Investigation of complex isotope patterns of ^{13}C-labeled plant metabolites by Mass Spectral Deconvolution

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Introduction

Isotope labeling and quantitative LC-MS profiling together offer a promising approach for unraveling dynamics of biosynthesis of specialized metabolites (natural products) in plants and other organisms. Isotope patterns and ratios in metabolites harbor valuable information tracing the biosynthetic history of specific compounds. This technique provides a promising tool for extending metabolic flux analyses to complex specialized metabolites in a wide range of organisms. However, the isotopic patterns of highly labeled metabolites can exhibit remarkable complexity, and therefore accurate and high-throughput data processing is necessitated when analyzing labeling of large numbers of metabolites in biological matrices. Here we demonstrate how a new approach based on spectral deconvolution achieves simultaneous quantification of complex isotope patterns of ^{13}C-labeled natural products.

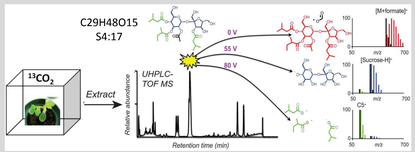

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Figure 1. Workflow of global isotopic labeling and profiling of plant specialized metabolites.

Methods

^{13}C labeling of plants and LC-MS analysis

Tomato (*S. lycopersicum* M82) specialized metabolites were labeled using hydroponic growth under $^{13}CO_2$ in a sealed growth chamber. Specialized metabolites (acylsugars, terpenes, and flavonoids) were extracted from leaf surface trichomes using methanol/water and analyzed. Assessments of ^{13}C incorporation in both molecular and fragment ions were generated using UHPLC and multiplexed nonselective collision-induced dissociation (CID)[1] on a Waters G2-S QTof in continuum mode. Mass spectra, including those of coeluting metabolites, were processed with commercially available MassWorks 4.0 software from Cerno Bioscience.

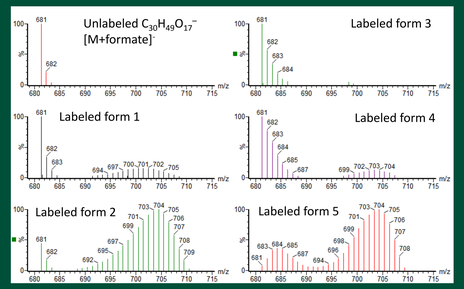

Figure 2. ESI (-) mass spectra of unlabeled acylsucrose S4:17($C_{30}H_{49}O_{17}$) and several labeled S4:17 show complex isotopic patterns. Different labeled forms 1-5 were detected from leaflet extract samples at different labeling days. The crude mixtures were analyzed using UHPLC-TOF MS. The unlabeled molecule was from a control plant grown under ambient atmospheric conditions (mostly $^{12}CO_2$)

Data processing for enrichment calculations

Raw mass spectra were exported from MassLynx to MassWorks and followed by comprehensive mass spectral calibration using monoisotopic ions of unlabeled compounds, including both mass error correction and mass spectral peak shape correction. After this calibration, quantification of ^{13}C enriched isotopologs was performed using MassWorks software.

Results & Discussion

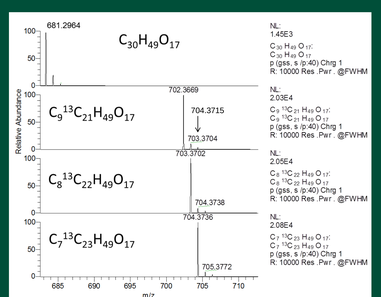

Figure 3. Challenge of accurate quantification of overlapping MS signals. The first spectrum is unlabeled acylsucrose S4:17, and the three below are deconvoluted labeled forms of S4:17 with different numbers of ^{13}C incorporated.

More than 20 acylsugars, rutin and tomatine were identified by their accurate masses, characteristic fragment masses, and chromatographic retention times in the LC-MS analysis. Owing to the diversity of those specialized metabolites, quantitative analysis of all the ^{13}C labeled isotopologs by integrated peak areas u using Quanlynx (Waters) which requires t time-consuming manual input. For instance, the most abundant acylsugar S4:17 has 29 carbons in the molecule (structure in Figure 1). When highly labeled, up to 29 isotopologs are detected in its mass spectrum. Figure 2 demonstrates the complexity of labeled S4:17 mass spectra. When fragmenting the molecular ion to achieve labeling information in metabolite substructures, investigating all the key fragments would add another layer of data processing complexity. In addition, quantification of all molecular and fragment isotopologs from complex mixtures without spectral deconvolution increases the likelihood of quantitative errors.

As illustrated through an example in Figure 3 of a hypothetical mixture with three different ^{13}C labeled metabolite compositions: $C_9{}^{13}C_{21}H_{49}O_{17}$, $C_8{}^{13}C_{22}H_{49}O_{17}$, and $C_7{}^{13}C_{23}H_{49}O_{17}$, the total signals (not shown in Figure 3) of the m/z 704 are primarily contributed from the three metabolites, *i.e.* A+2 peak of $C_9{}^{13}C_{21}H_{49}O_{17}$, A+1 peak of $C_8{}^{13}C_{22}H_{49}O_{17}$, and monoisotopic peak of $C_7{}^{13}C_{23}H_{49}O_{17}$. Assuming the three metabolites have similar abundances, quantification based only on the monoisotopic peak of $C_7{}^{13}C_{23}H_{49}O_{17}$ will miss roughly 10% and 4% from contribution from A+1 peak of $C_8{}^{13}C_{22}H_{49}O_{17}$ and A+2 peak of $C_9{}^{13}C_{21}H_{49}O_{17}$ respectively of the same nominal mass. In this study, we have taken a new way for simultaneous quantification of all isotopologs in the mixture. This approach assumes that the complex spectra observed are a linear combination of each possible ^{13}C labeled compounds and can be mathematically separated through spectral deconvolution. The process of the quantitative analysis is to compare calculated mixture spectra with measured complex spectra. To achieve accurate quantification, it is essential to conduct peak shape calibration. For example, if calculated spectra have Gaussian peak shape while measured spectra have undefined peak shapes, this peak shape difference will introduce about 5-10% errors in isotopologue quantification. As shown in Fig 4, the raw spectra (black) with distorted peak shape were calibrated to have symmetric and well-defined peak shapes (red) which will also be used to generate calculated spectra.

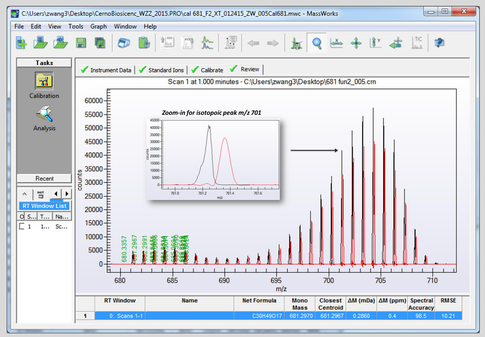

Figure 4. Calibrated (red) and raw data (black) mass spectra of labeled acylsugar S4:17 from LC-MS profiling. This spectrum indicated a complex mixture of slightly labeled and highly labeled S4:17 molecules. The peak shape is modified in calibration to fit for deconvolution analysis in the second step. The zoom-in isotopolog peak of m/z 701 shows the detailed peak shape before and after calibration.

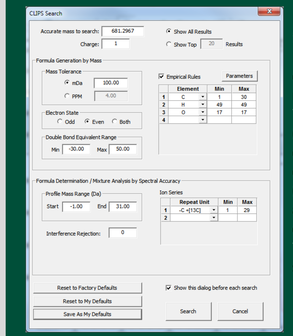

Figure 5. MassWorks CLIPS search for metabolite isotopolog analysis. For known compound, the elemental composition can be narrowed to the exact formula. The number of labeled element (^{13}C substitute for ^{12}C here) is defined for possible labeled composition of this compound (here, minimum 1 carbon and maximum 29 carbon can be labeled as ^{13}C). The search result for spectrum showed in Figure 4 is demonstrated In Figure 6.

Our work shows accurate acylsugar isotopolog enrichments, with the mol% of each labeled pattern calculated and showing agreement with manually organized peak integration. For example, the calibrated spectrum showed in the Figure 6 was CLIPS searched using the parameter shown in Figure 5. The search result yields the compositions of 30 different forms of S4:17 metabolites from the labeled mixture and their percentages (Table in Figure 6). The unlabeled $C_{30}H_{49}O_{17}$ is only 1.1% in the mixture and heavily labeled molecules like $C_7H_{49}O_{17}[^{13}C]_{23}$ (23 of 30 carbons are substituted with ^{13}C) accounts for >11%. Application of this approach measured enrichments of S4:17 at different leaf developmental stages, and supports our early discovery that about 40% of S4:17 molecules were newly synthesized within the first two days of labeling, with 60% unlabeled S4:17 fractions. Most of the labeled S4:17 had 16 to 24 carbons as ^{13}C. This type of composition information will help us to test our hypothesis that acylsugar biosynthesis proceeds through initial attachment of acyl groups at early developmental stages followed by partial hydrolysis and re-acylation.

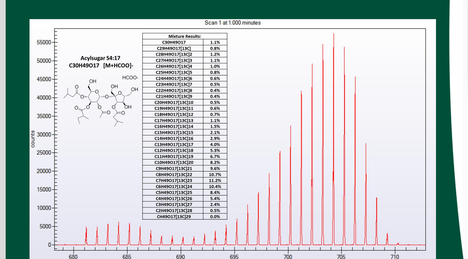

Figure 6. CLIPS search result of mixture composition and enrichment percentages of S4:17. The table shows abundance of each labeled form of compound S4:17 in the mixture.

Conclusions

1. Automated and simultaneous quantification of ^{13}C-labeling in plant metabolites containing up to 30 heavy isotopes by spectral deconvolution is achieved through MassWorks software.
2. Exploration of metabolic dynamics offers potential to improve our understanding of biosynthetic and catabolic contributions to metabolite fluxes.

References

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Acknowledgements

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