*NextGen Metabolomics, Inc, Ann Arbor, MI;* [2]*Department of Soil and Crop Sciences, Texas A&M, College Station, TX*

NextGen Metabolomics
IROA makes metabolic profiling easy

Abstract

The interest in metabolomics to understand fundamental biology and applied biotechnology, especially in the field of plant science, has driven technology development. This study describes the use of a combined analytical and bioinformatic metabolomics technology applied to the understanding of plant metabolism. The diurnal metabolome changes exhibited in a cultivar of wheat, TX8544, were determined using the IROA protocol. Metabolomics plays an important role in how an organism adapts to change, in this case the diurnal pattern of heat and light. Here an isotopically-defined standard wheat sample is added to the experimental sample and is analyzed as a single sample, reducing suppression, and sample-to-sample variance, including variance introduced during preparation and analysis.

Introduction

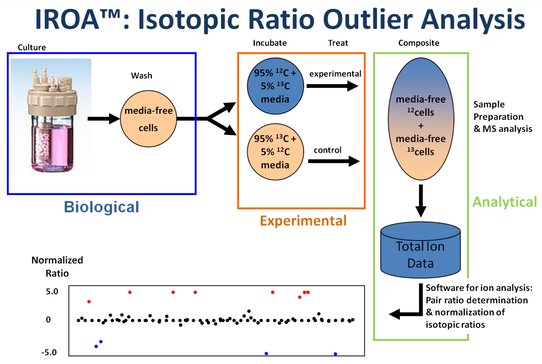

Figure 1. The global IROA process overview.

Global IROA Application

The most difficult aspect 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 global IROA labeling protocol (see Figure 1), utilizes isotopically-defined media in which all nutrients are labeled with either 5%^{13}C, "C12 IROA media" (experimental), or 95%^{13}C, "C13 IROA media" (control), so that all biological compounds carry a distinct molecular signature and these molecules can be distinguished from each sample set, experimental or control, as they have differing masses. Therefore, control and experimental samples can be analyzed as a single composite sample by LC-MS with all biological peaks uniquely paired, and natural abundance artifacts may be identified as such and discarded as they hold no distinct signature. With IROA, any biological compound will have two paired peaks; the peak from the C12-media is mirrored by a second peak from the C13-media (see Figure 2). The distance between these peaks readily identifies the number of carbons in the compound. In addition there are corresponding M^{+1} and M^{-1} peaks (and M^{+2} and M^{-2} etc. peaks) which are a mass difference of 1.00335 amu (the mass difference between a ^{13}C and ^{12}C isotope), giving 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 isotopically label the biological sample, the IROA "Phenotypic" Protocol (Figure 3) is applied, whereby 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 plant, fluid or tissue under study. Using an IROA-labeled Standard, all of peaks may be easily identified according to presence of their characteristic M-1 peak. The natural abundance metabolite peaks (paired to each Standard peak) may be readily identified because even though they do not carry any isotopic labeling, their exact mass and position are established relative to the Standard. Since the pooled Standard that is used is always the same for a given tissue or fluid, the compounds that are present in it have already been identified. Artifacts will have no match in the Standard and need not be considered in a final dataset. Whereas in a basic IROA dataset the ratio of the peak areas represents the relative deviation of the metabolic pool sizes brought about by the experimental condition, in a Phenotyping experiment the overall pattern of deviations from the Standard will define phenotype by difference from the Standard. It is possible to consider the Phenotyping experiment a very complex targeted analysis relative to the unbiased analysis of the global IROA experiment.

Theory & Discussion

Using the IROA Phenotypic Protocol, both control and experimental samples are analyzed as a pooled sample so that variability and ion suppression are removed, and fewer samples are required. Since the control Standard is labeled and because 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 by software algorithms allowing for a very dramatic reduction in data size. The number of carbons for each biochemical compound can be calculated from its mass spectra and when using accurate mass spectrometry the compound readily identified.

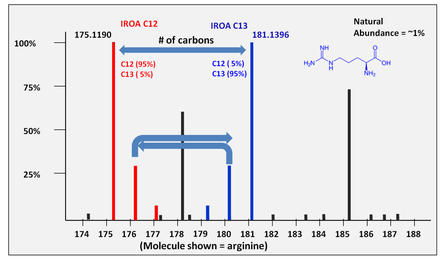

Figure 2. The IROA peaks. In the case of arginine, the ^{12}C M^{+} located at 175.1190 and its ^{13}C mate at 181.1396 clearly indicate a 6 carbon molecule. The corresponding M^{+1} and M^{-1} peaks are a mass difference of 1.00335 amu (the mass difference between a ^{13}C and ^{12}C isotope). Natural abundance peaks from exogenous sources do not have a ^{13}C counterpart and are not considered in the analysis .

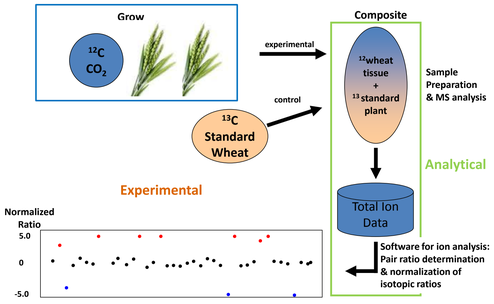

Figure 3. IROA Phenotyping protocol. Wheat stems or leaves are pooled with an isotopically-labeled wheat standard (95% 13C-enriched, IsoLife), processed, and analyzed by mass spectrometry. Software algorithms are used to sort the peaks, locate the M-1/13C ratio of the labeled metabolites and match the experimental M natural abundance peaks. The ratio of the paired peak areas are measured and normalized and outliers determined.

Results

Since plants typically grow in a diurnal light/dark cycle, this provide an amenable system to analyze the dynamics of changes in metabolism. This study describes the use of an analytical and bioinformatic metabolomics technology, "IROA Phenotypic Protocol" (Figure 3) applied to the understanding of the diurnal changes in TX8544, a Texas spring wheat cultivar. The IROA-labeled Standard used was wheat leaves derived from 7 week old *Triticum aestivum* grown using $^{13}CO_2$ as the only carbon source. Over 300 known metabolites were quantitated using the IROA method.

Among the metabolites measured, Figure 4 shows the paired peaks for leucine. The peaks produced using the IRO Phenotyping Protocol show the M^{-1}, M^{-2} characteristic of the IROA Basic peak. In this case the label was only 97.5%, however the ClusterFinder software was still capable of finding all of the IROA peaks. Being an IROA Phenotypic experiment, the C12 peak demonstrates a normal natural abundance M^{+1} peak. Rather than the typical IROA balanced U-shape (smile) peak, the IROA Phenotypic peak is unbalanced leading us to call it a "smirk".

In the light phase, photosynthetic carbon fixation occurs in the leaves and the stored carbon is converted into sugars and transported throughout the plant, whereas at night the plant remobilizes the sugars which are consumed during the night (see the diurnal pattern for sucrose, Figure 5).

Figure 6 shows the diurnal pattern for coniferyl glucose, a key constituent in the lignin biosynthetic pathway. It is known that circadian control may be one mechanism through which the lignin biosynthetic pathway is coordinated and metabolic lignification peaks during the day.

In addition to dark-light, temperature cycles are also are known to regulate metabolic patterns in plants. An up-regulation of the citric acid and urea cycles are known to have a role in colder temperatures, including during the night, as supported by the increase shown in succinate in the PM hours (Figure 7).

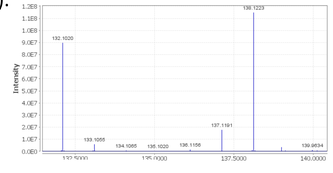

Figure 4. The IROA Phenotypic peak for leucine.

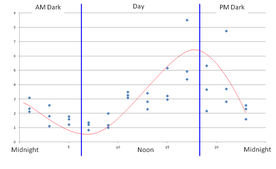

Figure 5. The IROA Phenotypic diurnal pattern for sucrose.

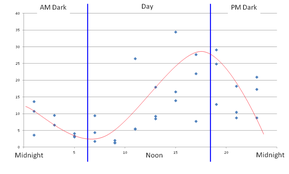

Figure 6. The IROA Phenotypic diurnal pattern for coniferyl glucose.

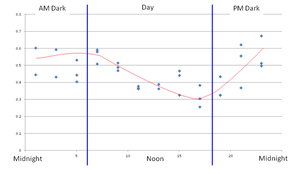

Figure 7. The IROA Phenotypic diurnal pattern for succinate.

Methods

Experiment design: TX8544 wheat was "clump" grown in pots from seed in a controlled greenhouse environment, with water applied as required (see picture upper right). At four weeks leaf tissue (leaves) was harvested at two hour intervals over a 24 hour period. At the time of collection the leaf was cut, placed into a manila envelope and immediately submerged in liquid nitrogen. Frozen samples were kept in a -80° freezer until lyophilization. Prior to analysis, individual samples were mixed with an exactly weighed, approximately equal isotopically-labeled wheat standard (95% 13C-enriched, IsoLife), processed, and analyzed by mass spectrometry to investigate metabolite changes.

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 ul 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 ul of distilled water for analysis.

LC-MS: Samples were stored at 20° C during mass spec analysis. Samples were stored at 20° C during mass spec analysis. Mass Spec analysis was performed on a Q-Exactive with an Open Accela autosampler and LC pump. 3 µl of sample was injected and chromatographed using a Thermo Scientific 1.9 micron Gold aq (150x2.1mm) column operated at 40 degrees. The negative mobile phase was A: distilled water with 0.1% Formic acid, and B: Acetonitrile (LC-MS grade) with 0.1% Formic acid. The gradient table was as follows:

No.	Time	A%	B%	µL/min
0	0.00	100.0	0.0	600.0
1	1.00	100.0	0.0	600.0
2	7.00	80.0	20.0	600.0
3	9.00	40.0	60.0	600.0
4	13.00	5.0	95.0	600.0
5	15.00	5.0	95.0	600.0
6	15.10	100.0	0.0	600.0
7	18.50	100.0	0.0	600.0

Data processing and analysis: The dataset was analyzed by the IROA ClusterFinder software. The peaks identified by ClusterFinder were compared against libraries of compounds for their IROA characteristics; C12 base peak, C12 M+1, C13 base peak, C13 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 C13 standard; in this case, an isotopically-labeled wheat standard (97% 13C-enriched, IsoLife). Therefore, these measurements represent the deviation of each metabolite relative to the isotopically-labeled standard.

Conclusions

The additional ability to understand structural aspects of compounds given through the use of distinct isotopic signatures, as seen in the mass spec, made it possible to assign names and understand structure to a degree not possible if only natural abundance peaks are present. All experimental biological peaks exhibited a C13-labeled pair and therefore artifacts were removed from consideration.

Summary

This was a proof-of-principle IROA Phenotypic study performed in wheat in which to understand the structural aspects of compounds through the use of distinct isotopic IROA signatures, as seen in the mass spec. It was shown that the IROA results are strong and clearly demonstrate diurnal metabolic patterns supported by other investigators. 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 C13-labeled pair and therefore artifacts 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).