

Identification of SUMO target by a novel proteomic approach in plants

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Abstract

Post-translational modifications (PTMs) alter chemically and physically the properties of proteins, including their folding, subcellular localization, stability, activity, and consequently their function. In spite of their relevance, studies on PTMs in plants have been rather limited. Protein modification with SUMO regulates several biological processes by affecting protein-protein interactions or changing the subcellular localizations of the target proteins. Here, we described a novel proteomic approach that combines two-dimensional liquid chromatography, immuno-detection and MS analyses, to identify SUMO targets in response to heat shock. By means of a sumoylation bacterial in vivo system, we validate that some of the targets identified are, in fact, labeled with SUMO.

Key words: mass spectrometry, plants, post-translational modification, proteomics, SUMO.

Introduction

Recent advances in sequencing technologies made available an increasing number of completed plant genomes. Attention now is driven towards addressing how information contained within DNA sequence databases can be used to elucidate the structure, function and control of biological systems. In the last years, following the availability of genomic sequences, many gene expression studies have been performed. However, it is becoming evident that analyses at the protein level are also needed to better understand biological processes. The relationship between protein amount and transcript level is not linear, thus quantitative proteome analysis has been proposed as alternative or, at least, complementary method to study gene expression at steady state or after environmental changes. An additional consideration when dynamic proteomes are studied, it is the fact that new protein isoforms can be generated by post-translational modifications (PTMs). These modifications alter protein properties, function, stability, subcellular localization or the ability to interact with other proteins or nucleic acids (Krueger *et al*, 2006). In general, the majority of the PTMs are reversible, producing equilibrium between the addition and removal of the modifying groups/peptides (Seo *et al*, 2004). However, in most cases, the equilibrium is clearly displaced toward the non-modified isoforms. This stoichiometric displacement makes PTMs, and also their targets, difficult to detect and to identify.

The Ubiquitin System is one of the major protein-modification systems required for the highly selective degradation of specific proteins in eukaryotic cells. In plants, the number of processes regulated by the ubiquitin pathway has increased very rapidly, including germination, flowering, cell division or response to environmental stresses (Seo *et al*, 2004; Smalle *et al*, 2004; Lechner *et al*, 2006; Stone *et al*, 2007; Dreher *et al*, 2007; Jurado *et al*, 2008). In an effort to identify ubiquitin targets, recent proteomic analyses have reported that several hundred proteins are likely targets of ubiquitin (Maor *et al*, 2007; Manzano *et al*, 2008). However, considering the very large number of E3 ubiquitin ligases identified in plants (Smalle *et al*, 2004; Jain *et al*, 2007), it is

reasonable to speculate that the number of target proteins would be much higher than the number reported, likely under different plant responses to environmental changes. Other proteins, called ubiquitin-like proteins, also modulate protein function in a reversible post-translational modification, but have been involved in several cellular regulatory pathways and responses to stress rather than to protein degradation. One of these ubiquitin-like proteins is SUMO (Small Ubiquitin-Related Modifier). Protein modification with SUMO (sumoylation) is implicated in a variety of cellular processes, including signal transduction, cell cycle, transcription regulation and DNA repair (Bossis *et al*, 2006). Proteomic approaches have been used to identify sumoylated substrates by purification of SUMO conjugates from *Saccharomyces cerevisiae* (Denison *et al*, 2005) or mammalian cell lines (Rosas-Acosta *et al*, 2005). In plants, the SUMO pathway plays an important role in controlling development and response to external stimuli (Downes *et al*, 2005; Hay *et al*, 2005; Miura *et al*, 2007; Catalá *et al*, 2007; Miura *et al*, 2009). Recent works using a proteomic analysis (Miller *et al*, 2010) or a two-hybrid screening (Elrouby *et al*, 2010) have reported the identification of several SUMO targets, some of them being validated in a bacterial in vivo system (Elrouby *et al*, 2010).

Here, we describe a novel proteomic strategy to identify proteins containing SUMO conjugates. This method is able to detect low abundant proteins modified with SUMO and allows comparative analyses of sumoylation under different conditions. In addition, it is also suitable to identify other PTMs, such as ubiquitination or phosphorylation. This approach combines entire proteome fractionation by two-dimensional liquid chromatography and immunoblotting detection of PTMs using specific antibodies, what increased the sensitivity of the method. The immuno-detected proteins were identified by MS analysis, and SUMO targets were validated by using a bacterial sumoylation system.

Experimental procedures

Chemicals

Urea, thiourea, sodium chloride, iminodiacetic acid, Tris, glycerol, SB3-10 [3-(Decyldimethyl-amonio) propanesulfonate inner salt], methanol G, 2-propanol, ammonium hydroxide, phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail, iodoacetamide, DTT, hexylene glycol, PIPES, Percoll, Miracloth, sucrose, ammonium bicarbonate and trypsin were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from J.T. Baker (Deventer, Holland). microBCA Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). N-octylglucoside was obtained from Melford (Suffolk, U.K.). 1,1,2-trichlorodifluoroethane was purchased from Panreac (Barcelona, Spain). PD-10 desalting columns were purchased from GE Healthcare Bio-Science (Uppsala, Sweden). CF start buffer (SB) and CF eluent buffer (EB) were commercialized by Beckman Coulter (Fullerton, CA, USA). The anti-phosphothreonine (clone H2), and anti-ubiquitin (clone P4D1) antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA), the anti-HA (clone 3F10) from Roche (Indianapolis, IN, USA), the anti-histidine Clontech and anti-SUMO from Santa Cruz. The chemiluminescence substrate and the ZipTipC18 columns were purchased from Millipore.

Plant material

We used *Arabidopsis thaliana* plants, Columbia-0 ecotype. Plants were grown in a controlled chamber at 22°C with a photoperiod of 16 hours light and 8 hours dark, on solid MG medium (MS salts plus 1% sucrose, 10 mM MES, pH=5.7 and 1% of plant Agar (Duchefa, The Netherlands). To generate transgenic plants overexpressing HA-SUMO (HA-SUMO^{OE}), the *SUMO1* (At4g26840) cDNA clone U17495 was obtained from The Arabidopsis Biological Resource Center (ABRC) and cloned into the binary vector pBA002, which contains a 35S promoter and 3 copies of the HA epitope (Kost *et al*, 1998). The SUMO coding region was fused in frame with the 3xHA epitope at the N-

terminus. Transformation of Arabidopsis was performed following the floral dip method (Clough *et al*, 1998).

Nuclei and chloroplast isolation and western-blot analyses

Arabidopsis nuclei were isolated from 10 day-old control and heat-shocked (30 min at 42°C) HA-SUMO^{OE} plants by the Percoll gradient method (Folta *et al*, 2000).

To analyze the presence of sumoylated or ubiquitinated proteins in chloroplasts, we purified these organelles. We used 20-day-old leaves from wild type or HA-SUMO^{OE} plants since the yield of isolated chloroplasts was higher and of better quality than those from 6-day-old seedlings. The leaves were chopped with a blade in Xpl buffer (3% sorbitol, 50 mM HEPES pH=7.5, 1 mM MgCl₂, 2mM EDTA, 2,5 ug BSA/ml, 5 mM sodium ascorbate) and incubated for 5 minutes on ice. Next, the homogenate was filtered through two layers of Miracloth. Intact chloroplasts were isolated as described by Weigel and Glazebrook, 2002, and total protein was extracted by adding 200 µl SDS-loading buffer containing 5% of β-ME and boiling them for 10 minutes.

Proteins were separated by SDS-PAGE and then transferred to PVDF membranes, which were analyzed by immunoblotting using antibodies against HA (to detect SUMO) or against ubiquitin.

Protein sample preparations and two-dimensional liquid chromatography analysis

Total protein was extracted from Arabidopsis seedlings or from nuclei preparations. Extraction was carried out in general lysis-denaturing buffer (6 M urea, 2 M thiourea, 10% glycerol (v/v), 50 mM Tris-HCl pH= 7.8), 2% (w/v) n-octylglucoside), containing 1x plant inhibitor cocktail and 1 mM PMSF. To extract proteins, 4 ml of general lysis-denaturing buffer were added to approximately 3 g of grounded plant materials. The solution was vortexed and incubated on ice for 30 minutes. Afterwards, it was centrifuged at 16.500 g at 4°C for 15 min and the supernatant transferred to a fresh tube. To further extract non-soluble proteins, the precipitated material was

resuspended in 2 ml of membrane lysis-denaturing buffer (general lysis-denaturing buffer plus 2.5% (w/v) SB3-10, 1x plant inhibitors cocktail and 1 mM PMSF). The mixture was vortexed, incubated on ice for 15 min, and centrifuged again at 16.500 g at 4°C for 15 min. The supernatant was combined with the first one and passed through a 0.2 µM sterile filter. The extracts were desalted and equilibrated with CF start buffer in a PD-10 desalting column following the manufacturer's instructions. Protein quantification was performed using the microBCA protein assay kit with different concentrations of BSA protein diluted in CF start buffer as standards.

Protein extracts (2.5 mg of protein) from Arabidopsis wild type or HA-SUMO^{OE} plants were subjected to 2D-LC analysis using the ProteomeLab PF2DTM instrument (Beckman-Coulter) and the procedure recommended by the manufacturer. The first dimension separation was carried out by chromatofocusing (CF) on a HPCF-1D column (250mm x 2.1 mm internal diameter, 300 amstrong pore size). The column was equilibrated at pH=8.5 with CF start buffer for 250 min at 0.2 ml/min. The pH gradient began after 20 min of sample injection when the CF eluent buffer at pH=4.0 moved through the column, decreasing the pH gradually from 8.5 to 4.0. Proteins were eluted according to their isoelectric points (pI) and, in the final step, the most acidic ones were eluted with 1 M NaCl, 0.2% n-octylglucoside. All fractions were collected in 96-wells plates using an automated collector.

All the different pH fractions collected from the first dimension were resolved on a Reverse Phase (RP) C18 column (HPRP column: 4.66 mm x 3.3 mm, 1.5 µm particle size). Two hundred µl of each fraction were run through the column in solvent A (0.1% v/v trifluoroacetic acid (TFA) in water) and then the proteins were eluted with a linear gradient (0-100%) of solvent B (0.08% v/v TFA in acetonitrile (ACN)) for 35 min. Separation was performed at 0.75 ml/min and the temperature column was maintained at 50°C. Eluted proteins were monitored by UV at 214 nm of absorbance. The different fractions of the first dimensions were collected in twelve 96-wells plates using an automated collector.

All chromatofocusing profiles were elaborated and compared by using the 32 Karat software (Beckman-Coulter). Quantitative analysis of the protein peak areas and heights were performed using the Mapping tools software (Beckman-Coulter).

Immunodetection assays

The complete proteome of Arabidopsis was collected in 96-microwell plates as described above and then transferred onto PVDF membranes (Millipore) by dot-blot. Membranes were blocked with 5% BSA in PBS buffer for 1 hour at RT and then incubated for 2 hours with monoclonal antibodies against phospho-threonine 1:500, against HA at 1:5000 to detect HA-SUMO, or against ubiquitin at 1:1.000 dilution in PBS 0.1% Tween-20. Afterwards, these membranes were washed 4 times in PBS 0.1% Tween-20 and, finally, incubated with anti-mouse IgG1 peroxidase-conjugated antibody or anti-rat (1:50.000 dilution in PBS 0.1% Tween-20). Detection of IgG-binding components was carried out by enhanced chemiluminescence (Millipore).

MS analysis

To identify Arabidopsis proteins modified by sumoylation, ubiquitination or phosphorylation, immunoreactive fractions were analyzed by MS. Eluted fractions were evaporated to a final volume of 10 μ l. Protein digestions were carried out by incubating the samples in 50 mM NH_4HCO_3 and 10 mM DTT at 60°C for 1 hour. The alkylation of the reduced sulfhydryl groups was performed by adding 55 mM iodoacetamide at 25°C for 30 min in the dark. Proteins were digested by adding 1.5 μ l of trypsin (125 μ g/ml) and incubating at 37°C overnight. The reaction was stopped with 1% of formic acid. Tryptic peptides were desalted and concentrated with ZipTipC18 columns according to the manufacturer's recommendation. Peptides were eluted in 0.1% TFA, 50% ACN for MALDI-TOF MS analysis, and with 1% formic acid, 50% methanol for electrospray MS analysis. To increase salt removal, samples were washed with 3-5 cycles of 0.1% TFA as wash solution. The solution was spotted directly onto a MALDI target and analyzed

by MALDI-TOF/TOF off-line coupled LC/MALDI-MS/MS. Mass spectrometry analysis were performed automatically with a 4700 Analyzer MALDI-TOF/TOF instrument (Applied Biosystems, CA USA). First, MS spectra of all spotted fractions were acquired in the positive reflector mode for peak selection (S/N>20, excluded precursor with 200 resolution) and further MS/MS spectra acquisition using the CID of selected peaks. The search of filtered peptides was performed in batch mode using GPS Explorer v3.5 software with a licensed version of MASCOT, in Swiss-Prot database. The MASCOT search parameters were: (1) species: *Arabidopsis thaliana*; (2) allowed number of missed cleavages (only for trypsin digestion): 1; (3) considered modifications: Cys as carboamidomethyl derivate and Met as oxidized methionine; (4) peptide tolerance: ± 150 ppm; (5) MS/MS tolerance: ± 0.4 Da, and (6) peptide charge: +1.

In vivo sumoylation in Escherichia coli system

To validate de sumoylated proteins that have been identified by MS analyses, we used the *E. coli* system that contains all components of the pathway (Mencia and de Lorenzo, 2005). The identified cDNAs were cloned into pET28b plasmid by restrictions enzymes, and verified by sequencing. We transformed all the plasmids, protein targets (pET28b-At3g50820, pET28b-At4g20260, pET28b-At3g18100, pET28b-At3g16420, pET28b-At1g22270), and system components (pBADE12 and pHRSUMO plasmids) into BL21(DE3) Rosetta competent cells. Protein expression was induced by adding IPTG at 0.5 mM to 50 ml of the cultures for 2 hours at 37°C and 15 hours at 30°C. In this system, human SUMO-1, Aos1 and Uba2, and mouse Ubc9 proteins are fused with a 6xHistidine tag in a polycistronic expression vector driven by a T7 promoter. Therefore, to analyze whether the target proteins are modified with SUMO, first we extracted total protein from bacteria and carried out histidine purification. Then, purified proteins were analyzed by Western blotting with anti-SUMO antibodies (Santa Cruz Inc) at 1:2000 dilution in PBS 0.1% Tween-20. Afterwards, these membranes were washed 4 times in PBS 0.1% Tween-20 and, finally, incubated with anti-rabbit IgG

peroxidase-conjugated antibody (1:25.000 dilution in PBS 0.1% Tween-20). Detection of IgG-binding components was carried out by enhanced chemiluminescence (Millipore).

Results and Discussion

Proteome separation by two-dimensional liquid chromatography

Post-translational modifications are known to affect protein functions. Although they have been subject of investigations for many years, the number of known proteins that are post-translationally modified is still limited. This is mainly due to the fact that PTMs are dynamic, reversible, have a fast turnover, and the stoichiometry of the modified proteins is low relative to the non-modified ones. In addition, the modifications can be removed during the protein purification process, making the identification of proteins containing PTMs difficult. To overcome some of these limitations and to uncover low abundant plant proteins that have been post-translationally modified, we have developed a new proteomic approach that combines: 1) protein extraction in denaturing conditions; 2) complete proteome fractioning by 2D-liquid chromatography using a high capacity system; and 3) a sensitive immuno-detection of PTMs (Supplemental Figure 1). This methodology has several advantages compared to the methodologies so far described since a large amount of total protein extract (up to 7 mg) can be loaded into the 2D-liquid chromatography system and the results are highly reproducible between different experiments. This reproducibility allows comparing protein profiles from different experiments with a high degree of confidence. In addition, PTMs are detected by immunoblotting, a highly sensitive technique allowing the identification of low abundance proteins containing specific PTMs. The immuno-detected proteins are identified by mass spectrometry (MS) or liquid chromatography MS-MS, and the peptide mass fingerprints used for searching proteins in databases using the MASCOT search-engine.

We have used the above described methodology to identify primarily proteins modified with SUMO, but the method is also suitable to identify other protein modifications such as ubiquitination or phosphorylation (see below).

Identification of SUMO targets

SUMO modification plays an important role in plant development as well as in plant response to different types of stress (Downes *et al*, 2005; Hay *et al*, 2005; Miura *et al*, 2007; Catalá *et al*, 2007; Miura *et al*, 2009; Miller *et al*, 2010; Elrouby *et al*, 2010). In this work, we generated Arabidopsis transgenic plants overexpressing SUMO1 fused to the HA tag and HA-SUMO conjugated proteins were detected by immunoblotting (Figure 1A). Total protein extracts from SUMO1^{OE} plants were fractionated by a 2D liquid chromatography (see methods) and fractionated proteins were transferred to PVDF membranes and analyzed by immunoblotting using the anti-HA antibody (Figure 2). To determine the identity of the proteins contained in the immuno-reactive fractions, some of them were analyzed by MS or, when indicated, by Liquid Chromatography MS-MS. These mass fingerprints allowed us to identify several putative SUMO targets (Table 1). In this work, we focused in showing the validity of this approach, rather than in the massive identification of SUMO modified proteins. To establish whether the identified proteins contained the sumoylation consensus sequence ψ -K-x-D/E (lysine is the modified amino acid, ψ a hydrophobic residue, and x any amino acid), their sequences were analyzed by using the SUMOplotTM program (<http://www.abgent.com/doc/sumoplot>). As shown in Table 1, the majority of the identified proteins showed a high score sumoylation consensus site. Proteomic analyses in yeast and mammals have uncovered a large number of transcription factors and DNA associated proteins that might be targets of the SUMO pathway, suggesting that sumoylation plays important roles in regulating DNA transcription (Denison *et al*, 2005; Rosas-Acosta *et al*, 2005; Miller *et al*, 2010). In Arabidopsis, PHR1 and ICE1, a MYB and a MYB-like transcription factors involved in phosphate

starvation and cold-acclimation responses respectively, are sumoylated by SIZ1 (Miura *et al*, 2005; Miura *et al*, 2007). Interestingly, we have identified the transcription factors MYB4R1 and a MAD box protein as candidates of sumoylation.

Unexpectedly, one of the identified candidate of sumoylation with high score corresponded to a chloroplast localized proteins (PSBO-2/PSBO1). Since the SUMO pathway machinery has not been described in chloroplast, we decided to analyze whether SUMO-modified protein are localized in these organelles. We purified chloroplasts from HA-SUMO^{OE} plants and their proteins were analyzed for the presence of HA-SUMO by immunoblotting with the anti-HA antibody. As shown in Figure 1B, chloroplasts contained proteins modified with SUMO. Furthermore, the molecular mass of the major protein identified by immunoblotting (40 KDa) is similar to the estimated molecular mass for the mature PSBO-1/2 proteins (35 KDa), suggesting that these proteins might be SUMO targets. Although it is possible that the chloroplast-enriched sample contain some cytoplasmatic, or other organelle, contamination, we found a noticeable increased of the 40 KDa protein immuno-signal respect to the free SUMO signal or other modified proteins in the chloroplast fraction (Figure 1B, lanes 3 and 4). This suggests that this 34 KDa SUMO-modified protein is located in chloroplasts. Since the sumoylation machinery has not been located in chloroplasts, it is most likely that these chloroplast proteins are modified with SUMO as precursors before being imported into this organelle. Recently, it has been reported several chloroplastic proteins as SUMO targets and some of them were shown to be modified in a bacterial system, but PSBO-1/2 proteins were not identified in this work (Elrouby *et al*, 2010). A large number of mitochondrial SUMO targets has also been identified (Braschi *et al*, 2009). Taken together, these data indicate that the SUMO pathway might function in other subcellular organelles besides the nucleus.

It was described that SUMO conjugates rapidly accumulated in response to heat stress in the nucleus (Saracco *et al*, 2007). For this reason, we decided to carry out comparative analyses of SUMO targets in heat shock treated (42°C for 30 minutes)

versus control plants. In this case, we performed nuclei isolation in order to enrich nuclear proteins. By using anti-HA antibody, we detected higher amount of HA-SUMO-modified proteins, in treated than in non-treated plants (Figure 3A). These two nuclear-enriched proteomes were fractionated and analyzed as described above. First, we analyzed the first dimension fractions of both samples by immuno-dot blot. Comparing the non-treated and treated nuclear enriched proteomes, we identified several differential spots that were specifically recognized by the anti-HA antibody (Figure 3B). We selected the fraction 30 for further fractionation using the HPRP-2D column. These 2D-fractions were further analyzed by immuno-dot blot using the anti-HA antibody (Figure 3C). The immune-reactive spots were analyzed by LC-HPLC MS/MS in order to identify the target proteins. The fingerprint search allowed us to identify two proteins PBP1 and GIGANTEA (GI) as SUMO targets in response to heat shock. PBP1, which is a SUMO conjugate in human cells (Vertegaal *et al*, 2006), has been recently identified as SUMO target in response to heat and H₂O₂-treatment in Arabidopsis (Miller *et al*, 2010). Arabidopsis PBP1 is a carbohydrate-binding protein (lectin), which may act as a chaperone that facilitates the correct polymerization of the PYK10 protein, a beta-glucosidase located in the ER bodies, when tissues are damaged. Using a bacterial sumoylation system we also showed that Arabidopsis PBP1 is modified with SUMO (Figure 4).

As commented above, we identified GI as SUMO target with high score. SUMO-plot analyses revealed that this protein contained two possible sites of SUMOylation. We cloned GI in two halves, each containing one site (GI-Nt and GI-Ct). Using the bacterial sumoylation system, we found that only the GI-Ct is modified with SUMO, indicating that the site located in the N-terminal part of the protein is not functional (Figure 4). Photoperiodic-pathway genes *GI*, *CONSTANTS* (*CO*) and *FLOWERING LOCUS T* (*FT*) promote transition from vegetative stage to flowering in response to a long-day photoperiod. Sumoylation-desumoylation in plants has been reported to be involved in flowering-time regulation. Several examples indicate that

SUMO homeostasis is important for flowering time regulation. For example, EARLY SHORT DAY FLOWERING 4 (ESD4) is a SUMO protease that negatively regulates transition to flowering (Murtas *et al*, 2003; Xu *et al*, 2007); SUMO E3 ligase SIZ1 is a negative regulator of transition to flowering that promotes *FLOWERING LOCUS C* (*FLC*) expression by repressing FLD activity through sumoylation (Jin *et al*, 2008). Our data indicates that GI, a component the flowering pathway, is also regulated by SUMO attachment. In addition, genetic and biochemical analyses have shown that SIZ1 is one of the most important E3 ligases of SUMO. SIZ1 is involved in phosphate starvation (Miura *et al*, 2005), cold acclimation (Miura *et al*, 2007), heat shock tolerance (Yoo *et al*, 2006), drought response (Catalá *et al*, 2007) and ABA signaling (Miura *et al*, 2009).

Sumoylation plays an important role in many plant responses to several stimuli and development. Recently, it have been reported the identification of several possible SUMO targets in Arabidopsis by affinity enrichment of sumoylated proteins combined with tandem MS analyses (Miller *et al*, 2010). In addition to this work, using a two-hybrid screening with ESD4, a SUMO protease, or SCE, a SUMO E2 conjugating enzyme, over 200 possible SUMO targets have been identified (Elrouby *et al*, 2010). The proteomic approach presented in this work is complementary to these works and may contribute to uncover new SUMO targets. Our approach allows a fine-tune comparative analyses of SUMO modification in different treatments/ stimuli/mutants that can be combined with other PTMs analyses, such as phosphorylation or ubiquitination, in the same experiment (see below).

Identification of phosphorylated and ubiquitinated proteins

A main advantage of the method described here is its suitability to study several types of PTMs *in vivo*. To evaluate its potential, the dot-blot membranes were also incubated with monoclonal IgGs against phospho-treonine or ubiquitin. Phosphorylation regulates many signaling pathways *in vivo*. However, taking into consideration the large number of kinases predicted in the Arabidopsis genome (Champion *et al*, 2004),

the number of phosphoproteins identified is lower than expected. This is likely due to the low stoichiometry of phosphorylated proteins *in vivo* and the fast turnover of the modification. Several proteomic approaches have been taken to identify phosphoproteins in plants. For instance, using immobilized metal affinity chromatography (IMAC) combined with LC-MS/MS more than 300 phospho-proteins were identified in Arabidopsis (Nühse et al, 2004) and rice (Whiterman *et al*, 2008a, 2008b). Recently, unique phosphorylation sites from 1346 proteins were identified by using protein extracts from Arabidopsis cultured cells and complementary phosphopeptide enrichment techniques coupled with mass spectrometry (Sugiyama *et al*, 2008). The method presented here can be used to identify low abundant proteins that can be phosphorylated in specific residues. In this work, we have only analyzed fractions 1 and 2 from the first dimension and we have found several spots that showed cross-reaction with a phospho-threonine antibody. Some of these immune-reactive fractions were analyzed by MS and the fingerprints obtained allowed us to disclose the proteins contained in these fractions (Table 2). The NetPhos2.0 program (<http://www.cbs.dtu.dk/services/NetPhos>) revealed, with a high score, several threonine residues that may be the phosphorylation sites (Table 2). Interestingly, one of the identified proteins, CRUCIFERIN3 (CER3), has already been described as a phospho-protein (Sugiyama *et al*, 2008), confirming the validity of our method. Since CER3 is a seed protein, the reason why we detected in our experiment using 7 day-old seedlings, is that very likely seeds were also pulled together with the seedlings. The other identified proteins have not yet been reported as phosphorylated, suggesting that our method is suitable to uncover new phosphorylation targets.

We have also used the method described here to identify ubiquitinated proteins (Table 3). In this case, to validate the system, some anti-ubiquitin immunoreactive spots were analyzed by MS and putative ubiquitinated proteins were identified. As in the case of SUMO targets, we have identified PSBO1 and PSBO2 proteins as ubiquitinated targets. To establish whether or not chloroplast proteins are ubiquitinated,

we isolated them and analyzed them by immunoblotting. Figure 1C shows that we were able to detect ubiquitinated proteins into the chloroplasts. As in the case of the SUMO pathway, the ubiquitination machinery has not been identified inside the chloroplast, therefore it is likely that ubiquitination of chloroplast precursor proteins take place in the cytosol prior to be translocated into these organelles. It has been described that chloroplast proteases are regulated by ubiquitination, but this regulation seems to occur in the cytoplasm (Shen *et al*, 2007a; 2007b). Interestingly, we found an ubiquitinated protein that migrated to a similar molecular mass (34 KDa) as the sumoylated protein described above (Figure 1B), suggesting that they might be the same protein but modified by two different tags. There are several examples where sumoylation acts antagonistically to ubiquitination by blocking the ubiquitin attachment sites. This competition is implicated in regulating gene transcription, chromatin structure, changes in protein-protein interaction, and subcellular localization (Gill *et al*, 2004; Johnson *et al*, 2004; Desterro *et al*, 1998; Bossis *et al*, 2005; Lin *et al*, 2004; Yang *et al*, 2003; Hietakangas *et al*, 2003). One of the best known examples of this competition is the regulation of the κ -B α protein, which undergoes poly-ubiquitination for 26S-dependent degradation or mono-sumoylation to prevent its degradation. In plants, ICE1 is also modified by SUMO and ubiquitin, indicating that this protein may be regulated in a similar fashion (Chinnusamy *et al*, 2007). Another example could be the lipase/acylhydrolase-GDSL protein. This protein has been identified as being ubiquitinated in *Arabidopsis* (Maor *et al*, 2007; Manzano *et al*, 2008). In this work, we have found that the lipase/acylhydrolase is modified with SUMO (Table 1), suggesting that it may be regulated by both peptide tags.

Concluding remarks

The proteomic approach described here represents a useful and sensitive methodology to analyze and identify PTMs targets. The use of this approach has several advantages: 1) proteins are extracted and analyzed under denaturing

conditions, reducing the removal of the PTM from the target proteins; 2) the combination of 2D-LC and immuno-detection allows the identification of low abundant modified proteins; and 3) the method is highly reproducible and comparable between different experiments. The use of this method will open the possibility to analyze proteome changes and to identify targets of PTMs during programmed development or in response to external stimuli. In addition, the use of this method is not restricted to plants and it could be used to analyze PTMs in any organisms.

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Table 1: Sumoylated proteins identified by MS.

Peptide Mass Fingerprint ⁽¹⁾ ms/ms ⁽²⁾ Total protein extract ^(a) Nuclei extract ^(b)	Ions score*	TAIR number	SUMOYLATION CONSENSUS SEQUENCE
PBP1 (PYK10-BINDING PROTEIN 1) ^{(1)(b)}	423	At3g16420	LKEE (K269)
GIGANTEA ^{(1)(b)}	171	At1g22770	LKPP (K226) AKDD (K975)
PSBO-2/PSBO2 (PHOTOSYSTEM II SUBUNIT O-2 oxygen evolving) ^{(1)(a)}	245	At3g50820	VKAD (K145)
PSBO-1 (OXYGEN-EVOLVING ENHANCER 33) ^{(1)(a)}	227	At5g66570	VKAD (K146)
DREPP plasma membrane polypeptide family protein ^{(2)(a)}	54	At4g20260	VKAE (K145)
MYB4R1 (myb domain protein 4R1 transcription factor) ^{(2)(a)}	3	At3g18100	LKQE (K364) LKKE (K495)
Pentatricopeptide (PPR) repeat-containing protein ^{(2)(a)}	3	At4g14820	IKPD (K341)
PBP1 (pYK10-binding protein 1) ^{(1)(a)}	121	At3g16420	LKEE (K269)
RuBisCO small subunit 2B (RBCS-2B) (ATS2B) ^{(1)(a)}	81	At5g38420	Low affinity
Ania-6a type cyclin ^{(2)(a)}	29	At2g01008	LKVE K131
Argininine-rich cyclin 1 ^{(2)(a)}	29	At2g26430	LKVE K76
Lipase/Acylhydrolase with GDSL-motif family ⁽²⁾	41	At1g54010	LKND (K189)
Putative AAA-type ATPase ^{(2)(a)}	9	At2g29080	IKLD (K509)
Protein phosphatase 2C-like ^{(2)(a)}	6	At5g53140	MKHP (K45)
Putative phosphatidylinositol/ phosphatidylcholine transfer protein ^{(2)(a)}	4	At2g21520	VKEE (K424)
MADS-box family protein ^{(2)(a)}	4	At3g041003	LKNP (K82)
TTL1 (TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 1) ^{(2)(a)}	1	At1g53300	FKLG (K511)

(*) Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event (MASCOT program). Sumoylation consensus sequences were calculated with the SUMOplot™ tool. The motifs and lisyne with high and low probability are indicated.

Table 2: Phosphorylated proteins identified by MS.

Peptide Mass Fingerprint	Ions score*	TAIR number	NetPhos prediction Sequence Score
Ribulose biphosphate carboxylase	153	At1g67090	None with significant score
CRU3 (CRUCIFERIN 3)	153	At4g28520	QATETIKSE 0.920 VFANTPGSA 0.925
Lipase/Acylhydrolase with GDSL-motif family	103	At1g54010	GRHNTEKAQ 0.955
Chloroplast lumen common family protein	45	At4g39470	KTHDTSVFD 0.664 RLQNTFKAK 0.550
SEX1 (STARCH EXCESS 1)	44	At1G10760	VTSTTVREV 0.986 VAVLTPDMP 0.934
Putative transformer-SR ribonucleoprotein	40	At1g07350	RRGRTPTPG 0.939 GRTPTPGKY 0.941 ARSSTSRSV 0.971
Transcription factor-related	39	At5g59230	SIPTTPVIV 0.838
EMB1796 (EMBRYO DEFECTIVE 1796)	39	At3g49240	LDDDTVRSV 0.827
Pleckstrin homology (PH) domain-containing protein	39	At5g12150	GRAFTLKAE 0.955 LRYTTSAEK 0.952 LMELTTRLD 0.935
Putative clathrin assembly protein	38	At4g32285	LREMPERI 0.946 VQRITSKLL 0.885

(*) Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event (MASCOT program).

Table 3: Ubiquitinated proteins identified by MS.

Peptide Mass Fingerprint combined ms/ms	Ions score*	TAIR number
PSBO-2/PSBO2 (PHOTOSYSTEM II SUBUNIT O-2); oxygen evolving	68	At3g50820
PSBO-1 (OXYGEN-EVOLVING ENHANCER 33)	62	At5g66570
SAM1 (S-adenosylmethionine synthetase)	58	At1g02500
Ribulose biphosphate carboxylase large chain precursor	192	At1g00490

(*) Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event (MASCOT program).

Figure legends

Figure 1: Immuno-detection of SUMO and ubiquitin modified proteins. **(a)** Total proteins (20 µg) from 6-day-old wild-type (1) or HA-SUMO^{OE} (2) seedlings were separated by SDS-PAGE and analyzed by immunoblotting using IgGs against the HA epitope to detect SUMO conjugates. Arrow indicates free 3xHA-SUMO1 and the bracket indicates HA-SUMO conjugates. **(b)** Total proteins (20 µg) from leaves of 21-day-old HA-SUMO^{OE} plants (3) or chloroplast-purified total proteins (4) were separated by SDS-PAGE and analyzed by immunoblotting using IgGs against the HA epitope to detect SUMO conjugates. Arrowhead indicates a 34 KDa protein modified with SUMO. **(c)** Total proteins (20 µg) from leaves of 21-day-old HA-SUMO^{OE} plants (5) or chloroplast-purified total proteins (6) were separated by SDS-PAGE and analyzed by immunoblotting using IgGs against ubiquitin. Asterisk indicates a 34 KDa protein modified with ubiquitin.

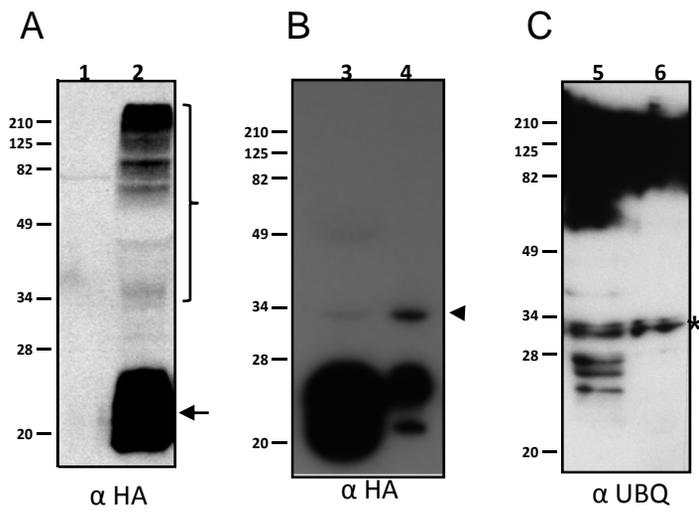
Figure 2: Identification of SUMO targets. **(a)** Protein extracts (2,5 mg) from HA-SUMO^{OE} plants were fractionated by using the PF2D ProteomeLab system, and the complete proteome was transferred to PVDF membranes by dot blot. Membranes were incubated with anti-HA antibody and the immunoreactive fractions were detected by chemiluminiscense. The immuno-blot corresponds to fraction 27, 28 and 29. **(b)** Representative example of the second dimension absorbance profile at 214 nm. This profile corresponds to the isoelectric-point fraction number 29 that has been separated in a Reverse Phase C18 column. Peaks marked with A, B and C correspond to the immunoreactive fractions showed in **(a)**. Proteins contained in these fractions were analyzed by MS to determine their identity (see table I).

Figure 3: Identification of SUMO targets in nuclei extracts. **(a)** Nuclear protein extracts (5 µg) from HA-SUMO^{OE} non-treated (1) and treated during 30 min at 42°C plants (2), were separated by SDS-PAGE and analyzed by immunoblotting using IgGs against the

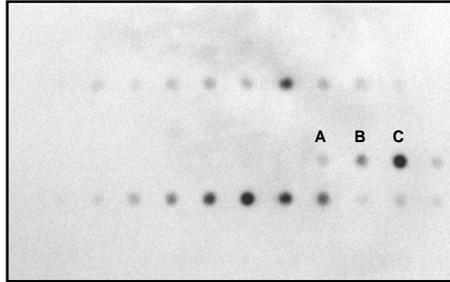
HA epitope to detect SUMO conjugates. **(b)** Nuclear protein extracts (0,5 mg) from HA-SUMO^{OE} non-treated (upper pannel) and treated plants (bottom pannel) were fractionated by using the PF2D ProteomeLab system in the first dimension, and all pH fractions were transferred to PVDF membranes by dot blot. The membrane was incubated with anti-HA antibody and the immunoreactive fractions were detected by chemiluminiscense. **(c)** Fraction number 30 was fractionated by using the PF2D ProteomeLab system in the second dimension and then was transferred to PVDF membranes by dot blot. Again the membrane was incubated with anti-HA antibody and the immunoreactive fractions were detected by chemiluminiscense. **(d)** Second dimension absorbance profile at 214 nm. This profile corresponds to the isoelectric-point fraction number 30 that has been separated in a Reverse Phase C18 column. Peaks marked with A, B, C and D correspond to the immunoreactive fractions showed in **(c)**. Proteins contained in these fractions were analyzed by MS to determine their identity.

Figure 4: Expression of sumoylation machinery with the specific targets in *E. coli*. Rosetta cells containing plasmids pET28b (1), pET28b-At1g22270-Ct (GI-Ct) (2), pET28b-At1g22270-Nt (GI-Nt) (3), and pET28b-At3g16420 (PBP1) (4), were incubated with 0.5 mM IPTG at 37°C during 2 hours, and at 30°C during 15 hours. Soluble proteins were purified by using Ni⁺ binding columns, separated in 11% acrylamide SDS-PAGE and electrotransferred to PVDF membranes. SUMO was detected by immunoblotting using anti-SUMO antibodies. Arrow heads mark the differential bands of GI-Ct or PBP1 that immune-reacted with the anti-SUMO antibodies compared with the control (1) and GI-Nt (3). Asterisk indicates degradation products of PBP1 that are also modified with SUMO.

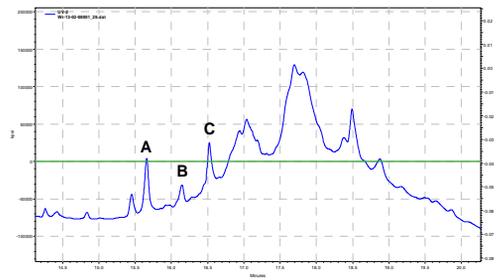
Lopez-Torrejón
Figure 1

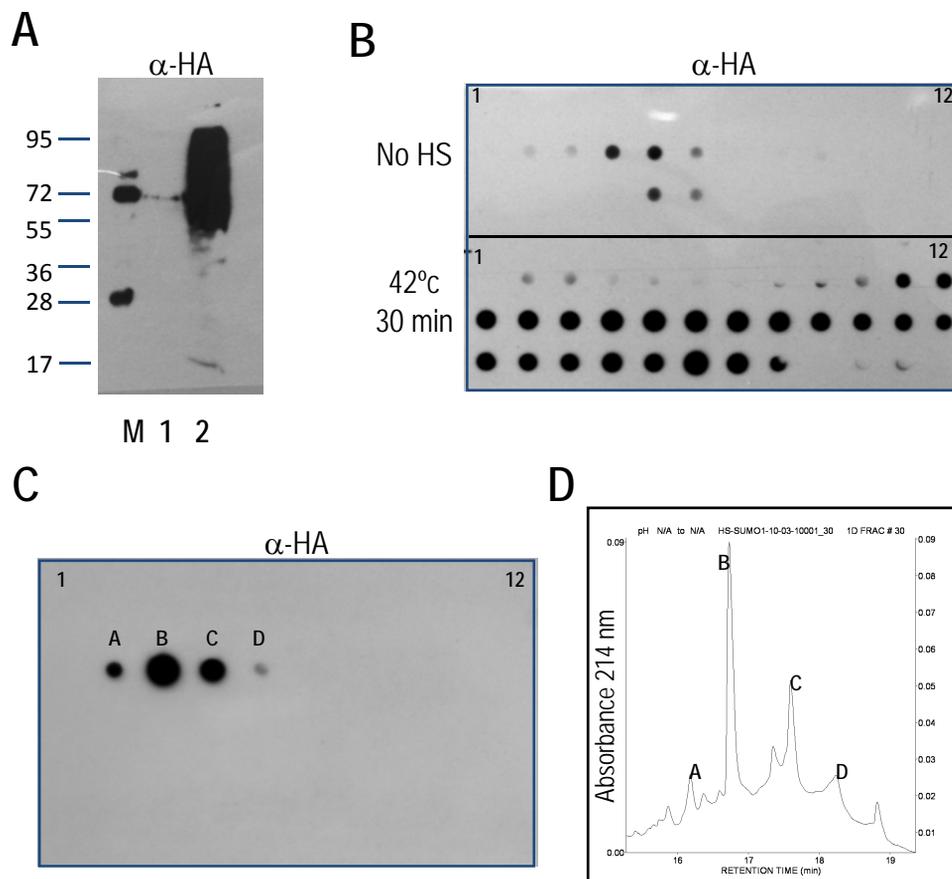


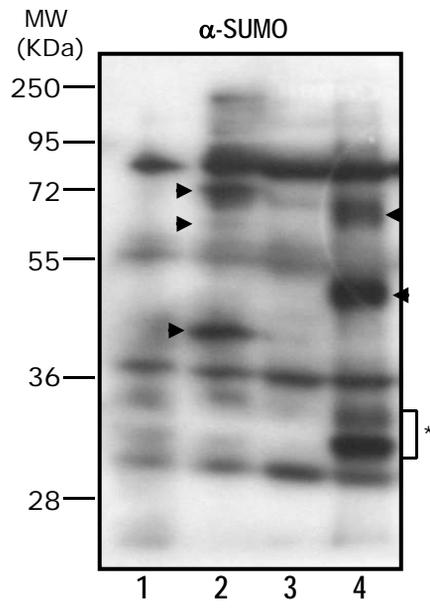
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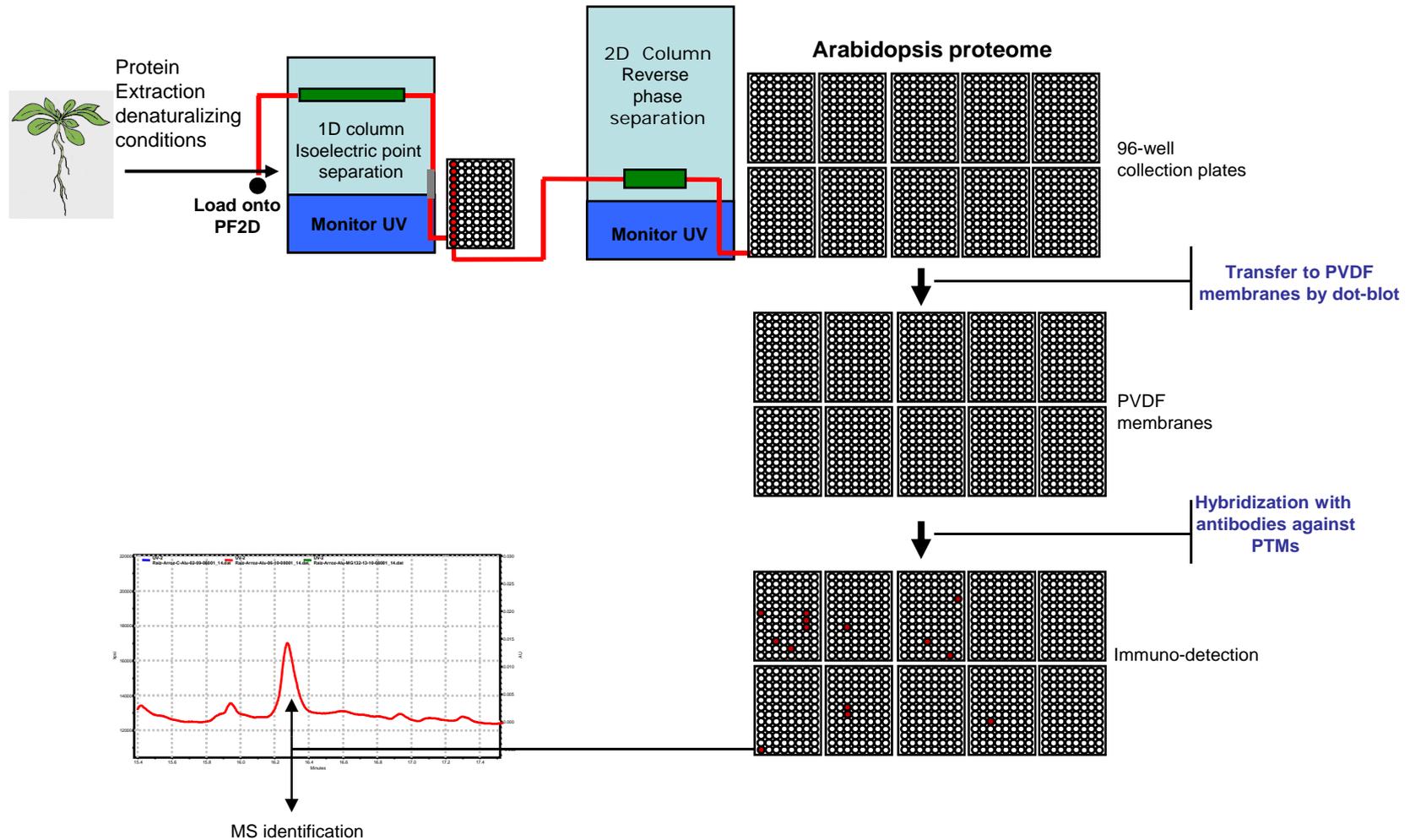


B









Supplementary figure1: schematic representation of the plant proteome separation using the PF2D system. The collected fractions are transferred to PVDF membranes that were hybridized with antibodies against different PTMs and detected by chemiluminescence. The positive immune-reactive proteins were identified by mass spectrometry (MS) or by liquid chromatography-MS-MS