SpikeTides™ for Subcellular Marker Proteins offer Improved Analysis of Complex Plant Samples

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The subcellular composition of tissue homogenates or organelle enrichments is frequently assayed by immunoblotting. However, for some plant organelles only a few, if any, antibodies are commercially available against established resident proteins, resulting in inaccurate estimates. Here, we demonstrate the utility of a suite of SpikeTides™ organelle markers in Arabidopsis and the viability of SRM assays as a more accurate and quantifiable alternative to immunoblotting.

Introduction

Accurate assessment of the subcellular composition of any biological sample is often prerequisite knowledge for the success of routine cell biology protocols, but it is not straightforward. Determinations by immunoblotting can be inaccurate, because results are not readily quantifiable (1) and, particularly in the plant sciences, the availability of antibodies against reliable organelle markers is not consistent throughout the cell. Furthermore, the number of non-model plant research species (2) is increasing rapidly, meaning that only few antibodies are currently available for certain species.

Despite the described shortcomings, immunoblotting of complex samples remains popular, as it is cheaper and more accessible than mass spectrometry-based techniques. Bridging the gap between accuracy and accessibility and developing adequate tools for future plant science research are therefore important tasks. To this end we have taken advantage of the latest developments in plant protein localization (3–5) to create a suite of 62 marker peptides representing up to 7 resident proteins for each of the 10 major subcellular compartments of Arabidopsis (3). The organelle marker proteins can be assayed on a range of mass spectrometers, i.a. by using SRM on a triple-quadrupole (QQQ)(6), or by using PRM on a triple-TOF or Orbitrap instrument (7). QQQ methods are robust, rapid and have relatively low costs, making out-sourcing of samples realistically affordable for non-proteomics research groups.

Here, we demonstrate the utility of SRM assays in determining the subcellular composition of complex samples by comparing SRM data to estimates from two alternative data-dependent techniques. Applicability and robustness is demonstrated by analyzing a broad range of plant material grown under standard- and low-light regimes. Marker peptides are available as a prefabricated set of heavy labeled non-quantified SpikeTides™ and can also be supplied as absolutely quantified SpikeTides™_TQL (8), permitting both relative and absolute quantification of the subcellular composition of complex plant samples.

Results and Discussion

Selection of subcellular compartment markers:

Few proteome-wide, quantitative studies have been performed in plants, so extensive investigations were conducted into appropriate subcellular markers (see methods section and Hooper *et al.*(3) for details). This resulted in the identification of 2 to 7 marker proteins for each of the following compartments: cytosol, endoplasmic reticulum, extracellular space, Golgi, mitochondria, nucleus, peroxisome, chloroplast, plasma membrane and vacuole, totalling 62 marker peptides, as detailed in Table S4 in Hooper *et al.*(3).

Performance of markers over a range of growth conditions:

The accuracy and reliability of markers at reporting changes in subcellular composition was tested by comparing SRM measurements to two different alternative methods. These consisted of data-dependent acquisition combined with subcellular localization information on several thousand proteins. Protein abundance was scored either by spectral counting or by a novel protein abundance score. Details of both methods can be found at Hooper *et al.*(3) and suba.live. Fig. 1 shows the relative abundance of subcellular compartments, as assayed by SRM, *vs.* the two data-dependent techniques. Protein extracts from five different tissues grown under standard-light or low-light/darkness conditions were compared.

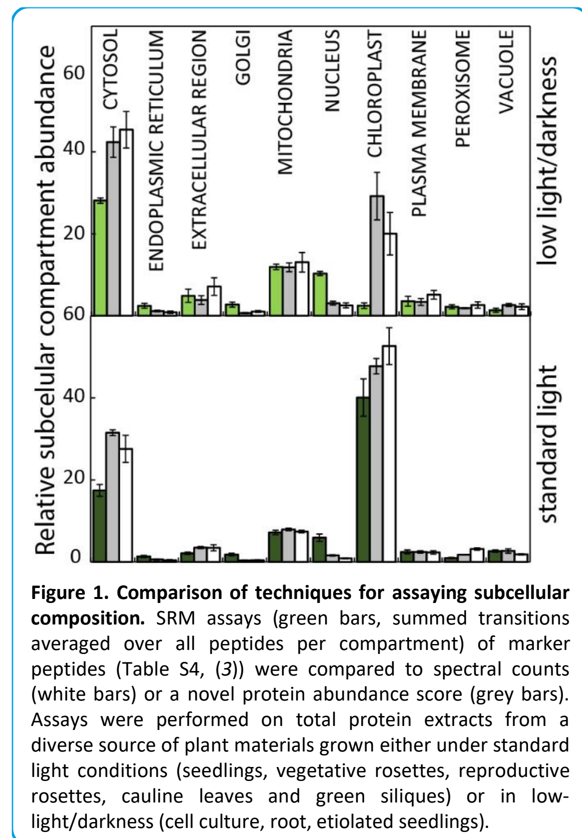

Figure 1. Comparison of techniques for assaying subcellular composition. SRM assays (green bars, summed transitions averaged over all peptides per compartment) of marker peptides (Table S4, (*3*)) were compared to spectral counts (white bars) or a novel protein abundance score (grey bars). Assays were performed on total protein extracts from a diverse source of plant materials grown either under standard light conditions (seedlings, vegetative rosettes, reproductive rosettes, cauline leaves and green siliques) or in low-light/darkness (cell culture, root, etiolated seedlings).

As demonstrated in Fig. 1, all three tested methods gave comparable estimates of subcellular composition with only three exceptions: the nucleus was somewhat over-estimated by SRM,

and the cytosol as well as the chloroplast of plants grown under low-light conditions were under-estimated by SRM. For these conditions, data available in Hooper *et al.*(*3*) will facilitate the selection of additional and/or more suitable markers. Overall, however, Fig. 1 shows that SRM markers provided a robust estimate of subcellular composition in almost all conditions.

Verification of SpikeTides™ markers:

SpikeTides™ peptide markers were spiked into protein extracts from an enrichment of Arabidopsis endomembranes. Application of SRM assay conditions led to indistinguishable SpikeTides™ and native peptide retention times (examples shown in Fig. 2). As anticipated, the native peptide to SpikeTides™ ratio was appreciably higher for enriched membranes i.e. the ER and Golgi (Fig. 2). This demonstrates the applicability of this approach to quantifying purity levels achieved during organelle enrichment experiments.

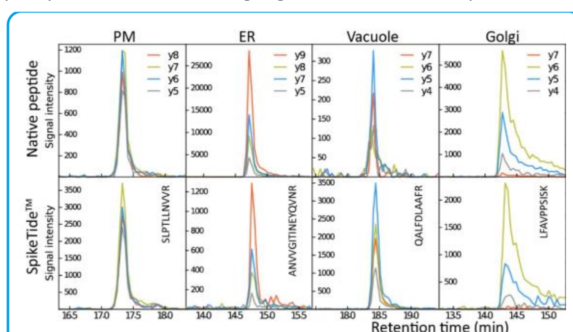

Figure 2. SRM assay results of selected SpikeTide™ and native compartment marker peptides. A mixture of SpikeTide™ peptides was spiked into enriched endomembranes from Arabidopsis cell culture. PRM assays were performed on an Orbitrap QExactive as described in Hooper *et al.*(*3*).

Conclusions and Future Perspectives

We have developed a set of SpikeTides™ peptides that allow the mass spectrometry based analysis of multiple, carefully selected, resident proteins from the 10 major plant subcellular compartments in Arabidopsis. SRM assays delivered comparable results to non-quantitative mass spectrometry assays over a wide range of growth conditions with only few exceptions. This demonstrates that SRM assays of SpikeTides™ organelle marker peptides offer a fully quantifiable alternative to immunoblotting when analyzing subcellular sample composition.

Selecting appropriate SRM organelle markers depends upon a sequenced and well-annotated genome, knowledge of protein localization, and/or use of proteins with close homology to established organelle residents from model species. Therefore, although this proof-of-concept study was conducted in Arabidopsis, it should be transferrable to many other species of interest (*10*), so will likely prove to be an important future research tool. A commercially available suite of organelle markers will encourage uptake of this alternative technique amongst non-proteomics research groups, and development in further species.

Materials & Methods

For the selection of subcellular compartment markers an initial screen filtered for proteins with high localization confidence, using data available in recent publications(*4*, *5*). Next, using ESTs and data from Wang *et al.*(*9*), the most abundant 15% of proteins was selected for each subcellular compartment so that markers were comparatively representative. Peptides were further screened for redundancy, known post-translational modifications, or appreciable changes in gene expression level under most growth conditions or in frequently analyzed tissues. Finally, suitable SRM transitions were compiled after assaying total protein extracts from leaves and cell-culture, using an Agilent 6460 QQQ.

For the verification of SpikeTides™ markers a mixture of SpikeTides™ (1 pmol) was spiked into protein extracts (10 ng) from an enrichment of Arabidopsis endomembranes. Previously developed SRM assay conditions were applied (representative results shown in Fig. 2, details available at www.peptideatlas.org (dataset identifier PASS00906) and Hooper *et al.*(3)).

Details of all experimental procedures and SRM transitions can be found at Hooper *et al.* (3).

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