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Plant protein phosphorylation monitored by capillary liquid chromatography–element mass spectrometry

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Abstract

Many essential cellular functions such as growth rate, motility, and metabolic activity are linked to reversible protein phosphorylation, since they are controlled by signaling cascades based mainly on phosphorylation/dephosphorylation events. Quantification of global or site-specific protein phosphorylation is not straightforward with standard proteomic techniques. The coupling of capillary liquid chromatography (μLC) with ICP-MS (inductively coupled plasma-mass spectrometry) is a method which allows a quantitative screening of protein extracts for their phosphorus and sulfur content, and thus provides access to the protein phosphorylation degree. In extension of a recent pilot study, we analyzed protein extracts from the model organisms *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* as representatives for multicellular and unicellular green photosynthetically active organisms. The results indicate that the average protein phosphorylation level of the algae *C. reinhardtii* is higher than that of *A. thaliana*. Both the average phosphorylation levels were found to be between the extreme values determined so far for prokaryotes (*C. glutamicum*, lowest levels) and eukaryotes (*Mus musculus*, highest levels). Tissue samples of *A. thaliana* representing different stages of plant development showed varying levels of protein phosphorylation indicating a different adjustment of the kinase/phosphatase system. We also utilized the μLC–ICP-MS technology to estimate the efficiency of a novel phosphoprotein enrichment method based on aluminum hydroxide, since the enrichment of phosphorylated species is often an essential step for their molecular characterization.

Keywords: Protein; Phosphorylation; Phosphoprotein; Plant; Algae; *Arabidopsis thaliana*; *Chlamydomonas reinhardtii*; Proteomics; Element mass spectrometry; ICP-MS; Phosphorus; Sulfur; HPLC; MOAC; Enrichment

Reversible protein phosphorylation is considered as one of the functionally most important covalent protein modifications [1,2]. It is involved in a variety of vital cellular functions, including internal and receptor-mediated signaling cascades as well as many steps of metabolic regulation. Accordingly, the average protein phosphorylation level in a cell is directly connected to the status of different functions [3]. For instance, the growth rate of tissues may be reflected in the average relative abundance of phosphorylated isoforms, since in proliferating cells compared to resting cells, often kinases are upregulated with concomitant downregulation of phosphatases. Activation of tyrosine kinases was found to be particularly correlated with various forms of cancer [4]. In agreement with these observations, elevated phosphorus levels have been reported for tumor compared to control tissue [5].

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Since the majority of these studies are focused on mammals or bacteria, knowledge about protein phosphorylation in plants is still highly fragmentary. Phosphoproteins in plants are either homologous to eukaryotic phosphoproteins or they are unique plant proteins. Representatives of the latter are proteins involved in photosynthesis [6,7], but also include other plant-specific proteins, for instance those involved in phytochrome signaling, which is a light-regulated pathway of growth control [8].

The mass spectrometric methods ESI-MS (electrospray ionization) and MALDI-MS (matrix-assisted laser desorption/ionization) contribute increasingly to the pinpointing of protein phosphorylation sites [9]. Today, major challenges in phosphoproteomics arise from three main areas:

(i) Due to the low abundance of regulatory active phosphoproteins in combination with a generally substoichiometric phosphorylation, sensitivity is a general challenge for phosphorylation analysis. This underlines the need for efficient enrichment of phosphoproteins [10,11] and/or phosphopeptides [12–15]; (ii) manual control of search-engine generated phosphopeptide hits appears to be mandatory, but guidelines for this task are still emerging [16,17]; (iii) relative and absolute quantification of phosphoproteins and of site-specific phosphorylation is still challenging. Most quantification methods rely on stable-isotope labeled internal standards [18–20] or chemical modifications, but label-free methods have been proposed for this purpose as well [21,22].

Recently, element mass spectrometry was introduced as an alternative tool for spotting of phosphorylation sites and determination of phosphorylation stoichiometries [23,24]. This method is based on hyphenation of capillary liquid chromatography (μLC) with inductively coupled plasma-mass spectrometry (ICP-MS) and was successfully applied both on the peptide and protein level [25,26]. The element-selective ICP-MS detector allows for screening of phosphorus-containing compounds in the LC eluate, and the signal intensity is directly proportional to the phosphorus content. Moreover, simultaneous monitoring of the sulfur trace can serve as an internal measure for protein amount. In case the number of the sulfur-containing amino acids cysteine and methionine is known, the protein phosphorylation stoichiometry can be determined via the ratio phosphorus to sulfur. In a recent report, we could show that this method is not restricted to well-defined single peptides or proteins [27]. The phosphorylation stoichiometry of enzymatically digested protein mixtures can be calculated by integration of all peaks in the phosphorus and sulfur trace, respectively. By this method, a ''global'' phosphorylation state of a cellular cytosolic proteome could be determined for the first time and different protein phosphorylation levels in prokaryotic and eukaryotic samples could be observed.

In the present work, we extended this approach to the analysis of *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* as examples for photosynthetic organisms. Photosynthesis is restricted to plant parts above ground, and growth may vary significantly between different compartments, e.g., resting seeds and roots. Therefore, we analyzed different tissues of *A. thaliana* in search for variations of their proteomic phosphorylation patterns and stoichiometry. In addition, ICP-MS is ideally suited to monitor purification and enrichment procedures of phosphorylated species, which are methods indispensable for pinpointing of modified sites by molecular MS methods such as ESI- or MALDI-MS. As an example, we used the described methodology to monitor the enrichment efficiency of the newly developed MOAC approach [10], which aims at extraction of intact phosphoproteins.

Materials and methods

Materials. Modified trypsin was obtained from Roche Diagnostics. All other chemicals and solvents were obtained from Sigma–Aldrich or Roth in the highest purity available. HPLC-grade water was used throughout. Zip–Tips were obtained from Millipore.

Growing of plants and cell cultures. *Arabidopsis thaliana* plants (ecotype Columbia) were grown in a Percival growth chamber for 5 weeks at 20 °C at a 12-h day/night rhythm. Light intensity was adjusted to 130 mE. *C. reinhardtii* (wildtype CC-125) cells were grown for 7 days in 16/8 h light/dark regime in liquid culture containing TAP Medium [28]. Cells were harvested in the light period and centrifuged for 10 min at 4000 rpm. The supernatant was discarded and the pellet was used for the extraction of proteins. *A. thaliana* cell cultures derived from leaves were grown in JPL medium [29] as described before [30]. Every seven days, 10% of the culture was transferred to a new flask to maintain the cell culture line. Cells were harvested just before transfer (after seven days of growth in new media).

Protein extraction. Plant material was frozen by addition of liquid nitrogen and ground in a pre-chilled mortar. Up to 1.6 g of ground material (leaves, seeds, roots, flowers, or cell cultures) or the pellet from about 100 mL of cell suspension was mixed with 5 mL extraction buffer (50 mM MES, 20 mM imidazole, 60 mM NaF, 0.2 M K–aspartate, and 0.2 M Na–glutamate, pH 6.1) containing freshly added β-mercaptoethanol (1–5%) and 15 mL TE (Tris EDTA, pH 7.5) buffered phenol. The mixture was incubated for 30 min at 4 °C and centrifuged for 8 min at 4000 rpm. The upper phase (containing the proteins) was transferred into a fresh tube, washed once with extraction buffer, and proteins were precipitated out of the phenol phase overnight with five volumes ice-cold acetone.

Phosphoprotein enrichment. MOAC enrichment was performed as previously described [10]. The solution and binding buffer (DB) consists of 0.2 M Na–glutamate, 0.2 M K–aspartate, 30 mM MES, 20 mM imidazole, 0.25% CHAPS, and 8 M urea, and was adjusted to pH 6.1 with HCl prior to use. The elution buffer (EB) consists of 0.1 M K–pyrophosphate and 8 M urea, and was adjusted to pH 9.0 with H_3PO_4 prior to use. Briefly, protein pellets were washed twice with ice-cold methanol, dried for 30 min at room temperature and dissolved in DB using vigorous vortexing for 5 min. 100 μL of each sample was saved for analysis of samples without enrichment. $Al(OH)_3$ (Sigma A-1577) was pre-equilibrated with 1.8 mL DB, centrifuged at 10,000 rpm and the supernatant was discarded. Up to 1 mg protein was loaded onto 80 mg of $Al(OH)_3$. The mixture was rotated at 4 °C for 30 min and centrifuged for 1 min at 10.000 rpm. Five washing steps with 1.8 mL MB each followed by rotation and centrifugation were performed accordingly. Proteins were eluted by incubation with 0.8 mL EB at room temperature, centrifuged at 10.000 rpm for 2 min and precipitated from the resulting supernatants and the initial samples (see above) by methanol/chloroform precipitation.

Tryptic digestion and protein purification. 1D-SDS–PAGE was performed according to standard procedures with commercially available precast gels (NuPAGE gels, Invitrogen) using potentiostatic conditions (120–200 Volt). For purification of protein precipitates from low-molec-

ular impurities, about 40–50 µg total protein lysate was applied per gel lane and electrophoresis was stopped when the front marker had migrated about 2–3 cm (10–15 min). Lanes (about 1 cm × 0.3 cm) were cut out of the gels and digested with trypsin. Reduction with DTT (45 min at 54 °C) and alkylation with iodoacetamide (30 min at room temperature) was applied prior to in-gel digestion at 37 °C over night using modified trypsin (Roche). Proteolytic peptides were extracted 2–3 times with 5% formic acid and desalted with RP-C_{18}-micropipet tips (Zip–Tips, Millipore).

µLC-ICP-MS analysis. Capillary liquid chromatography was performed either with a dual syringe pump (ABI 140B, Applied Biosystems) with external solvent split or a dual piston pump with built-in solvent split (Ultimate, LC-Packings). A 200 µm i.d. column (15 cm length, Grohm) filled with reversed phase C_{18} adsorbens (5 µm particle size, 300 Å pore size, Vydac) was used for on-line gradient LC at a flow rate of 2 µL/min on column. Peptides were separated by a linear gradient from 2% acetonitrile/0.065% TFA to 80% acetonitrile/0.1% TFA within 30 min. The LC column outlet was directly coupled to the ICP-MS via a capillary-based microconcentric nebulizer mounted on a low-volume spray chamber as interface (CEI-100, CETAC). A double-focusing sector-field ICP-MS (Element 2, Thermo Electron) at a resolution of 4000 (medium resolution) was used as element-selective detector. The instrument was tuned via infusion of a multi-element standard with a syringe pump.

Data acquisition and processing. Peak tops (20% mass window) were monitored using the electric scan mode. The scan cycle time was 200–400 ms per run for monitoring ^{31}P and ^{34}S with a duty cycle of nearly 50% per element. Monitoring ^{34}S instead of ^{32}S was preferred, because a slightly better performance in terms of signal-to-noise is usually achieved. Data evaluation was performed after ASCII export to the graphics software Origin, including background subtraction if necessary. Sensitivity factors of ^{31}P and ^{34}S were determined by repeated injection of a mixture of phosphoserine and cysteine followed by subsequent elution with varying solvent composition. The corresponding correction function for the respective gradient was calculated from the experimental values by a polynomial fit. For calculation of the global phosphorylation degree, the integrated area of the ^{34}S trace after sensitivity correction was taken as a measure for the protein content. The obtained intensity value was corrected for natural abundance of ^{34}S and divided by the average number of sulfur atoms per protein. Based on genomic data [31], the abundance of sulfur-containing amino acids in the respective proteome is 4.28% in *A. thaliana* and 4.00% in *C. reinhardtii* (based on codon usage). This is equivalent to 19 sulfur atoms per protein on average for both species, assuming an average protein size of 50 kDa (about 450 amino acids).

Results and discussion

Determination of proteomic phosphorus-to-sulfur ratios by µLC–ICP-MS

In order to obtain a value for a global protein phosphorylation level, some precautions have to be considered. Basically, proteins have to be extracted from the respective tissue sample and separated from interfering substances, predominantly those with phosphorus or sulfur, i.e., oligonucleotides, phospholipids, and small metabolites. The major fraction of these compounds is removed efficiently during the extraction procedure (see Materials and methods for details), but further purification steps were performed in order to ensure their complete removal. The extracted protein mixture was subjected to a short 1D-SDS gel electrophoresis step, whereby impurities migrate with the front and are thus eliminated. The protein-containing gel part is cut into pieces, proteins are in-gel digested with trypsin and the resulting peptides are extracted and desalted using RP-C_{18} micropipet tips.

These samples are then injected onto a reversed phase capillary column and peptides are subsequently eluted by an acetonitrile gradient with an ICP-MS coupled on-line as detector. Monitoring of ^{31}P and ^{34}S allows screening for phosphorylated and sulfur-containing peptides simultaneously. Since a complex peptide mixture is injected, no complete separation of peptides is achieved by one-dimensional chromatography. Instead, peptides elute in a defined retention time window according to their polarity, but separated from residual small phosphorus- and sulfur-contain-

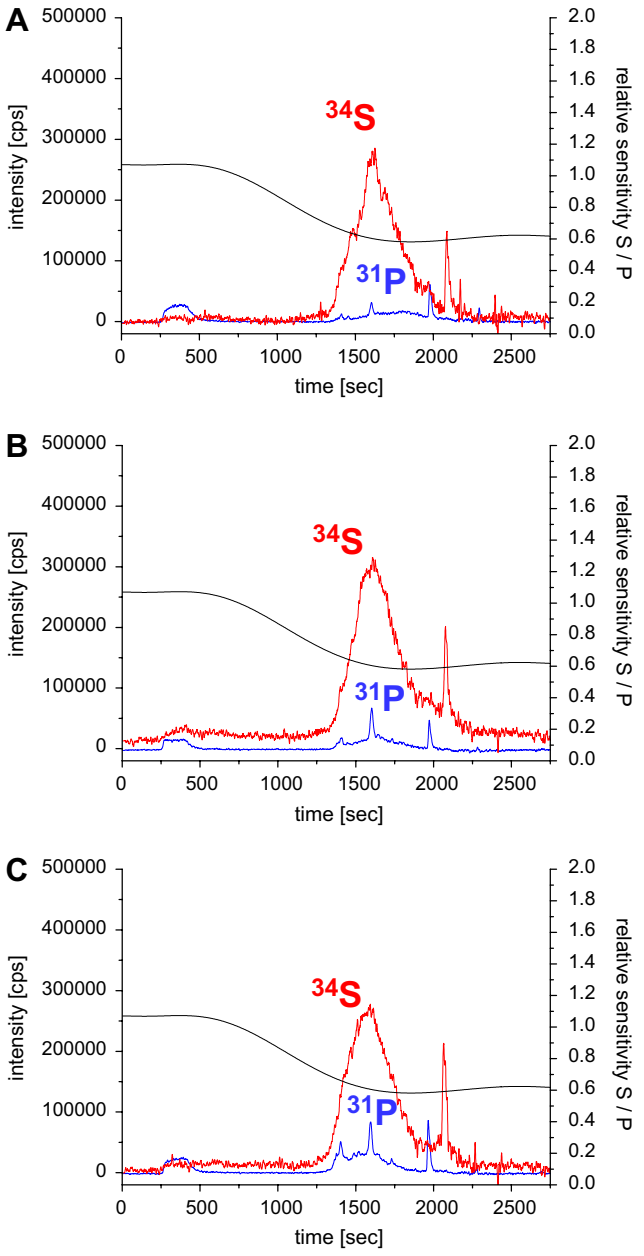

Fig. 1. Analysis of enzymatically digested protein extracts from *A. thaliana* by µLC–ICP-MS with monitoring of ^{31}P and ^{34}S. Samples were collected from different tissues: (A) leaves; (B) flowers; (C) cell cultures.

ing species if present. Fig. 1 shows three example chromatograms of different *A. thaliana* protein extracts, which have been prepared and analyzed as described above. Expectedly, all three samples show similar peak patterns reflecting the origin from the same species. Nevertheless, small but explicit differences between the tissues can be noticed as well.

It is visible at first glance that protein phosphorylation in these plant samples is rather low. Intense sulfur signals (note that ^{34}S has only 4.29% natural abundance) can be observed at retention times typical for peptides (20–40% acetonitrile). In contrast, only weak phosphorus signals, including a few distinct peaks, characterize the ^{31}P trace in the identical retention time window. This is in sharp contrast to protein extracts of mouse cells, where similar abundances of ^{31}P and ^{34}S have been observed [27].

Quantification of cellular protein phosphorylation levels

For quantitative considerations, the element-specific response of ICP-MS has to be corrected. One of the advantageous features of ICP-MS is that the sensitivity for a given element is usually independent of its chemical form and that only a moderate matrix influence has to be considered. The most important matrix effect is caused by different organic loads into the plasma. Consequently, the corresponding element traces have to be corrected for response variations in relation to LC solvent composition. This is achieved by infusion of standards at different solvent compositions and subsequent calculation of a sensitivity function ^{34}S to ^{31}P (included in Figs. 1 and 3), which then is used to calculate the correct molar element ratios in the sample [27].

Table 1 gives the average relative ^{31}P to ^{34}S intensities (median ± mean deviation) after sensitivity correction and the corresponding total P to S ratios for different tissues of *A. thaliana* and *C. reinhardtii* samples. A number of biological replicates (*N*) were analyzed in order to assess sample-to-sample variations and to give a first approximation of the precision of the method.

In order to convert the molar P to S ratios into a stoichiometric protein phosphorylation degree, the mean number of sulfur atoms per protein has to be taken into account.

Table 1
Phosphorylation stoichiometries (ratios of ^{31}P to ^{34}S and P to S) in protein extracts from individual tissues of the plant *A. thaliana* and from the green algae *C. reinhardtii* as determined by μLC–ICP-MS

Organism	Compartment	*N*	Ratio $^{31}P/^{34}S$ (mol/mol)	Ratio P/S (mol/mol)
Arabidopsis thaliana	Leaves	7	0.068 ± 0.010	0.0029 ± 0.0004
	Cell culture	6	0.152 ± 0.050	0.0065 ± 0.0022
	Seeds	5	0.028 ± 0.006	0.0012 ± 0.0002
	Roots	4	0.110 ± 0.015	0.0047 ± 0.0007
	Flowers	4	0.079 ± 0.007	0.0034 ± 0.0003
Chlamydomonas reinhardtii	Cell culture	3	0.306 ± 0.017	0.0131 ± 0.0007

This number was estimated using the corresponding genome data, assuming that the abundance of amino acid codes in the genome is reflected in the respective proteome. It was calculated that proteins with a mean molecular weight of 50 kDa contain on average 19 sulfur-containing amino acids (cysteine and methionine) in both species. Normalization of the sulfur signal intensities by the number of sulfur atoms gives direct access to the protein molarity and thus to the desired global protein phosphorylation degree (see Fig. 2).

The experiments for the first time delivered information about plant protein phosphorylation on a whole organism scale. The quantitative evaluation reveals the overall low protein phosphorylation level in the plant *A. thaliana*, which is in the order of 0.07 mol phosphorus per mol protein (with variations between 0.02 and 0.15). As illustration, this is equivalent to every 15th protein being completely phosphorylated at one site. However, number and stoichiometry of phosphorylation may vary significantly in the protein pool, which is supported by the occurrence of a few distinct abundant peaks in the ^{31}P chromatograms.

The obtained values are slightly higher compared to prokaryotic cells, but considerably lower compared to eukaryotic mouse cells (*Mus musculus* 3T3 cells), where nearly 0.7 mol phosphorus per mol protein was found [27]. Since proteins are heated before SDS–PAGE and acidic conditions are employed during analysis, the observed phosphorus content may be caused exclusively by heat- and acid-stable *O*-phosphorylation at serine, threonine, and tyrosine. In contrast, labile phosphorylation sites at aspartate and histidine, which play a major role

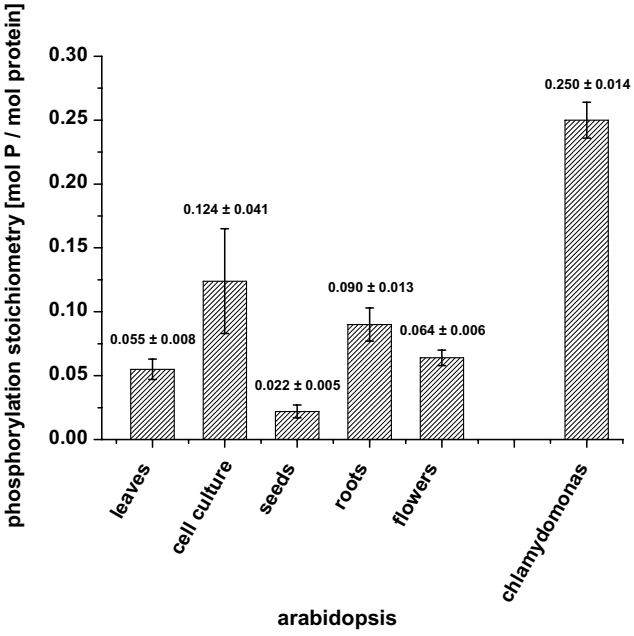

Fig. 2. Average global protein phosphorylation stoichiometry (median ± mean deviation) as obtained by μLC–ICP-MS for different tissues from *A. thaliana* and for *C. reinhardtii*.

in prokaryotes (phosphorelays) [32], are probably not detected. The low phosphorus signal in *C. glutamicum* [27] is in accordance with this assumption and may reflect different utilization of signaling cascades in prokaryotes and eukaryotes. The comparably low phosphorylation level detected in *A. thaliana* may, therefore, point to a lower extent of *O*-phosphorylation in plants versus mammals. The extent of labile phosphorylation sites in plants remains unclear, but a number of plant-typical processes such as plant hormone response and circadian rhythms have been found to be regulated by phosphorelays employing histidine kinase pathways [33]. Interestingly, protein samples from the green algae *C. reinhardtii* showed an intermediate protein phosphorylation degree between the values for the plant *A. thaliana* and for mouse cell culture.

It is instructive to compare our results with recent claims that about 30% of eukaryotic proteins are phosphorylated [1,3]. Our data for a variety of tissue types are in the same order of magnitude. This suggests that a certain phosphorylation level is indeed crucial throughout different kingdoms of life. However, the significant variations of at least one order of magnitude point to the fact that this level may be adapted to the needs of the respective life form, compartments (see next chapter) and environment.

Tissue-dependent variations and their implications

Besides signaling, metabolism is a major cell function depending on protein phosphorylation. For instance, growth rates of cells should influence the respective protein phosphorylation level. No variations between different growth conditions (logarithmic and stationary phase) were found in the bacterium *C. glutamicum* (data not shown), but the phosphorylation level of 0.02 mol P per mol protein might be too low to observe significant differences. However, varying growth rates and metabolic activities may occur in different tissues of higher organisms. Such variations were indeed found in protein extracts of different *A. thaliana* tissues (see Fig. 2). Interestingly, these variations in the global phosphorylation degree give insights into the importance protein phosphorylation might have during different developmental stages.

Significantly lower phosphorylation than in all other tissues was found in seeds, in accordance with their minimized metabolism before germination. Nevertheless, the low degree for seed tissue surprises because of the necessity for nutrient storage. Apparently, the stored phosphorus is not bound to proteins to a large extent in seeds. Indeed, it is known that the major source of phosphorus in seeds is phytic acid [34], which probably explains why plants do not have to rely on protein-bound phosphorus for seed germination.

Phosphorylation varies only slightly between samples from leaves, flowers, and roots. Besides a somewhat elevated level in root samples, plant parts above and below ground show no obvious and statistically firm differences, which might be directly correlated to photosynthetic or phytochromic activity. Many enzymes in the photosynthesis chain are membrane-bound and are probably not completely covered by the employed protein extraction method. Therefore, photosynthesis-related phosphoproteins might be underrepresented. However, it is not clear whether these enzymes contribute to a significant extent to the overall phosphoprotein pool. It might be that the vast majority of phosphate is bound to proteins involved in signaling and metabolic pathways, e.g., respiration. These mechanisms are very similar in most eukaryotic organisms.

The highest phosphorylation level in *A. thaliana* was observed in cell cultures, which may reflect the generally increased metabolism of cultured cells. It appears to be consistent with findings about activation of kinases during mitosis that rapidly dividing tissue has a higher degree of phosphorylation, which could hold for the slightly increased values in root samples as well. Interestingly, the cell culture data of *A. thaliana* showed noticeable higher sample-to-sample variations (ranging from 0.08 to 0.25 mol P per mol protein) in comparison to the plant-derived samples. Surprisingly, such pronounced variations were not found in case of *C. reinhardtii* cell cultures.

Compared to *A. thaliana* the green algae *C. reinhardtii* showed higher phosphorylation levels of about 0.25 mol phosphorus per mol protein. While *A. thaliana* cell cultures are immotile, *C. reinhardtii* has a flagellum and is motile. Since flagellar dependent movement of prokaryotes as well as of eukaryotes seems to be inherently linked to protein phosphorylation [35,36], this may explain the higher degree of phosphorylation in *C. reinhardtii* compared to *A. thaliana* cell cultures. This assumption is supported by the fact that seven flagellar proteins could be identified after MOAC enrichment and that a very recent study on protein phosphorylation in *C. reinhardtii* shares the impression that protein phosphorylation is quite abundant in flagella of *C. reinhardtii* [37].

Monitoring of phosphoprotein enrichment efficiency

Enrichment of phosphorylated compounds is usually inevitable to safely identify phosphorylation sites, especially in case of low phosphorylation stoichiometries or proteins with low copy numbers. Whereas several protocols exist and are applied to phosphopeptide enrichment, enrichment on the protein level is still rarely described in the literature. A novel method termed MOAC (metal oxide affinity chromatography) employs aluminum hydroxide as solid phase, and the method was successfully applied to identify phosphorylation sites [10,11]. Enrichment of phosphoproteins was monitored by a phosphoprotein-specific staining procedure, which proved the efficiency. However, quantitative evaluation of results obtained by staining methods is limited, since these exhibit a limited dynamic range and moderate reproducibility. In contrast, μLC–ICP-MS with phosphorus to sulfur

monitoring is much better suited for measuring the enrichment efficiency, due to its wide linear dynamic range and due to the ratio measurement, which should not be affected by sample preparation. For that reason, we analyzed samples from *A. thaliana* and *C. reinhardtii* by μLC–ICP-MS before and after enrichment with MOAC (see Fig. 3).

The ICP measurements confirmed an enrichment of phosphorylated proteins by the MOAC procedure. This is evident from a comparison of the ^{34}S trace to the ^{31}P trace in Fig. 3. After enrichment (Fig. 3B and D) the area under the ^{31}P trace relative to the ^{34}S trace is considerably higher than before enrichment (Fig. 3A and C), strongly indicating an increase of peptide bound phosphate. It was possible to calculate an enrichment factor by comparing the molar P/S ratio before and after enrichment for the samples. The enrichment factor was calculated by dividing the molar P/S ratio after enrichment by the initial P/S ratio. This factor was found to vary between about 2 and 20, leading to a final degree of phosphorylation between 0.4 and 1.0 mol phosphorus per mol protein in the enriched fraction (see Table 2). In a simplified picture, this means that 40–100% of the enriched proteins are phosphorylated completely at one site.

The enrichment factor varies between tissue types and organisms, which indicates the presence of phosphopro-

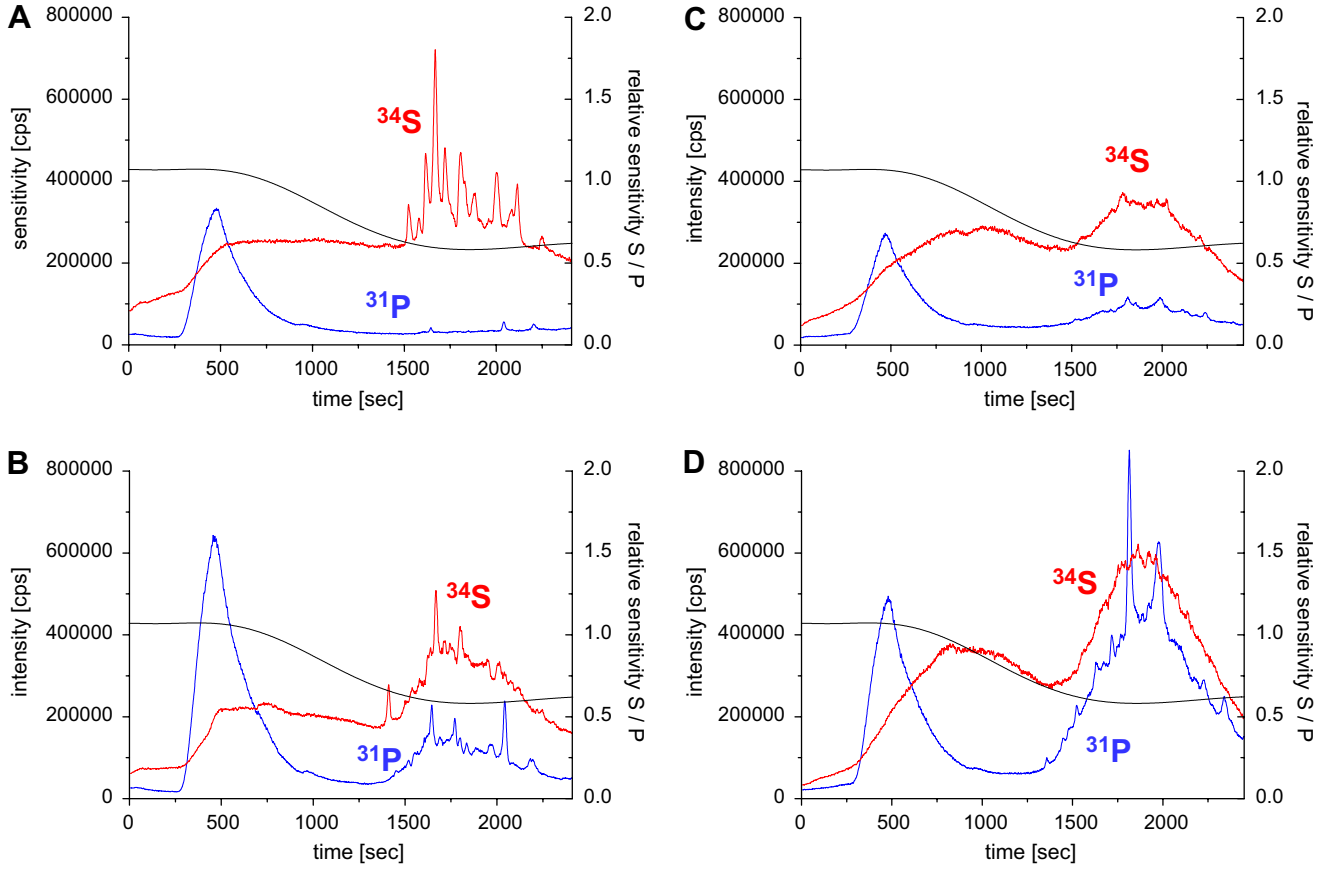

Fig. 3. μLC–ICP-MS analysis of digested protein extracts before and after phosphoprotein enrichment by metal oxide affinity chromatography (MOAC). Left row: *A. thaliana* seeds (A) before and (B) after MOAC enrichment; right row: *C. reinhardtii* (C) before and (D) after MOAC enrichment.

Table 2
Protein phosphorylation stoichiometries of selected *A. thaliana* and *C. reinhardtii* samples before and after phosphoprotein enrichment with aluminum hydroxide (MOAC) and corresponding enrichment factors

Organism	Compartment	Ratio before enrichment (mol/mol)		Ratio after enrichment (mol/mol)		Enrichment factor
		P/S	P/protein	P/S	P/protein	
Arabidopsis thaliana	Leaves	0.0040	0.075	0.0221	0.42	5.5
	Cell culture	0.0076	0.145	0.0321	0.610	4.2
		0.0133	0.253	0.0301	0.572	2.3
	Seeds	0.0010	0.019	0.0228	0.433	22.8
		0.0011	0.021	0.0228	0.434	20.7
Chlamydomonas reinhardtii	Cell culture	0.0141	0.267	0.0527	1.000	3.7

tein-specific and mixture-specific effects. Thus the main benefit of the MOAC procedure is the support of pinpointing of unknown phosphorylation sites. In conclusion, the demonstrated monitoring of the MOAC procedure by μLC–ICP-MS allows confirmation and monitoring of phosphoprotein enrichment, which gives an independent success control and is helpful for further method development intended to improve the applied protocols.

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