PROS AND CONS OF PLANT NUCLEAR PROTEIN ENRICHMENT

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Abstract: Nuclear proteome contains important regulatory proteins. To improve the detection of these proteins, Percoll gradient-based fractionation techniques have been developed and optimized. However, owing to the ever increasing sensitivity of identification methods based on liquid chromatography and mass spectrometry, the time and material consuming fractionation methods may no longer be necessary. Here, we show that a Percoll-based nuclear protein fractionation of tomato leaf proteome increased the number of detected proteins, but at least some nuclear proteins were lost or depleted in the process.

Key Words: nuclear proteome, LC-MS, proteome fractionation

INTRODUCTION

The nucleus is a complex and heterogeneous organelle that is composed of two main structural parts: nuclear envelope and nucleoplasm. The later contains most of the cell's genetic information but also other molecules, including proteins that play a central role in regulating gene expression (Petrovská et al. 2015). The identification and characterization of these regulatory proteins presents a challenge. They are usually in a low abundance and subjects of post-translational modifications. The enrichment of phosphorylated or acetylated proteins (e.g. Černý et al. 2013b) may increase the detection limits, but the most effective technique to isolate nuclear proteins seems to be a cell fractionation. The standard protocol consists of a homogenization of tissues, pelleting, elimination of contaminating organelles and separation on a density gradient. Plant extracts are more complicated and require an additional filtration step after a homogenization to remove large debris (e.g. Sikorskaite et al. 2013). The extraction of nuclei is a time consuming step and may have a negative impact on proteome quality. For instance, even the addition of protease inhibitors does not necessarily prevent a residual protease activity during filtration and pipetting steps. Further, the efficiency of the extraction protocol is not comparable to that of phenol extraction or acetone/TCA precipitation and thus the amount of starting material has to be considerably larger. Here, we compare results of an optimized protocol for nuclear protein extraction and standard protein precipitation protocol in the analysis of tomato leaf proteome.

MATERIAL AND METHODS

Plant material

Leaves of 4-week-old tomato (*Solanum lycopersicum*, cv. moneymaker) were collected, frozen in liquid nitrogen, homogenized (Retsch Mill MM400), aliquoted and stored at -80 °C.

Protein extraction

Total protein extracts were prepared by acetone/TCA/phenol extraction (Carpentier et al. 2005, Černý et al. 2014, Novák et al. 2015) from 200 mg of ground tissue. In brief, the homogenized tissue was washed with 1.5 ml acetone (4 °C, 30 min), clarified by centrifugation, washed with 10% (w/v)

TCA in acetone, 10% (w/v) TCA in distilled water then 80% (v/v) acetone, resuspended in 0.8 ml SDS buffer [2% (w/v) SDS, 30% (w/v) sucrose, 5% (v/v) β-mecraptoethanol, 5 mM EDTA, 100 mM Tris, pH 8.0], and protein was extracted by 0.4 ml buffer-saturated phenol. Phenolic phase was collected and protein was precipitated overnight in 1.6 ml ice-cold 100 mM ammonium acetate in methanol (-20 °C). Protein pellets were washed with 1.0 ml 80% (v/v) acetone in distilled water and dried. Nuclei were extracted on percoll gradient from 2 g of ground tissue as described previously (Sikorskaite et al. 2013). In brief, 1 g of homogenized tissue was extracted in 5 ml of NIB buffer [10 mM MES-KOH (pH 5.4), 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 250 mM sucrose, 0.1 mM spermine, 0.5 mM spermidine, 1 mM DTT], decanted through two layers of pre-wetted cheesecloth, treated with Triton X-100 (dropwise to 0.5%), agitated for 20 min at 4°C and centrifuged at 1000 × g for 10 min. The pellet was resuspended in 10 ml of NIB and nuclei were purified using Percoll/sucrose density gradient (2.5 M sucrose, 60% Percoll for isolation; 35% Percoll for washing). Nuclear proteome was precipitated with 10% (w/v) TCA in acetone and washed 1.0 ml 80% (v/v) acetone in distilled water and dried. The resulting protein pellets were solubilized (100 mM ammonium bicarbonate, 8 M urea) and digested with an immobilized trypsin (Promega) overnight and desalted by C18 SPE (Černý et al. 2013a).

LC-MS proteome analysis

Analyses were performed using a gel-free shotgun protocol based on nano-HPLC and MS/MS (Baldrianová et al. 2015). Briefly, tryptic digests were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 40 cm column (0.075 mm inner diameter; NanoSeparations) and a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) directly coupled to a CaptiveSpray nanoESI source (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with up to a 120-min, 4% to 40% acetonitrile gradient. Spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s.

Protein identification

The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016). In brief, recalibrated MGF files were searched against Tomato protein database (ITAG 2.4; 8/2014) by Sequest HT, MS Amanda and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation.

RESULTS AND DISCUSSION

Identification of tomato proteins

Total protein extracts and nuclear enriched extracts were prepared in 14 replicates each. To increase the proteome coverage, MS spectra were processed by a combination of three complementary search algorithms and the resulting data obtained from all replicates were combined. Altogether, 1,711 and 1,199 protein groups were identified in total protein extracts and nuclear protein extracts, respectively (Figure 1).

Enrichment of nuclear proteins does not necessarily improve their detection

The overlap in identified proteins between total protein and nuclear extracts is high, representing 51% and 74% of all identified proteins, respectively (Figure 1A). A similar distribution is also reflected on a peptide level (45% and 65%; Figure 1B). Further, identification of 139 proteins unique to nuclear extracts is based only on a single identified peptide and these are thus not suitable for a quantitative analysis. In depth analysis of our data showed that at least some nuclear proteins are significantly enriched in nuclear extracts, including DNA-directed RNA polymerase II (not detected in total protein extracts) or three proteins of histone family H1 (200×), H4 (not detectable in total protein extracts) and H2B (200×). However, 12 histones and four histone-associated proteins were detected only in total protein extracts (Figure 2). Similarly, only four of eight detected 14-3-3 proteins were found in nuclear extracts. This shows that the nuclear protein enrichment may in fact have a negative impact on the detection of at least some of the nuclear proteins. To our knowledge, this is the first report of its kind in tomato proteomics and we can not exclude that this is a consequence of a tomato specific protease

activity. Standard protocols for nuclei extraction consists of several incubation steps at 4 °C and employ protease inhibitors. If the inhibition efficiency was lower (as is often the case) quality of the proteome would suffer.

Figure 1 Identification of proteins in total protein extracts (blue) and nuclear extracts (orange)

A) Identified proteins *B) Identified peptides*

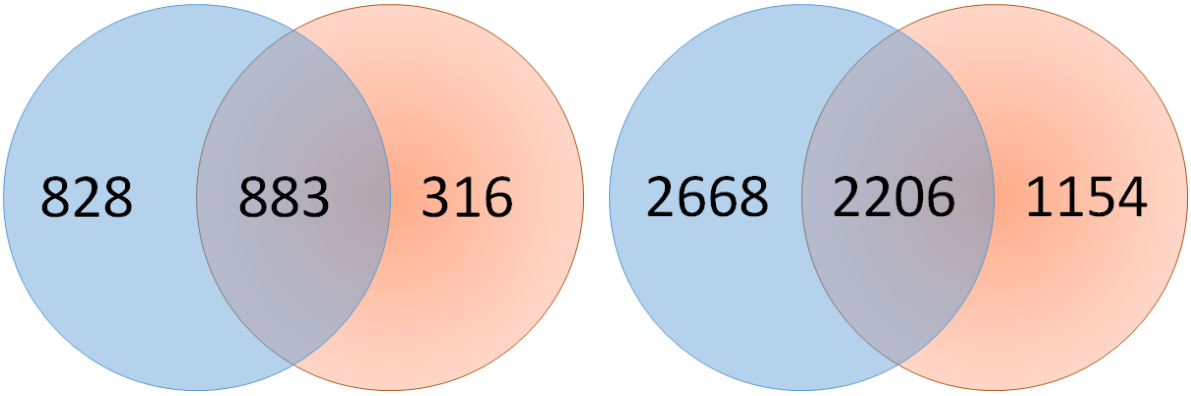
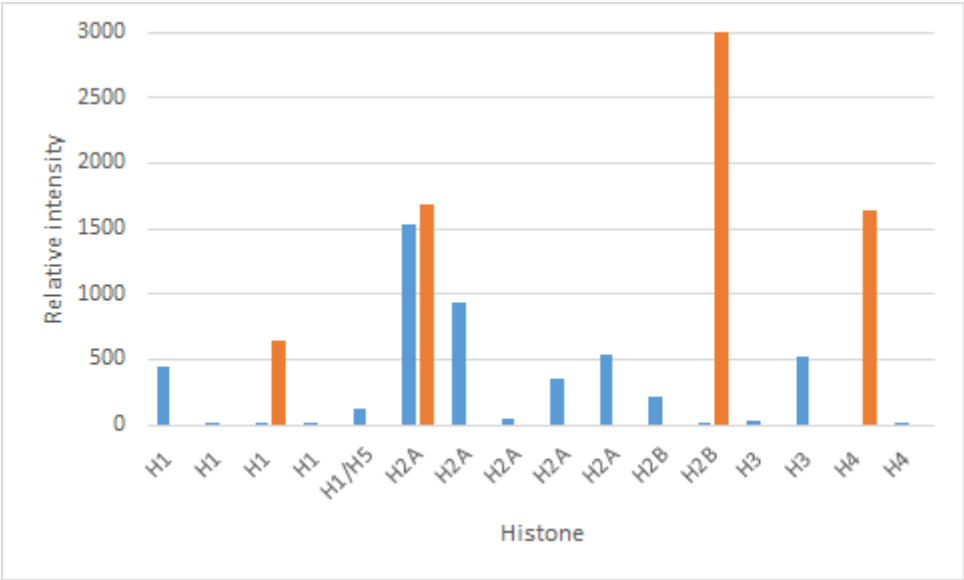


Figure 2 Relative abundances of detected histones in total protein extracts (blue) and nuclear extracts (orange) based on sum of all assigned peptide spectral matches



CONCLUSION

Analysis of nuclear protein extracts increased the number of identified proteins and peptides in tomato leaves by ~16% and ~19%, respectively. Insufficient annotation of tomato proteome does not allow to easily assess the localization of all 316 proteins unique to nuclear extracts. However, based on the profiles of several well-known nuclear proteins we conclude that the established Percoll-based protocol shows disproportionate yields in nuclear proteins and may even decrease the detectability of some. This would imply that the acetone/TCA total protein extraction is superior in the qualitative analysis.

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