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Evaluation of the Potential Risk of Analyzing Phosphopeptides with Easy-nLC 1200-Coupled Mass Spectrometers

Xiaoxiao Duan, Yuanya Zhang, Xiahe Huang, Xiao Ma, Hui Gao, and Yingchun Wang*

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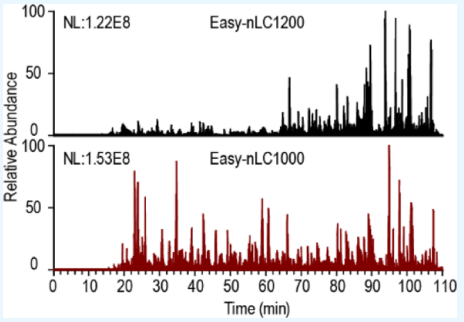
ABSTRACT: Liquid chromatography−mass spectrometry (LC−MS) is a major tool for the large-scale qualitative and/or quantitative analysis of protein phosphorylation in cells or tissues. The performance of LC is pivotal for the success of phosphoproteomics in both sensitivity and reproducibility. Here, we report that the widely used Easy-nLC 1200 has poor performance in analyzing phosphopeptides, particularly in terms of sensitivity and reproducibility, whereas its predecessor, Easy-nLC 1000, has a much better performance. Therefore, we suggest that Easy-nLC 1200 is not appropriate for LC−MS-based proteomics analysis for samples with a limited amount, particularly phosphopeptides from plants.



INTRODUCTION

Protein phosphorylation is a post-translational modification that is involved in numerous signaling and regulatory mechanisms. High-throughput mass spectrometry-based phosphoproteomics is a powerful tool to study protein phosphorylation toward understanding these mechanisms.[1,2] However, there are several challenges in the phosphoproteomic study. First, phosphoproteins are generally in low stoichiometry compared to their unphosphorylated counterparts, necessitating their pre-enrichment biochemically before being subjected to LC−MS analysis.[3] Second, phosphopeptides are usually not as efficient as unphosphorylated peptides in ionization during LC−MS analysis, resulting in reduced sensitivity in their MS detection.[4] Third, the labile character of the phosphoester bond usually alters the fragmentation pattern of phosphopeptides, resulting in poor spectrum quality and increased difficulty in spectral annotation.[5] Finally, phosphopeptides were often lost during front-end online HPLC separation due to the formation of phosphopeptide−metal ion complexes, with metal ions on the surface of the HPLC flow path,[6−8] resulting in an unpredictable chromatographic pattern and reduced experimental reproducibility and sensitivity. Additional challenges exist when analyzing phosphoproteomes from plant samples because a variety of metabolites and pigments may interfere with protein digestion and the enrichment of phosphopeptides.[9] Moreover, phosphoproteins are overall much less abundant in plants than in animals. It is also an important challenge to prepare a plant phosphopeptide sample with the amount commensurate with that usually used for animal samples for LC−MS analyses.[10]

Notwithstanding these challenges, recent technological advances in nano-flow reversed-phase LC−MS coupled with the increased efficiency of phosphopeptide enrichment from complex peptide mixtures improved the number of identified phosphosites to an unprecedented level in a single-shot phosphoproteomics.[11−13] The performance of LC is critically important for reproducible separation and detection of phosphopeptides by MS with high sensitivity. Currently, the most widely used LC−MS system for proteomics and phosphoproteomics is perhaps the Easy-nLC 1200 (or its predecessor Easy-nLC 1000) coupled with the Orbitrap series mass spectrometer. Thus, it can be estimated that the quality of the phosphoproteomics data for many research studies was largely dependent on the performance of the system.

RESULTS AND DISCUSSION

Recently, we tried to develop a working protocol for single-shot phosphoproteomics of *Arabidopsis* seedlings using an Easy-nLC 1200 coupled with an Orbitrap Fusion Lumos. To our surprise, after numerous tries, we failed to achieve an optimal experimental condition that allows reproducible identification of phosphosites from the same amount of starting plant material. The inconsistent base peak patterns from the resulting chromatograms indicate that the poor chromatographic performance is probably the major cause (Figure 1a). As a control, an older version of the instruments, that is, Easy-nLC 1000 coupled with Orbitrap Elite, showed a much better performance in both chromatography and

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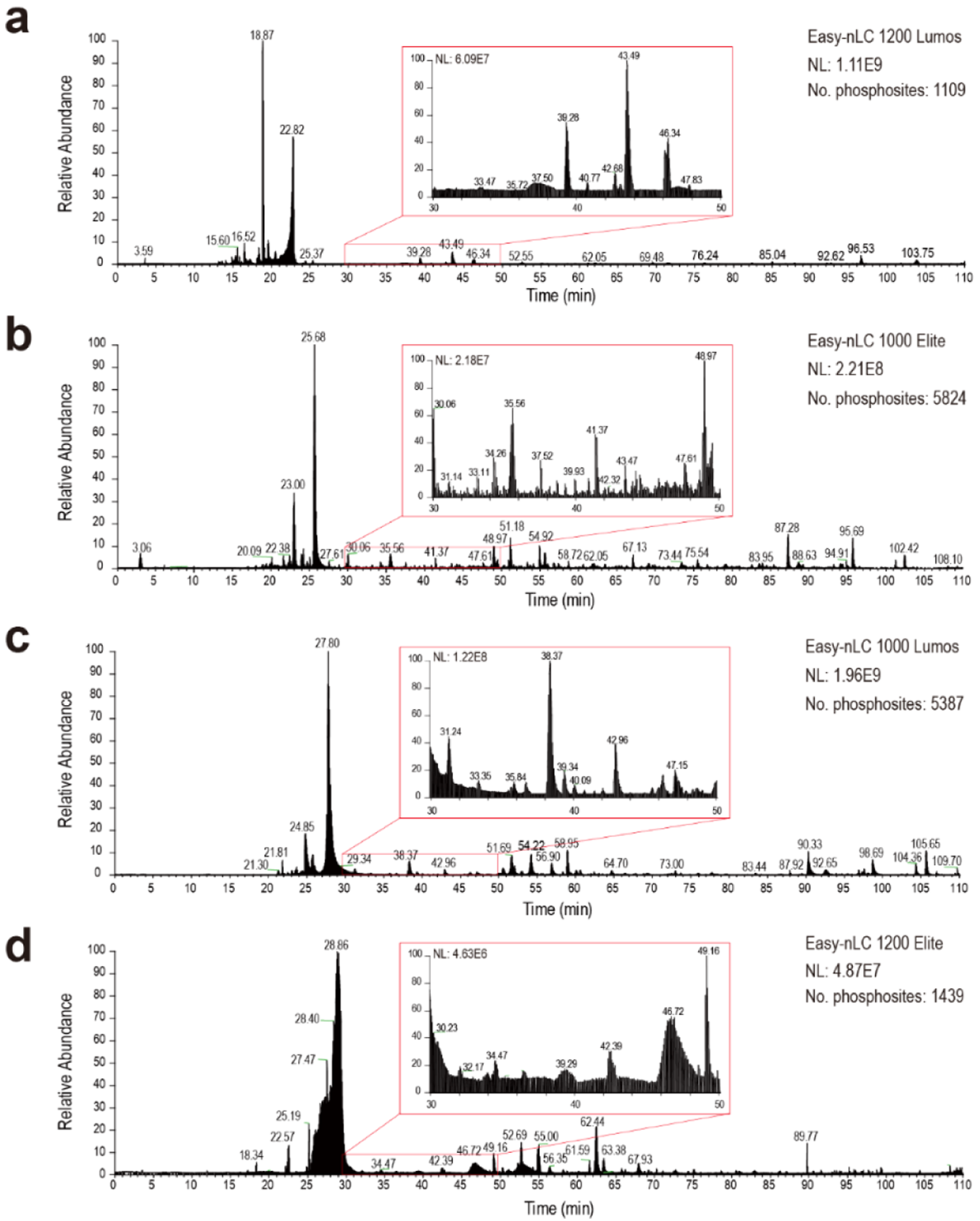

Figure 1. Base peak chromatograms of plant phosphopeptides analyzed with different LC−MS setups. Phosphopeptides enriched from 300 μg proteins extracted from *Arabidopsis* seedlings were analyzed by (a) Easy-nLC 1200 coupled with Lumos, (b) Easy-nLC 1000 coupled with Elite, (c) Easy-nLC 1000 coupled with Lumos, and (d) Easy-nLC 1200 coupled with Elite. A representative region in each chromatogram was magnified and is shown in the box with the red frame.

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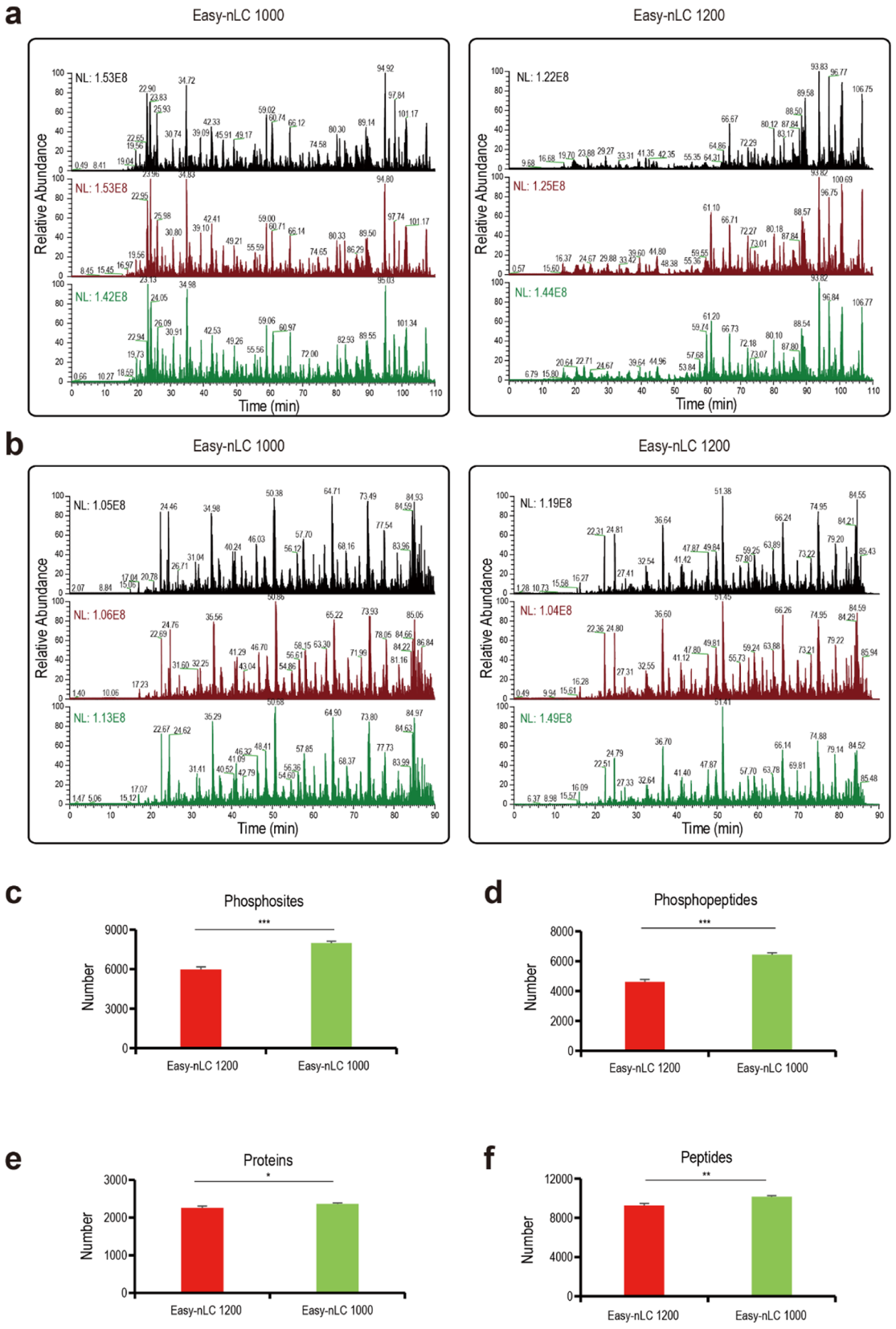

Figure 2. Performance comparison of the two LC systems for online separation of phosphopeptides or total peptides from human 293T cells for MS analysis. Phosphopeptides enriched from 600 μg of the tryptic digest of 293T WCLs or tryptic peptides from 2 μg of 293T WCLs were analyzed using Orbitrap Elite coupled with either Easy-nLC 1200 or Easy-nLC 1000 UHPLC at the front-end. (a,b) Triplicate base peak chromatograms of phosphopeptides (a) or peptides (b) generated with Easy-nLC 1000 (left panels) or Easy-nLC 1200 (right panels) at the front-end. (c–f) The bar graphs show the number of phosphosites (c), phosphopeptides (d), protein groups (e), and peptides (f) identified with the indicated LC for the front-end separation. Error bar: standard deviation, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

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identification of phosphosites in otherwise same experimental conditions (Figure 1b and Table S1). The observation suggests that Easy-nLC 1200 probably has some defects in the separation of phosphopeptides. Indeed, after switching the LC of the two instruments, the performance of the Orbitrap Lumos coupled with Easy-nLC 1000 was greatly improved in both the number of identified phosphosites and the reproducibility (Figure 1c and Table S1), whereas Orbitrap Elite displayed a much poorer performance compared with that obtained before switching the LC (Figure 1d). This result further confirms the defect of Easy-nLC 1200 in the separation of phosphopeptides. To ensure if such a defect is a unique case to our instrument or a more general problem, we sent phophopeptide samples to two other labs and analyzed with an Easy-nLC 1200 coupled with Lumos and an Easy-nLC 1200 coupled with Orbitrap Eclipse using the similar experimental conditions. Again, a poorer chromatographic performance, a lower number of identified phosphosites, and a poorer MS/MS identification rate were observed compared with the results obtained using Easy-nLC 1000 coupled with Orbitrap Lumos (Figure S1).

Phosphopeptides enriched from plant tissues may contain impurities such as phenolic compounds, starches, oils, pigments, and secondary metabolites that could affect the LC performance.[14] Thus, we also compared the performance of the two LC systems in analyzing phosphopeptides enriched from human 293T cells. Again, Easy-nLC 1000 showed a much better performance (Figure 2a,c,d). Notably, we found that the lower the starting material, the poorer the performance of Easy-nLC 1200 when compared with that of Easy-nLC 1000 (Figure S2a,c,d and Table S2).

Since an Easy-nLC 1200-coupled Orbitrap series mass spectrometer is a widely adopted instrumental setup for proteomics analysis, it is a surprise that such a defect has not been reported. We reasoned that the adverse result caused by the defect could be trivial when the instruments were used for analyzing the total tryptic digests of protein mixtures. Indeed, when 2 μg of tryptic digests of 293T whole cell lysates (WCLs) was analyzed using Orbitrap Elite that is coupled with either Easy-nLC 1200 or Easy-nLC 1000, an excellent chromatographic performance was observed for both LC systems as indicated by the base peak separation and the reproducibility among the triplicate experiments (Figure 2b). Nevertheless, a slight but statistically significantly better performance was still observed for Easy-nLC 1000 because more peptides and proteins can be identified with Easy-nLC 1000 ($p < 0.05$) (Figure 2e,f). Similar results were also observed when the starting material was reduced from 2 μg to 400 ng (Figure S2b,e,f and Table S3).

CONCLUSIONS

Easy-nLC 1200 was designed for operating with a higher pressure, and thus, several parts in the flow path were made with stainless steel such as the connectors, which are made from plastic polyether ether ketone (PEEK) material in Easy-nLC 1000. It was reported that the metal surface in the flow path of the LC can adsorb acidic peptides, particularly phosphopeptides bearing a negatively charged group.[15−17] The adsorption of phosphopeptides by the metal surface in the flow path could lead to decreased sensitivity and low reproducibility between runs. The problem could not be easily detected when large amounts of the samples are loaded because the metal surface in the flow path can be saturated by the adsorption of a small fraction of the peptide samples, and the effect of the adsorption-caused sample loss is not apparent. For phosphopeptides enriched from plant tissues, a much lesser amount can be obtained from the same amount of starting material compared with that from animal tissues or cell lines, and the adsorption could lead to more significant sample loss and obvious sensitivity decrease. Simply increasing the starting material of plant tissues could not alleviate this problem because of the simultaneous increase of the interfering pigments and secondary metabolites.[14] Therefore, we conclude that Easy-nLC 1200 is not appropriate for reversed-phase LC−MS (with 0.1% formic acid in the mobile phase of the LC)-based proteomics analysis for samples with limited amounts, particularly phosphopeptides from plants.

METHODS

Sample Preparation. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium high glucose with Glutamax (Hyclone, 31966-021) and supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (Invitrogen, 15140-122). The cell culture was incubated at 37 °C with 5% CO_2. Cells at an approximately 90% confluence were washed twice with PBS and then lysed with 4% SDC lysis buffer. The lysate was boiled for 10 min at 100 °C. The protein concentration was determined by the BCA assay (Thermo Fisher Scientific, 23225). The proteins were digested with 1:50 (w/w) trypsin at 37 °C overnight. An aliquot of the tryptic digest was desalted with C18 StageTip and then freeze-dried with a SpeedVac and stored at −80 °C before use, and the rest of the tryptic digest would be used for the enrichment of the phosphopeptides.

Arabidopsis (Col-0) seedlings were ground with liquid nitrogen in a mortar before extraction with 4% SDS lysis buffer. The proteins were digested, and enrichment of the phosphopeptides was performed as previously reported.[4]

Phosphopeptide Enrichment. Phosphopeptides were enriched from the tryptic digest (before desalting) using titanium dioxide beads (TiO_2; GL Sciences, 5010-21315) according to the protocol described by Humphrey *et al.*[18] Plant phosphopeptides were enriched according to the protocol described by Huang *et al.*[4]

Nanoflow LC Tandem MS. The trap column was prepared by first creating a Kasil frit at one end of a 10 cm length capillary with 200 μm inner diameter/360 μm outer diameter. The frit was created using the method described by Cortes *et al.* using a Kasil formamide frit kit (Next Advance Corporation, NY, USA).[19] Briefly, the frit was prepared by rapidly dipping the capillary in a well-mixed 30 μL of Kasil 1624, 10 μL of Kasil 1, and 10 μL of formamide and curing at 100 °C for 16 h. The 30 cm analytical column (150 μm inner diameter) for phosphopeptide separation was packed in-house according to a previous report,[20] and a 10 mg/mL slurry of 1.9 μm AQ-C18 beads (Dr. Maisch, Beim Brückle, GER) in methanol was used to pack both the trap column and analytical column under 4 MPa nitrogen.

The peptides or phosphopeptides were analyzed using an Easy-nLC 1000 UHPLC system or Easy-nLC 1200 UHPLC system coupled to an Orbitrap Elite or Orbitrap Lumos instrument (Thermo Fisher Scientific, NY, USA). For the phosphoproteomics' comparison, we used a 110 min gradient at a flow rate of 600 nL/min. Buffer B of the gradient was composed of 95% (vol/vol) ACN/0.1% (vol/vol) formic acid and buffer A was composed of 0.1% (vol/vol) formic acid. The

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gradient was ramped from 2 to 21% buffer B in 75 min, to 34% buffer B in 24 min, to 100% buffer B in 6 min and kept for 5 min. For the analysis of the peptides from the WCLs, a 90 min gradient was ramped from 3% B to 8% B in 10 min, to 20% B in 60 min, to 30% B in 8 min, to 100% B in 2 min and kept for 10 min.

The plant phosphopeptides were analyzed using an Orbitrap Lumos instrument. A range of 375-1500 and an Orbitrap resolution of 60,000 (at m/z 200) were used. Precursor ions with charges of +2 to +7 were isolated with an isolation window of 1.6 and fragmented by high energy dissociation with 32% collision energy. The dynamic exclusion with a duration of 30 s was used. For full MS scans, the automatic gain control (AGC) target and maximum injection time were set at 1×10^6 and 50 ms, respectively. For the MS2 scans, an ion trap was used as a detector, and the ion trap scan rate was set at rapid. The AGC target and maximum injection time were set at 2×10^4 and 50 ms, respectively.

For the Orbitrap Elite instrument, a range of 300−1800 m/z and an Orbitrap resolution of 240,000 (at m/z 400) were used for the full MS scan. The mass spectrometers were operated in a data-dependent acquisition mode to automatically isolate and fragment Top20 multiply charged precursors according to their intensities. Precursor ions with charges of +2 to +7 were fragmented by collision-induced dissociation with 35% collision energy. Dynamic exclusion with a duration of 30 s was used. The AGC target was set at 1000 for the MS2 scans. The raw files of the MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD034396 (http://www. proteomexchange.org).

Data Analysis. The database search was performed in the MaxQuant environment (version 1.6.3.4).[21] The UniProt human proteome database containing 20,244 entries or the TAIR *Arabidopsis thaliana* proteome database containing 35,386 entries was used for the database search. Variable modifications were set such as oxidation of methionine, protein N-terminal acetylation, and phosphorylation of serine, threonine, and tyrosine residues for the phosphopeptide samples. Carbamidomethylation of cysteine was set as the fixed modification. The maximal number of missed cleavages was set at 2, and the minimum peptide length was set at 7 amino acids. The false discovery rates for both peptide and protein identifications were set at 1%. Other parameters were set up using the default values.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05616.

Comparison of the performance of the three front-end LC systems from different labs for LC−MS analysis and comparison of the performances of the two front-end LC systems for online separation of phosphopeptides or tryptic peptides from 293T WCLs for MS analysis (PDF).

Results of the single-shot LC−MS runs with different instrumental setups for the phosphopeptides prepared from *Arabidopsis* seedlings; results of the single-shot LC−MS runs for the phosphopeptides prepared from different amounts of 293T WCLs using Orbitrap Elite coupled with different LC systems at the front-end; and results of the single-shot LC−MS runs for tryptic peptides from different amounts of 293T WCLs using Orbitrap Elite coupled with different LC systems at the front-end (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

Yingchun Wang − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; University of Chinese Academy of Sciences, Beijing 100049, China*; Email: ycwang@genetics.ac.cn

Authors

Xiaoxiao Duan − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; University of Chinese Academy of Sciences, Beijing 100049, China*; orcid.org/0000-0001-6277-4018

Yuanya Zhang − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China*

Xiahe Huang − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China*

Xiao Ma − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; University of Chinese Academy of Sciences, Beijing 100049, China*

Hui Gao − *State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; University of Chinese Academy of Sciences, Beijing 100049, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c05616

Author Contributions

X.D. and Y.Z. are joint first authors. X.D. and Y.Z. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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