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GC-EI-TOF-MS analysis of *in vivo* carbon-partitioning into soluble metabolite pools of higher plants by monitoring isotope dilution after $^{13}CO_2$ labelling

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Abstract

The established GC-EI-TOF-MS method for the profiling of soluble polar metabolites from plant tissue was employed for the kinetic metabolic phenotyping of higher plants. Approximately 100 typical GC-EI-MS mass fragments of trimethylsilylated and methoxyaminated metabolite derivatives were structurally interpreted for mass isotopomer analysis, thus enabling the kinetic study of identified metabolites as well as the so-called functional group monitoring of yet non-identified metabolites. The monitoring of isotope dilution after $^{13}CO_2$ labelling was optimized using *Arabidopsis thaliana* Col-0 or *Oryza sativa* IR57111 plants, which were maximally labelled with ^{13}C. Carbon isotope dilution was evaluated for short (2 h) and long-term (3 days) kinetic measurements of metabolite pools in root and shoots. Both approaches were shown to enable the characterization of metabolite specific partitioning processes and kinetics. Simplifying data reduction schemes comprising calculation of ^{13}C-enrichment from mass isotopomer distributions and of initial ^{13}C-dilution rates were employed. Metabolites exhibited a highly diverse range of metabolite and organ specific half-life of ^{13}C-label in their respective pools (^{13}C-half-life). This observation implied the setting of metabolite specific periods for optimal kinetic monitoring. A current experimental design for the kinetic metabolic phenotyping of higher plants is proposed.
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Keywords: *Arabidopsis thaliana* Col-0; *Oryza sativa* IR57111; ^{13}C-carbon; $^{13}CO_2$-carbondioxide; Dynamic flux analysis; Electron impact ionization (EI); Gas chromatography (GC); Metabolite profiling; Stable isotope dilution; Time-of-flight mass spectrometry (TOF-MS)

1. Introduction

The labelling of plants with CO_2 using both radioactive (e.g. Calvin, 1956, 1964) or stable isotope tracing (e.g. Schaefer et al., 1975, 1980; MacLeod et al., 2001; Schwender et al., 2004) has been used since decades utilizing the main entry points of CO_2 into plant metabolism, namely ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) and phosphoenolpyruvate carboxylase (EC 4.1.1.31). Over time, CO_2 tracing yielded ground-breaking biological insights into photosynthetic carbon assimilation, photorespiration and metabolism and, thus, into essential life-sustaining physiological mechanisms on earth.

With the increasing availability of stable isotopes, methods for the *in vivo* labelling of plants are conceivable. Successful methods for the complete and saturating ^{13}C-labelling were reported previously using a microbial model, such as *Saccharomyces cerevisiae* (Birkemeyer et al., 2005). In this work we present and characterize a method for the full *in vivo* ^{13}C-labelling of higher plants.

Abbreviations: EI, electron impact ionization; GC, gas chromatography; MS, mass spectrometry; MST, mass spectral tag; RI, retention time index; TOF, time-of-flight.

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With this prerequisite in place isotope dilution after $^{13}CO_2$ labelling was monitored. The kinetic measurement of isotope decay from ^{13}C to ^{12}C can be performed under ambient atmospheric conditions, thus allowing sampling with minimal experimental disturbance. As a consequence experiments with extended kinetic monitoring of CO_2-dilution under diverse regimes of environmental conditions are now conceivable. Thus we aim to contribute to the ongoing discussion and development of empirical flux estimations in the field of plant physiology (e.g. Fernie et al., 2005; Baxter et al., 2007). We specifically intend to work towards dynamic flux estimations (Ratcliffe and Shachar-Hill, 2006) as a tool for the phenotypic analysis of gene function in higher plants.

Besides nuclear magnetic resonance (NMR), mass spectrometric (MS) analysis has traditionally been used for flux analyses, for example MALDI-TOF (e.g. Wittmann and Heinzle, 2001), quadrupole based GC–MS (e.g. Dauner and Sauer, 2000), ion trap mass spectrometry (e.g. Klapa et al., 2003) or LC triple quadrupole mass spectrometry (e.g. van Winden et al., 2005). We employed the widely applied gas chromatography electron impact ionization time-of-flight mass spectrometry, in short GC-EI-TOF-MS technology, for metabolite profiling (e.g. Wagner et al., 2003; Lisec et al., 2006; Erban et al., 2006). GC-EI-TOF-MS is a non-scanning mass spectrometric technology, which allows simultaneous monitoring of a mass range and high acquisition rates of 10–500 mass spectra s^{-1}. Thus apparent fragment ratios are not subject to artefacts caused by the temporal offset of the sequential mass recording, which is inherent to mass scanning technologies, such as the quadrupole or ion trap GC–MS. The feasibility of mass isotopomer monitoring of methoxyaminated and silylated derivatives by GC–MS metabolite profiling has been demonstrated earlier (Roessner-Tunali et al., 2004; Baxter et al., 2007). The decision to use this specific mode of derivatization and analytical monitoring was made in view of the high synergy to be expected of method development and metabolite identification efforts in the metabolite profiling field (e.g. Schauer et al., 2005a). Substantial instrumental progress has also been made, for example by GC×GC-TOF-MS (e.g. Sinha et al., 2004a,b; Kell et al., 2005) implementation. Furthermore, a highly versatile tool box of metabolite fractionation and chemical derivatization schemes awaits exploration (Kopka, 2006a).

In the following study we perform a technological assessment of the combination of mass isotopomer analysis using the GC-EI-TOF-MS profiling method and monitoring of isotope dilution after $^{13}CO_2$ labelling. We specifically address the use of populations of replicate, genetically identical plants for flux studies and describe fundamental technological requirements. First results are presented, which demonstrate the feasibility and potential but also the current limitations of this novel combination of techniques.

2. Results and discussion

2.1. GC-EI-MS fragmentation analysis

The electron impact (EI) fragmentation pattern of trimethylsilylated and methoxiaminated metabolite derivatives (analytes), which are observed in routine metabolite profiles (e.g. Erban et al., 2006; Fiehn et al., 2000a; Lisec et al., 2006; Roessner et al., 2000) delimit the potential of this profiling method for the multi-parallel analysis of metabolic fluxes using ^{13}C-stable isotope dilution. Specifically the targeted retrieval of quantitative mass isotopomer information and the calculation of isotope enrichment from mass isotopomer distributions require the thorough interpretation of GC-EI fragmentation patterns and the knowledge of the sum formula of each analyzed mass fragment. As suggested previously (Birkemeyer et al., 2005), we used mass spectral tags (MSTs; cf. the definition made by Desbrosses et al., 2005 refined by Kopka, 2006b) with ambient isotopic composition and MSTs from *in vivo* ^{13}C-labelled material for the interpretation of mass spectral fragmentation patterns (Fig. 1). This interpretation effort was based on the detailed EI fragmentation patterns of trimethylsilylated and methoxiaminated carbohydrates (DeJongh et al., 1969; Laine and Sweeley, 1973; MacLeod et al., 2001; Sanz et al., 2002) or amino acids (Abramson et al., 1974; Bergström et al., 1970; Leimer et al., 1977), which have been previously published. Furthermore, interpretation was supported by the mass shifts observed upon *in vivo* ^{13}C-labelling (Fig. 1).

The fragmentation patterns of 58 analytes, comprising approximately 100 electron impact fragments, were analyzed. Representative sugars, organic acids, amino acids, amines and polyols were chosen (cf. Supplementary file 1). This selection represents approximately 7.6% of the current non-redundant Golm Metabolome Database compendium (GMD; Kopka et al., 2005; Schauer et al., 2005a; http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html). The sum formula of each mass fragment was deduced from known general fragmentation reactions and available references (see above). For further referencing all obtained fragment information was linked to the mass spectrum identifier system used by GMD (MPIMP-ID). The ^{12}C- and ^{13}C-monoisotopic masses and the respective number of carbon atoms, which originate from the carbon-backbone of each metabolite and are not introduced by chemical derivatization reagents, were empirically determined from ambient and fully *in vivo* labelled MSTs (Fig. 1). In agreement with the GC-EI-TOF-MS instrument specifications, monoisotopic masses are given at full mass unit precision.

Mass fragments comprising the full metabolite carbon-backbone, such as M^+ (molecular ion) or $M-15^+$ (i.e. a mass fragment generated from M through a loss of 15 a.m.u.), were mostly present at low intensities and in some cases below detection limit. Through our effort we now offer alternative fragment ions for mass isotopomer

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