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Recent advances in stable isotope-enabled mass spectrometry-based plant metabolomics

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Methods employing isotope labeled compounds have been an important part of the bioanalytical canon for many decades. The past fifteen years have seen the development of many new approaches using stable (non-radioactive) isotopes as labels for high-throughput bioanalytical, 'omics-scale' measurements of metabolites (metabolomics) and proteins (proteomics). This review examines stable isotopic labeling approaches that have been developed for labeling whole intact plants, plant tissues, or crude extracts of plant materials with stable isotopes (mainly using ^{2}H, ^{13}C, ^{15}N, ^{18}O or ^{34}S). The application of metabolome-scale labeling for improving metabolite annotation, metabolic pathway elucidation, and relative quantification in mass spectrometry-based metabolomics of plants is also reviewed.

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Introduction

Researchers engaged in the application of metabolomics in a wide range of disciplines have identified both analytical and informatics limitations that, when addressed, will yield a dramatic improvement in information quality and quantity [1]. Grand challenge areas have emerged from the metabolomics community for methodological improvement including: first, metabolite annotation; second, metabolite quantification; third, metabolic pathway elucidation; fourth, high resolution spatial localization; and, fifth, temporal analysis including metabolite turnover and flux [2]. In this review we will examine how stable (non-radioactive) isotopes have been used as mass labels for mass spectrometry (MS)-based metabolomics in order to make analytical improvements in these challenge areas. Most often, metabolomics research uses MS, nuclear magnetic resonance (NMR) spectroscopy, or some combination of the two methodologies, although other spectroscopies and approaches are occasionally employed. Stable isotopic labeling techniques are also employed for NMR [3]; it is worth following both MS and NMR disciplines as procedures used for labeling can be found in either. General MS-based metabolomics approaches and concepts have been reviewed elsewhere [4].

The metabolomic analysis of plants and plant-derived materials particularly benefits from isotope-enabled methodology for several reasons. First, plant metabolomes are large and chemically diverse and they are under-represented in public database resources, which are often more heavily populated with clinical, pharmaceutical, or petrochemical related compounds. Plant metabolite annotation can, therefore, be difficult and error prone and thus benefits from the additional information provided by using isotopic labeling strategies. Furthermore, quantification can be much more error prone due to interference from complex plant matrices much of which can be controlled using stable isotopes. Second, it is possible to provide a plant with relatively inexpensive isotopically labeled nutrients and completely substitute a specific element with nearly 100% enrichment of a stable isotope. A number of plant metabolomics specific reviews have been published fairly recently [1,5].

Metabolic turnover and flux analysis are important techniques that rely heavily on labeling information. Metabolic flux analysis can be performed in many different ways depending on the types of analytical techniques used and the constraints and assumptions employed. We will not cover this in depth here, as there are excellent recent reviews on this topic [6••,7••].

In Box 1 are definitions for commonly used terms in the field of metabolomics and mass spectrometric analysis of stable isotope labeled metabolites. Here we will examine recent work using various different stable isotopic labeling strategies to improve the depth and precision of plant metabolomics studies with a particular focus on challenges associated with metabolite annotation, quantification and metabolic pathway elucidation.

Using isotopes as labels

Stable isotopic labeling is sometimes confused with radioactive (unstable) isotopic labeling (using ^{3}H, ^{14}C, ^{35}S, ^{32} or ^{33}P), which has historically been used to great effect for metabolic pathway elucidation and turnover or

Box 1 Definitions

Isotopes are atoms of the same element with different numbers of neutrons so that different isotopes of the same element have different masses.

Isotopic abundance indicates the relative amount of an isotope present in a chemical; this often refers to a specific atom or atoms in a molecular structure.

Isotopic envelope refers to the complete set of mass spectral peaks observed for all of the isotopomers of a chemical.

Isotopomers refer to chemically identical molecules differing only in their isotope composition. Each molecule with *n* carbon atoms has a total of 2*n* possible stable carbon isotopomers.

One **mass isotopomer** is the set of isotopomers having the same molecular mass. Each molecule with *n* carbon atoms has $n + 1$ carbon mass isotopomers.

Metabolic flux refers to the net flow of substance (moles/time) through the reactions and compartments or metabolic processes.

Metabolic labeling refers to methods where the endogenous biosynthetic apparatus of living cells is used to incorporate chemical or isotopic tags into biomolecules.

Metabolome is a term conceptualized by extrapolation from ‘genome’, and refers to the complete set of metabolites in a biological system often as they correspond to a complete set of genes.

Metabolomics is the characterization of the metabolome.

A **monoisotopic peak** is a peak in an isotopic envelope that measures a single isotopomeric form of a chemical; this usually refers to the lowest *m/z* peak of the envelope, which (if populated) measures only isotopomers completely composed of the lightest stable isotopes of each element at every atom (i.e. ^{1}H, ^{12}C, ^{14}N, ^{16}O, ^{31}P and ^{32}S).

Natural isotopic abundance refers to the variable composition of isotopes in naturally occurring substances. It is typically dominated by the lightest stable isotopes of each element and has slightly different amounts of the heavier stable isotopes depending on cosmic origin and depletion and enrichment from isotope effects that depend on a given substance’s chemical history.

Stable isotopes are atom types of the same element with nuclear configurations that do not break down spontaneously through the process of radioactive decay.

Tracers are labeled chemicals that are used to help understand the reactions that occur in metabolism.

flux analysis. Use of radioisotopes has limitations in its application due to biological perturbations [8], safety concerns and incompatibility with MS and NMR. Stable isotopes such as ^{2}H, ^{13}C, ^{15}N, ^{18}O, and ^{34}S, which have one or two more neutrons than the most abundant naturally occurring isotopes (^{1}H, ^{12}C, ^{14}N, ^{16}O, and ^{32}S) have been widely used as labels for MS analysis as they can easily be distinguished by mass. While radioisotopes are detectable as signal from their radiation without any contribution of noise from unlabeled compounds, stable isotopically labeled compounds are detected with the same sensitivity as their unlabeled counterparts, which results in relative advantages and disadvantages between the two labeling types. The comparison between radioisotope labels and stable isotope labels and the complementarity of their utility has been reviewed recently [9•].

Labeling metabolites with stable isotopes at a metabolomics scale

Simultaneous introduction of stable isotopes into large numbers of metabolites can be accomplished using either *in vivo* or *in vitro* approaches. Figure 1 summarizes common labeling approaches in these two categories. While *in vitro* labeling is performed following metabolite extraction, *in vivo* labeling strategies make use of metabolic labeling [10] where endogenous metabolism is used to propagate label from some entry point into and throughout the metabolic network. This is accomplished easily in plant cell culture by reformulation of growth media to include labeled nutrients [11].

In several *in vivo* labeling approaches (see Figure 1a) an intact plant is provided with labeled nutrients (such as $^{13}CO_2$ or $K^{15}NO_3$) normally obtained from the environment or growth medium in unlabeled form. With this method, labeling can be performed in bursts, for a short time, or for longer given adequate growth chamber equipment. While different isotopes of the same element at the same position within a molecular structure may be regarded as chemically indistinguishable (having identical electronic configurations), differences in mass due to different numbers of neutrons between isotopes alter the frequencies of molecular vibrations resulting in different rates for some chemical transitions (bond breaking/forming and rehybridization), or what is more generally referred to as ‘isotope effects’ [12]. The magnitude of isotope effects for ^{13}C, ^{15}N, ^{18}O, ^{34}S are small enough that they generally do not either perturb the biological system (when compared with unlabeled) or have significant analytical consequences within typical error of metabolomics measurements. Deuterium (^{2}H) labeling, on the other hand, can cause significant isotope effects and can be toxic to the organism depending on the chemical form incorporated and the isotopic enrichment [13] and thus requires proper considerations of these limitations.

Labeling with ^{13}C is most generally useful due to the omnipresence of carbon in metabolites, although ^{15}N has been used for metabolic studies of nitrogen metabolism [14]. Comprehensive metabolic labeling of plants with ^{15}N is facile and has been used widely for quantitative proteomics [15] and protein turnover [16•,17•]. Labeling with $^{13}CO_2$ typically requires an enclosure to maintain an atmosphere of $^{13}CO_2$ around the plant. Labeling can be accomplished on a fairly short term (2 h) using a simple enclosure [18]. Labeling with $^{13}CO_2$ can also be accomplished under field conditions for pulse-labeling experiments for organisms as large as trees [19]. Growth enclosures have been constructed for longer term labeling experiments that require additional functionality to

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