Relative and absolute quantitative shotgun proteomics: targeting low-abundance proteins in Arabidopsis thaliana

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Abstract

The plant system is a highly dynamic structure on all molecular levels, transcripts, proteins, and metabolites. Thus, protein analysis has to cope with a highly dynamic range of concentrations. A severe problem is the detection of low-abundance proteins in the presence of housekeeping proteins. Basically three approaches are facilitated to measure protein abundance in a comprehensive manner: 2DE and one- or multi-dimensional shotgun proteomics, with or without stable-isotope labelling. These comparative techniques allow for the identification of altered protein levels compared with a reference state. However, they are limited to the analysis of medium/high-abundance proteins. Using stable-isotope dilution techniques it is possible to target the quantitative analysis to low-abundance proteins and to measure absolute concentrations of proteins. Based on multi-dimensional non-gel shotgun proteomics in Arabidopsis thaliana, a list of tryptic peptides comprising >1000 proteins was generated. A strategy for quantitative plant proteomics is proposed using this master-list for selecting signature peptides of proteins. To prove the concept, a liquid chromatography–high-resolution triple quadrupole multiple reaction monitoring–mass spectrometry technique is described to determine the absolute amount of a low-abundance sucrose synthase isoform out of an ultra-complex A. thaliana protein extract.

Key words: Absolute quantitation, Arabidopsis, high resolution, linear ion trap, multiple reaction monitoring (MRM), plant, quantitative proteomics, relative quantitation, shotgun proteomics, single reaction monitoring (SRM), stable-isotope labelling, SUSY, triple quadrupole.

Introduction

Mass spectrometry (MS) offers the opportunity to generate large amounts of protein sequence-dependent data that, combined with genomic information, gives the potential for high throughput analysis of the plant proteome (Zivy and de Vienne, 2000; Roberts, 2002; Whitelegge, 2004; Agrawal and Rakwal, 2005; Glinski and Weckwerth, 2005b). Facing proteomics, the ultimate benefit to biology will be determined by the reliability of relative and absolute protein expression measurements. Currently, three main techniques are used for quantitation in proteomics: two-dimensional (2D)-PAGE, stable-isotope labelling, and stable-isotope labelling–free shotgun proteomics (Fig. 1). A common argument in favour of 2D-PAGE is that a comparison can readily be made between two gels and thus proteome differences can be detected. However, 2D-PAGE is still problematic because of reproducibility, varying staining efficiency of individual gels, and bias against some protein classes such as membrane proteins. In shotgun proteomics a complex protein sample is tryptically digested and analysed using liquid chromatography–mass spectrometry (LC-MS) techniques (Yates, 2004). The heterogeneous sample with proteins of different chemical and physical behaviour is broken down to a mixture of peptides easily adaptable to classical reversed-phase LC-MS techniques (Fig. 1). However, the increased complexity of the sample due to the sheer number of tryptic peptides is a challenge to chromatographic and mass spectrometric resolution. The advantages of shotgun proteomics are the capacity for throughput and less bias against protein classes, as in the case of 2D-PAGE (Washburn et al., 2001). Nevertheless, shotgun proteomics is not a quantitative technique per se. The intensity of a peptide peak depends linearly on the concentration of the peptide. However, different peptides have different propensities...
for ionization. Therefore, two different peptides present in equimolar amounts may show substantially different intensities in the mass spectra. In the case of label-free shotgun proteomics, this results in a relative quantitative analysis used, for example, for an integrative metabolite/protein profiling approach (Weckwerth, 2003; Weckwerth et al., 2004; Morgenthal et al., 2005). Recently, a simple and fast method for the rough estimation of relative protein abundance has been described by Liu et al. (2004). They found that the number of tandem mass spectra ('spectral count') collected from a peptide mixture displayed perfect linearity with respect to concentration. By contrast, percentage sequence coverage and number of peptides per protein did not show as good a linear correlation as a spectral count. Nevertheless, most quantitative techniques rely on modifying one of the samples with a stable isotope, which changes the molecular mass but not the mass spectrometric and chromatographic behaviour. Quantitative differences are then determined directly as the difference in peak area between the two peptides in the mixed sample. There are several approaches for labelling peptides with stable isotopes (Fig. 1): metabolic labelling using isotope-enriched or -depleted media (Oda et al., 1999; Ong et al., 2002; Whitelegge et al., 2004); proteolytic labelling of peptides using H$_2^{18}$O/H$_2^{16}$O (Yao et al., 2001); protein/peptide derivatization with ICAT (isotope-coded affinity tag-labelling) (Smolka et al., 2001) or methanol/HCl (Goodlett et al., 2001); and, for instance, ITRAQ$^\text{TM}$ that uses a multiplex set of four amine-specific isobaric reagents (Ross et al., 2004) allowing four-way relative and absolute quantitation.

Besides the relative quantification of proteins, there is a strong need for the analysis of low-abundance proteins and the determination of absolute quantities of proteins in ultra-complex mixtures. The highly dynamic proteome is a great challenge. As a promising approach stable-isotope dilution techniques in combination with shotgun proteomics are emerging (Barr et al., 1996; Gerber et al., 2003;

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**Fig. 1.** General scheme of the most-used quantitative approaches for proteomics. Starting with the comparison of two, A and B, or more samples, relative quantification is achieved in all three cases. (1) Comparative proteomics using traditional 2DE. Protein staining enables the comparison of relative protein abundance. Protein identification is achieved by cutting out the protein spot, tryptic digestion, and analysis using mass spectrometry. A step further is the use of extracted ion traces for signature peptides to quantify the proteins in a spot. This technique is complementary to protein staining in the gel and circumvents the problem of overlapping protein spots. (2) Differential stable-isotope labelling. Owing to a mass shift introduced using different stable isotope-labelled linkers the intensity ratios of peptide isomers in an MS analysis can be determined. The ratio depicts the differences in abundance in the two samples. (3) Direct quantification using shotgun protein LC-MS analysis. Peptides are quantified by integration of extracted ion traces and normalization to internal standards (Weckwerth et al., 2004; Morgenthal et al., 2005) or averaging the spectral count per protein (Liu et al., 2004). For details, see text. Using the direct quantitative approach the measurement of many samples and statistical data mining becomes feasible (Weckwerth et al., 2004; Morgenthal et al., 2005).
Zhang et al., 2004; Pan et al., 2005). Proteins of interest are tryptically digested in the presence of synthetic peptide standards of known concentration with an incorporated stable isotope \(^{13}\mathrm{C},^{15}\mathrm{N}\). These standards are identical to the analyte peptides of interest but are distinguished by mass difference. Stable isotope-labelled and unlabelled peptides co-migrate during chromatography and absolute quantification is achieved by comparison of the peak area abundances of the internal standard peptide with the corresponding native counterpart due to, for example, multiple reaction monitoring (MRM) via tandem MS (Barnidge et al., 2003).

Since abundance and metabolic turnover of a protein are not correlated, many key enzymes are less abundant and therefore difficult to analyse. Sucrose synthase (SUSY) is a key enzyme involved in sucrose metabolism. This enzyme catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Its activity has been studied in various plants and has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Hesse and Willmitzer, 1996). Several studies (proteomic studies included) indicate that SUSY exists in both a cytosolic and a plasma membrane/bacteroid membrane-associated form (Komina et al., 2002; Wienkoop and Saalbach, 2003). In the model plant Arabidopsis, the complete genome sequence reveals six putative members of the SUSY gene family (Barratt et al., 2001). Isoform At5g20830 and At5g49190 in leaves are known to be highly stress responsive (Dejardin et al., 1999). To date, no data relative to the other four isoforms are available (Baud et al., 2002).

Here a strategy for absolute quantitative one-dimensional (1D) high-throughput shotgun proteomics is proposed, starting with a master-list of signature peptides identified in a shotgun proteomics experiment (Wienkoop et al., 2004) (Fig. 2). Based on the analysis of a signature peptide for low-abundance SUSY isoform At3g43190, this protein is detected and quantified out of an ultra-complex protein mixture of entire Arabidopsis tissue. SUSY is usually not detectable in a typical non-targeted 1D shotgun analysis of a complex plant protein extract. The targeted analysis allows not only low-abundance proteins to be quantified but also enables the throughput analysis of many samples. The proposed method can be expanded for quantitative examinations of many proteins in a single run.

**Materials and methods**

**Generation of a protein master-list for the identification of signature peptides**

Arabidopsis thaliana Col 0 plant protein was analysed using a multi-dimensional chromatographic approach coupled to ion trap LC-MS. The whole procedure is described in a recent study by Wienkoop et al. (2004).

**Preparation and LC-MS analysis of a stable isotope-labelled internal standard peptide for SUSY**

A specific internal SUSY (At5g43190) peptide standard HVSNLDRLEA*RR was synthesized by stable-isotope \(^{13}\mathrm{C}/^{15}\mathrm{N}\) alanine labelling (Thermo Electron, Ulm, Germany). LC analysis of the triple charged precursor ion was performed. To achieve the most sensitive and specific signal possible in the presence of a complex matrix, four single reaction monitoring (SRM) transitions were monitored for the native and internal standard peptides and the best chosen: native 489.17→484.6 and standard 490.5→484.6 \((\mathrm{m/z})\) with an optimized collision energy of 15.

**Protein purification and sample digestion of Arabidopsis plant tissue**

Hydroponic A. thaliana Col 0 cultures were grown in phytotrons under controlled light, gas, and temperature conditions. Arabidopsis tissue of 7-week-old plants was harvested and proteins were extracted according to Weckwerth et al. (2004). For tryptic digestion the ultra-complex protein mixture was solubilized in 10% ACN/25 mM Ambic/10 mM CaCl/8 M urea and divided into two portions. One portion was spiked with internal standard peptide (25 fmol peptide per 1 μg protein). In the first step of digestion 1:100 LysC was used for 5 h at 37 °C followed by a second digestion step.
with 2 µl trypsin beats per 10 µg protein (Poroszyme, Applied Biosystem, Darmstadt, Germany) for 16 h at 30 °C. Prior to trypsin digestion the sample was diluted to an end concentration of 2 M urea using the following buffer: 10% ACN/25 mM Ambic/10 mM CaCl.

Non-targeted and targeted peptide analysis using nano LC–ion trap-MS and nano-LC–triple quadrupole-MS
A nano HPLC system (Agilent 1100) was used for controlled nano-flow rates (300 nl min\(^{-1}\)). Samples (20 µg) were loaded onto a 75 µm ID RP column (Zorbax 300SB-C18, 3.5 µm, Agilent) coupled on-line with the MS. A 90 min gradient was performed from 40% to 100% MeOH, 0.1% FA. MS/MS was performed using a ThermoFinnigan (San Jose, CA, USA) mass spectrometer (LTQ ion trap). MRM was performed using a high-resolution TSQ Quantum triple quadrupole (ThermoFinnigan). On the LTQ a triple play and a combination of triple play, together with a pseudo-SIM-MS/MS scan sequence according to Venable \textit{et al.} (2004) was performed on both precursor ions (489.17 and 490.5) with a mass window of 10 m/z. The pseudo-SIM-MS/MS sequence was programmed between 46 and 58 min during the gradient. The TSQ tuning and MRM of the target peptides were essentially performed according to Glinski and Weckwerth (2005a). Q1 was kept at 0.3 resolution and Q3 at 0.7 m/z with a dwell time of 50 ms (Glinski and Weckwerth, 2005a). For quantitation, a calibration curve was achieved using different amounts of standard peptide in the protein mixture from 0 to 0.5 pmol (0/10/50/100/250/500 fmol) end concentration.

Results and discussion
Generation of signature peptides for proteins
A master-list of proteins was generated using a multi-dimensional chromatographic approach as demonstrated in Fig. 2. Total leaf protein was extracted and fractionated using ion exchange chromatography as described in Wienkoop \textit{et al.} (2004). Each fraction was analysed using a two-dimensional chromatographic system comprising a strong cation-exchange column coupled to a silica-based reversed-phase C18 monolithic column. The chromatographic system was downscaled to nano-flow ESI to achieve highest sensitivity. Based on this analysis a master-list of proteins was obtained. From this list, peptides were chosen as signature peptides for proteins based on the highest score for identification. For proof of concept study, a SUSY isoform was chosen known to be of very low abundance. This protein was only detected in the first and the third quadrupole enhances the selectivity and sensitivity of the analysis. Several SRM modes can be performed in one chromatographic separation. Thus, the simultaneous analysis of dozens of target-peptides can be performed in a single run (Glinski and Weckwerth, 2005a). Using internal standard peptides it is also possible to generate calibration curves for the absolute quantitation of peptides.

Targeted analysis and absolute quantitation of SUSY out of an ultra-complex protein sample
For absolute quantitation of biological samples stable-isotope dilution techniques are standard methods. Nevertheless, quantification of less abundant proteins out of ultra-complex plant protein mixtures such as crude extracts from \textit{Arabidopsis thaliana} using a non-gel 1D shotgun proteomics approach has never been shown before.

SUSY, a key enzyme involved in sucrose metabolism in plants was chosen for initial absolute quantification studies. Its activity has been studied in various plants. However, specific isoforms have not been distinguished. Yet, the SUSY isoform At3g43190 was chosen, due to mass spectrometric peptide sequence information acquired by multi-dimensional peptide identification, as described above (Wienkoop \textit{et al.}, 2004). The tryptic peptide HVSNLD-RLEARR was picked for standard synthesis since it reached highest spectrum quality. The native peptide can easily be distinguished from the chemically identical counterpart via a 4 Da mass shift and identical retention time (52 min; Fig. 3). However, fragmentation patterns are of great advantage for confirmation. In the first step, the quality of the internal standard peptide has to be evaluated such that the replacement of the residue containing stable isotopes after solid-phase peptide synthesis must be nearly 100%. Thus, no residual peak without stable-isotope label should be monitored, otherwise the absolute amount of the native peptide would be falsified (Fig. 4). For peak integration, three replicates of 20 µg digested \textit{Arabidopsis} protein extract with and without 500 fmol spiked standard peptide each were analysed via LC-MRM-MS using a high-resolution triple quadrupole mass spectrometer (TSQ, Thermoelectron) (Fig. 3). Increasing the resolution of the first and the third quadrupole enhances the selectivity of the SRM. Thereby the signal to noise ratio is increased and, thus, also the sensitivity/purity of detection and quantification (Glinski and Weckwerth, 2005a). With the standard curve (Fig. 5), the absolute amount of the peptide (1.58% mass of total protein) was determined to be 4.5 fmol µg\(^{-1}\) protein mixture, which correlates to 2.25 fmol mg\(^{-1}\) fresh weight.

Non-targeted analysis using a ‘pseudo-SIM-MS/MS’ scan on an ion trap MS enhances signal detection of low-abundance peptides in an ultra-complex sample
The analysis of the same protein mixture under exactly the same HPLC settings as for the triple quadrupole...
instruments performed with data-dependent scanning on a linear ion trap (LTQ, Thermoelectron) showed no detectable peak at 52 min, corresponding to the native SUSY-peptide ion trace (Fig. 6A). However, identification of a purified protein of even less than 10 fmol is usually possible but seems to be limited within an ultra-complex protein mixture due to ion suppression. Even under extended gradient conditions, SUSY could not be detected. Intriguingly, after insertion of a data-dependent MS/MS segment with a mass window of 10 Da, including the SUSY-peptide precursor \( m/z \), a peak was found at 52 min, which could be identified as the

**Fig. 3.** High-resolution triple-quadrupole MS (TSQ) analysis of the sucrose synthase within an ultra-complex Arabidopsis protein mixture. (A) Sample (20 \( \mu \)g protein mixture) without internal stable isotope-labelled standard peptide. (B) Sample (20 \( \mu \)g protein mixture) spiked with internal stable isotope-labelled standard peptide.

**Fig. 4.** Control of purity for the stable isotope-labelled peptide. The upper trace (489.17 \( m/z \)) is the native peptide ion; the lower trace (490.5 \( m/z \)) is the stable isotope-labelled peptide ion. Measurement was made with TSQ Quantum.
SUSY-peptide (Fig. 6Bii). This scan-mode can be interpreted as a pseudo-SIM or pseudo-SRM on a very wide mass window. Consequently, a range of precursor ions is selected for fragmentation including the targeted ion. The higher sensitivity can be explained by the fact that all fragment ions are summed up to a total ion intensity signal by contrast to a SRM scanning process on a triple quadrupole MS. Thus, the data-dependent MS/MS scan technique without dynamic exclusion, by contrast with full scan triple play analysis with dynamic exclusion, seems to be very powerful for searching for low-abundance proteins in a complex protein matrix and provides enough scans for quantification. However, the typical wide precursor selection mass window for an ion trap spectrum decreases selectivity by contrast to an SRM on a triple quadrupole MS (Glinski and Weckwerth, 2005a). Comparing signal/noise intensities of both analyses between TSQ and LTQ the triple quadrupole MS analysis was about 2-fold higher (see also Glinski and Weckwerth, 2005a).

**Conclusion**

One-dimension shotgun protein analysis with stable-isotope dilution using internal standard peptides provides a powerful tool for high throughput quantitative proteome analysis of low-abundance proteins and absolute quantitation. These data will enable the different proteins and their concentrations in the plant tissue to be compared. However, for some very low-abundance proteins it may be necessary to scale-up the initial sample loading and extend...
the chromatography to two- (or more) dimensional fractionation prior to analysis (Wienkoop et al., 2004).

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References


