Hydroponic Isotope Labeling of Entire Plants (HILEP) for Quantitative Plant Proteomics

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Overview

- HILEP is a quantitative method to study the dynamic changes of the proteome of whole plants and multi-organism phytosystems.
- HILEP employs metabolic labeling of plants grown hydroponically in presence of ^{14}N or ^{15}N salts.
- HILEP was successfully applied to Arabidopsis plant submitted to oxidative stress.
- An automatic protein quantitation analysis pipeline was developed with mzXML raw data and mgf peak list files, Mascot, the freely available TransProteomic Pipeline (TPP) and MicrosoftExcel software.
- See also poster # ThPH 117

Introduction

Quantitative analysis by mass spectrometry (MS) is a major challenge in proteomics as the correlation between analyte concentration and signal intensity is often poor due to varying ionisation efficiencies in the presence of molecular competitors. However, relative quantitation methods that utilise differential stable isotope labelling and mass spectrometric detection are available. Many drawbacks inherent to chemical labelling methods (ICAT, iTRAQ) can be overcome by metabolic labelling with amino acids containing stable isotopes (e.g. ^{13}C and/or ^{15}N) in methods such as Stable Isotope Labelling with Amino acids in Cell culture (SILAC). SILAC has also been used for labelling of proteins in plant cell cultures (1) but is not suitable for whole plant labelling. Plants are usually autotrophic (fixing carbon from atmospheric CO2) and, thus, labelling with carbon isotopes becomes impractical. In addition, SILAC is expensive.

Recently, *Arabidopsis* cell cultures were labelled with ^{15}N in a medium containing nitrate as sole nitrogen source. This was shown to be suitable for quantifying proteins and nitrogen-containing metabolites from this cell culture (2,3).

Labelling whole plants, however, offers the advantage of studying quantitatively the response to stimulation or disease of a whole multi-cellular organism or multi-organism systems at the molecular level. Furthermore, plant metabolism enables the use of inexpensive labelling media without introducing additional stress to the organism. And finally, hydroponics is ideal to undertake metabolic labelling under extremely well-controlled conditions.

We demonstrate the suitability of metabolic ^{15}N hydroponic isotope labelling of entire plants (HILEP) for relative quantitative proteomic analysis by mass spectrometry. To evaluate this methodology, *Arabidopsis* plants were grown hydroponically in ^{14}N and ^{15}N media and subjected to oxidative stress.

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HILEP Methodology

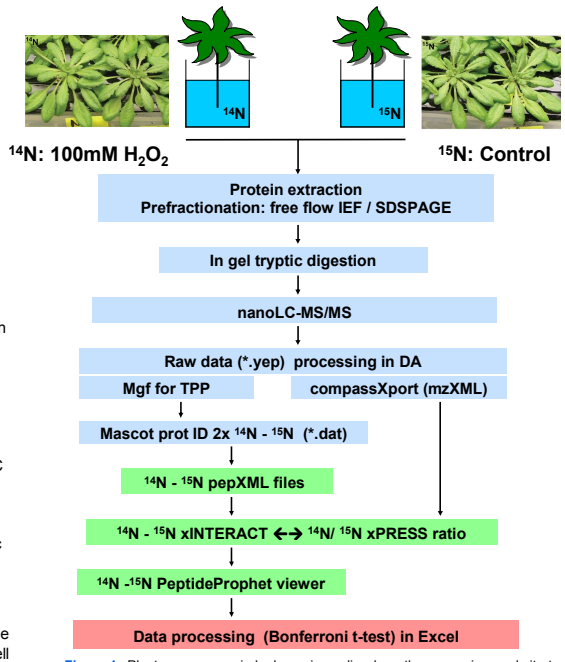

Figure 1.. Plants were grown in hydroponic media where the ammonium and nitrate nitrogen sources were replaced by the equivalent 98%+ ^{15}N-labelled components. ^{14}N or ^{15}N hydroponically grown *Arabidopsis* plants were then subjected to oxidative stress by spraying leaves of 7 weeks old plants with 100 mM hydrogen peroxide. Total protein extracts and intercellular washing fluids (IWF) were separated on SDS PAGE or free flow isoelectrophoresis (IEF)-SDS-PAGE, followed by in gel tryptic digestion. Peptides from tryptic digests were separated by reverse phase liquid chromatography on a 100 min 2-50% ACN gradient (Dionex Ultimate™ HPLC, LC Packings. Peptides were then detected and analysed by MS/MS with an Esquire HCT ion trap (Bruker Daltonics) or a 7 tesla Apex Qe FTICR mass spectrometer (Bruker Daltonics). Raw LC-MS/MS datasets were converted to mzXML using CompassXport. DataAnalysis software (Bruker Daltonics) was used for peak and compound detection, deconvolution and to export peak lists as MGF files. The MGF files were then converted by an AWK script (DataAnalysis2TPP) to be compatible with the freely available Trans-Proteomic Pipeline,(TPP,4). MGF files were then submitted to two Mascot MS/MS Ions Searches against ^{14}N masses and ^{15}N masses. At this stage peptides from identified proteins could be manually quantified using retention time and m/z information. For automation using TPP, Mascot ^{14}N and ^{15}N results were converted to pepXML by Mascot2XML, merged by xINTERACT and evaluated by PeptideProphet and XPRESS. The TPP components are indicated in green. Quantified peptides were exported and f to Excel from the TPP PepXML Viewer, and then formatted using an AWK script (5). Only proteins with 3+ unique and isoform-specific peptides (ion score > identity score) were considered for quantitation. To estimate the significance of each relative protein quantitation result, a two-sided heteroscedastic (Welsch's) *t*-test was applied and *p*-values were then corrected (Bonferroni) for the number of hypotheses tested in each experiment.

Results

No phenotypic differences were observed between hydroponically grown ^{15}N and ^{14}N plants.

Pooled ^{14}N and ^{15}N protein extracts were fractionated by free-flow IEF and/or SDS-PAGE (Fig. 2). 2D separation increased the identification and quantitation protein coverage.

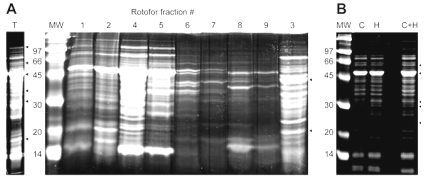

Figure 2. SDS-PAGE: Arabidopsis plants treated with 100 mM H2O2 (H, ^{14}N), control (C, ^{15}N) and pooled in a ratio of 1:1 (H+C).
20µg protein of total extract (T), and 10 free-flow IEF fractions.
Apoplastic Intercellular Washing Fluids (IWFs) proteins (5 µg).

The mass spectral data reveals that the high percentage of 98+% of ^{15}N in the nitrogen source is well reflected in the isotope envelope of labelled proteolytic peptides.

^{14}N and ^{15}N peptide pairs are distinct allowing the separate integration of each isotope envelope for quantitation (Fig. 3, 4).

Figure 3. Ion trap MS **(A)** and MS/MS **(B)** spectra of the peptide FEETLYGTSR containing 13 nitrogen atoms. Both the ^{14}N (*m/z* 602) and ^{15}N (*m/z* 608) peptide were selected for MS/MS, as shown in **(B)**. Similar b- and y-ion series were observed for both precursor ions.

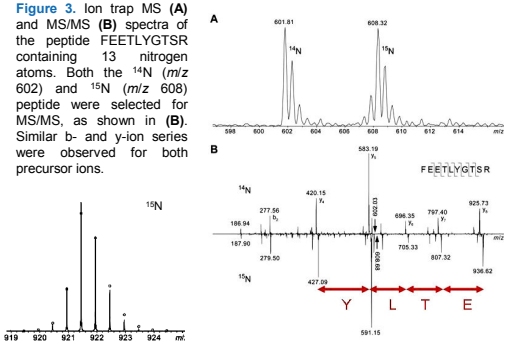

Figure 4. Least-squares fit (circles) to an FTICR mass spectrum of the peptide LEGDRESTLGFVDLLR Rubisco fragment. Such isotope distribution estimate the presence of 98.1%^{15}N.

The influence of outliers on the $^{14}N/^{15}N$ ratios average can be attenuated or avoided by :

- Using at least 3 different unique peptides for quantitation
- Using the median of $^{14}N/^{15}N$ peptide ratios rather than average.
- Discriminating mixed ions with accurate mass and highly resolved FTICR MS data combined with Ion Trap MS/MS data (Fig. 5).

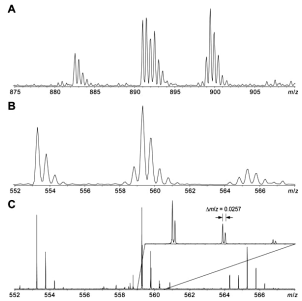

Figure 5. Ion trap and mass spectra of overlapp and ^{15}N species as reve the isotope pattern. **(A)** envelope of the EGPPVFEQPEMTYEK overlapping with th envelope of another identified peptide. In cases (e.g. SVGDLTS the mass difference is to to be resolved in an ion t The two overlapping differing by 0.05 Da spectrum were detected resolving power of achieved by a 7 tesla mass spectrometer **(C)**.

The potential of the HILEP technology and the proposed automatis analytical workflow was illustrated for intercellular washing fluid pro extract (IWF) of plants submitted to oxidative stress, separated on PAGE. Data extracted from ProteinProphet typically gave 150-200 identifications with a probability > 0.95% and a false positive rate b 1%. For instance, endochitinases (PR3), a glucanase (PR2), GSTs protease, lectins seemed upregulated, whereas lipases, a galactos a xylosidase, a germin like protein were down regulated. However, fraction of these were significantly differently expressed from RuBis (Tab. 1).

Table 1. Up and down regulated proteins from intercellular washing fluid ($^{14}N/^{15}N$ peptide ratios were extracted from PeptideProphet viewer. Abund protein ratio of H_2O_2 / control significantly different from RuBisCO ratio as calcu with Bonferroni corrected t-test.

		20 h N14	SD	20 h N15	SD	40 N1
AtCg00490	(RuBisCO large subunit) -	**1.00**	0.34	**1.00**	0.27	**1.**
At5g17920	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransfer	**0.73**	0.06			
At5g67360	Subtilisin-like protease precursor (Cucumisin-like serine protease)	**0.54**	0.08	**0.55**	0.11	
At1g42970	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precurs	**1.63**	0.23	**1.71**	0.16	
At3g57260	Glucan endo-1,3-beta-glucosidase (PR-2)	**1.27**	0.09			**1.**
At2g13360	Serine--glyoxylate aminotransferase (EC 2.6.1.45)					**0.**
At1g29670	Lipase\hydrolase					**0.**
At1g29660	Lipase\hydrolase, putative;	**0.58**	0.04			**0.**
At5g64570	Beta-xylosidase	**0.43**	0.05	**0.49**	0.06	**0.**
At5g08380	Alpha-galactosidase-like protein			**0.60**	0.08	
At3g18490	CND41 chloroplast nucleoid DNA binding protein-like			**0.59**	0.08	
At3g57240	Beta-1, 3-glucanase	**0.42**	0.05	**0.47**	0.23	
At1g09340	Putative RNA-binding protein					**1.**
At2g28470	Putative beta-galactosidase precursor (EC 3.2.1.23)			**0.65**	0.07	
At1g76160	putative pectinesterase Multicopper oxidase	**0.45**	0.03	**0.45**	0.03	
At1g21670	contain WD40-like r Repeat domain			**0.58**	0.04	

References

1. Gruhler A, Schulze WX, Matthiesen R, Mann M, Jensen. 2005. *Mol Cell Proteomics* 1697-1709
2. Engelsberger WR, Erban A, Kopka J, Schulze W X . 2006. *Plant Methods* 2: 14 .
3. Benschop JJ, Mohammed S, O'Flaherty M, Heck MJ, Slijper M, Menke FL. 2007. *M Proteomics*. Feb 21; [Epub ahead of print].
4. Transproteomic pipeline (TPP) web site:http://tools.proteomecenter.org/.
5. HTML link to AWK scripts will shortly be on http://www.ms-utils.org/.