

# ELUCIDATING PROTEIN POSTTRANSLATIONAL MODIFICATIONS USING COMBINATION OF RECOMBINANT PROTEIN SPECTRAL LIBRARY AND *IN SILICO* DESIGNED SRM ANALYSIS

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**Abstract:** Posttranslational modifications (PTMs) of proteins represent fascinating extensions of the dynamic complexity of proteomes of living cells, but also present a difficult obstacle in the proteome analysis. Identification and mapping of PTMs in proteins have improved dramatically, but to comprehend complex mechanisms and biological functions, one must address also very low abundant proteins. Here, we demonstrate *in silico* derived analysis of a low abundant target of ubiquitination and the MS/MS identification of the predicted ubiquitination sites.

**Key Words:** posttranslational modifications, ubiquitination, proteomics, mass spectrometry

## INTRODUCTION

The protein PTM analysis is a challenging task that requires an advanced methodology (Černý et al. 2013b) and usually also a deal of luck. Here, we employed *in silico* analysis and state-of-the-art mass spectrometry to determine position of a known PTM. Our model is a 60 kDa protein that is regulated by proteasome mediated degradation pathway.

## MATERIAL AND METHODS

### Plant material, cultivation and total protein extraction

The protein of interest was extracted by immunoprecipitation from transgenic *Arabidopsis thaliana* line bearing its fusion with GFP under 35S promoter. In parallel, recombinant protein (native sequence) was expressed and purified from *E. coli*. Protein ubiquitination *in vivo* was confirmed by Western blot analysis. Material for LC-MS analysis was prepared as described previously (Černý et al. 2013a). The resulting peptides were then analyzed online by nanoflow C18 reverse-phase liquid chromatography using a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo) directly coupled to a nanoESI (electrospray ionization) source CaptiveSpray (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker)(Baldrianová et al. 2015; Novák et al. 2015), or TSQ Quantiva triple quadrupole (Thermo). The SRM method was designed by Skyline 3.1 (MacCossLab Software; <https://skyline.gs.washington.edu>).

## RESULTS AND DISCUSSION

Protein ubiquitination is an important PTM in plant hormone signalling and thus the sites of ubiquitination are interesting candidates for the site-directed mutagenesis. The sequence of our protein of interest contains 20 lysine residues and it is not clear which are involved in its regulation. Its amount *in planta* is relatively low, but by employing a 35S overexpressor and immunoprecipitation, we were able to detect its ubiquitination (Figure 1). However, the protein amount for an untargeted LC-MS was not sufficient to elucidate the PTM's position (the protein coverage was below 10%). We have prepared a recombinant version of our protein and the resulting MS/MS tryptic peptide library covers 78% of the protein sequence. The remaining 22% cannot be reached by a trypsin digestion. We tested

our library and 42 peptides based on SRM designed and optimized for recombinant protein could be traced in immunoprecipitated samples, though the intensities of some indicated the presence of additional PTM(s) (Figure 2).

Figure 1 Western blot validation of protein ubiquitination in vivo. Overlay of a consecutive staining by anti-GFP (orange) and anti-ubiquitin (blue), the relative molecular mass is indicated

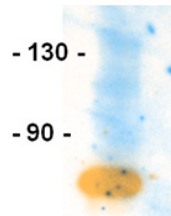
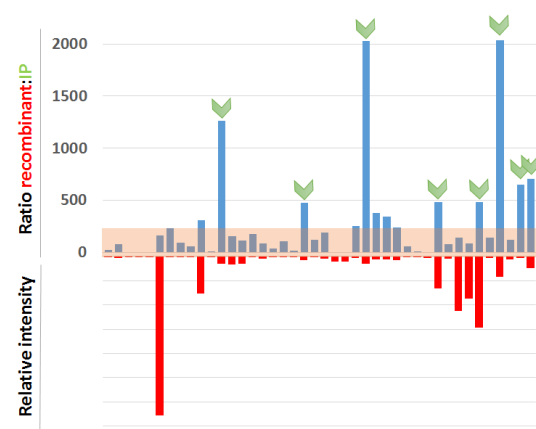
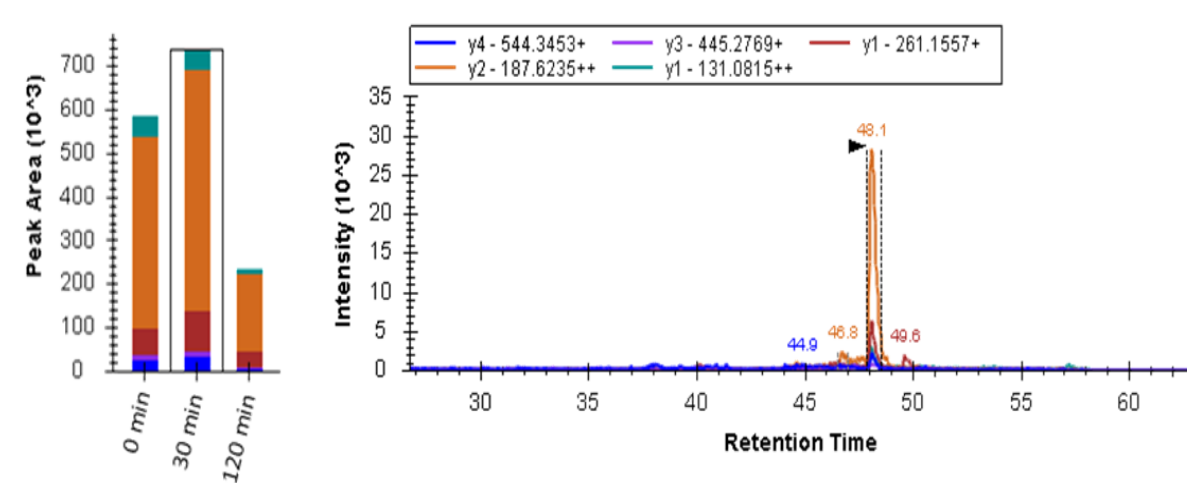


Figure 2 Peptide intensities. Recombinant peptide intensities (red) and the comparison of peptide abundance in recombinant immunoprecipitated protein from transgenic Arabidopsis seedlings



As the next step, we designed SRM for predicted ubiquitination sites. Altogether, SRM for over 1,400 peptides were tested. The analysis pointed out four ubiquitination sites. To provide further evidence, we used the TUBE1-agarose matrix (Tandem Ubiquitin Binding Entities) to enrich ubiquitinated proteins from plant extracts and we were able to detect at least one of the determined ubiquitination sites (Figure 3).

Figure 3 Targeted analysis of identified ubiquitination site in enriched plant protein extracts. Bar plot Seedlings were pretreated with proteasome inhibitor MG-132 and sampled 0-120 min after the induced degradation. The bar plot indicates the ubiquitinated peptide accumulation and the consequent protein degradation



## CONCLUSION

In conclusion, we were able to pinpoint in vivo ubiquitinated peptides of a low abundant protein. This study demonstrates the potential of modern technology and we believe that this approach could be the next level in elucidating complex PTMs in cell signalling pathways.

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