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Identification of Zinc-Responsive Proteins in the Roots of *Arabidopsis thaliana* Using a Highly Improved Method of Two-Dimensional Electrophoresis

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Short Communication

Zinc (Zn) is an essential micronutrient for various physiological and metabolic processes in plants, although it is toxic in excess. To understand better Zn-responsive proteins, we developed a highly improved method of isoelectric focusing (IEF) in which whole lysate from Arabidopsis roots is subjected to IEF without any desalting steps. In this method, samples extracted with lysis buffer containing 1.5% SDS can also be directly applied to IEF. By applying this method to Zn-treated Arabidopsis roots, 10 up-regulated and 17 down-regulated proteins were identified, 15 of which showed a significant correlation with previously reported transcriptomic data.

Keywords: *Arabidopsis thaliana* • 2-D gel electrophoresis • IEF • Microsomal fraction • SDS • Zn stress.

Abbreviations: 2-D, two-dimensional; DTT, dithiothreitol; IEF, isoelectric focusing; IPG, immobilized pH gradient; LC-MS, liquid chromatography–mass spectrometry; MS, Murashige–Skoog; TCA, trichloroacetic acid.

Zinc (Zn) is an essential micronutrient in all organisms. Plants have evolved homeostatic mechanisms to maintain Zn within tolerated concentrations for optimal biochemical and physiological functioning. The cellular Zn homeostasis is controlled by transport, in which Zn is bound by chelators, such as phytochelatins and metallothioneins, and then sequestrated inside the vacuole (Clemens 2001). Excess Zn harms the growth of plants, presumably due to competition with other biologically important ions, which ultimately leads to reduced biomass, leaf chlorosis and root growth inhibition (Haydon and Cobbett 2007, Kawachi et al. 2009). When exposed to excess Zn, *Arabidopsis thaliana* accumulates substantially higher concentrations of Zn in roots than in shoots (Arrivault et al. 2006).

Since the establishment by O'Farrel of two-dimensional (2-D) gel electrophoresis combining isoelectric focusing (IEF) and SDS–PAGE, it has been used as an excellent separation method for proteins (O'Farrel 1975). The utility of this technique has increased recently, particularly in proteomics studies (Robertson et al. 2008, Minami et al. 2009). However, depending on the protein sample, it is often difficult to obtain a well-resolved 2-D gel pattern. Plant seedlings are particularly poor protein sources for IEF analysis because of the presence of non-proteinaceous contaminants specific to plants, such as polyphenols, lipids, organic acids, terpenes or pigments, which can lead to irreproducible results on protein separation in 2-D gel electrophoresis (Wang et al. 2003, Zukas and Breksa 2005). For these reasons, desalting steps such as trichloroacetic acid (TCA) or acetone treatment are a prerequisite to obtaining a well-resolved 2-D gel pattern. However, we encounter an increasing loss of proteins during these desalting steps because of proteolysis or aggregation. Therefore, a direct application of extracted proteins to IEF is highly desirable to improve quantification and reproducibility of the results.

In this study, we developed an IEF method that does not require any desalting steps and uses protein samples dissolved in buffer containing SDS. Moreover, we show that adding SDS to the samples considerably improved the resolution of 2-D gel images and the number of detectable spots. Finally, to evaluate this new IEF method combined with SDS–PAGE, we investigated the Zn-responsive proteins in Arabidopsis roots.

To examine the effect of excess Zn on Arabidopsis growth, plants were grown on either standard MS medium (containing 30 μM $ZnSO_4$) or MS medium supplemented with various concentrations of $ZnSO_4$ (**Supplementary Fig. S1**). Arabidopsis shoot and root growth was significantly affected

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by Murashige–Skoog (MS) medium containing >300 μM $ZnSO_4$ (**Supplementary Fig. S1A**). The concentration of chlorophyll in shoots was reduced by up to 30%, and the growth of roots was inhibited by up to 70% (**Supplementary Fig. S1B, C**). Based on these results, Zn-responsive proteins were compared between plants grown on standard MS medium and those grown on MS medium supplemented with 300 μM $ZnSO_4$ (hereafter referred to as Zn treated).

Microsomal fractions were prepared as the insoluble proteins from roots grown for 10 d. The supernatants from ultracentrifugation during the preparation of the microsomal fractions were used as the soluble protein fractions.

There are two major methods for IEF analysis. The first is a filter paper method in which a protein sample is loaded on a pre-hydrated immobilized pH gradient (IPG) gel before IEF analysis (Koga 2008; **Fig. 3A, B**). In the second method, an IPG gel is rehydrated together with the protein sample (Fukao et al. 2002, Fukao et al. 2003; **Fig. 3C**). In principle, the desalting of protein samples removes ionic impurities and leads to better focusing of protein spots, although a great loss of proteins occurs. We hypothesized that the filter paper method could allow a good spot resolution without any desalting steps; placed at both the acidic and basic ends of the gel, the filter paper may be sufficient to trap the ionic impurities and decrease molecular congestion within the IPG gel.

To check the validity of our hypothesis, soluble proteins were further concentrated by ultrafiltration and then subjected to IEF, using the filter paper method, without any desalting. With this method, we obtained a very well-resolved 2-D gel pattern and 1,243 spots were detected in **Fig. 1A**, whereas only 459 spots were detected by the sample rehydration method (data not shown). Moreover, seven proteins that were up-regulated and three that were down-regulated in response to excess Zn were reproducibly obtained in three independent experiments (**Fig. 1**; **Table 1**). Based on these results, we determined that the filter paper method is suitable for 2-D gel electrophoresis of samples prepared without any desalting.

Why is the filter paper method effective at separating protein samples prepared without desalting? In the filter paper method, unlike the sample rehydration method, a sample is loaded after rehydration of the IPG gel (**Fig. 3B, C**). Therefore, proteins and ionic impurities enter the rehydrated IPG gel during IEF. Desalting probably occurs at an early point in IEF, because the current immediately increased to approximately 200–300 μA and then decreased to <100 μA within an hour in our experiment. Ionic impurities would migrate faster than proteins in the IPG gel because of their lower molecular weight and thus should reach and adsorb to filter paper at either the acidic or basic end (**Fig. 3B**). The migration of proteins probably starts afterwards; in fact, the proteins were actually not well focused without the filter paper, probably because the impurities were not removed from the IPG gel

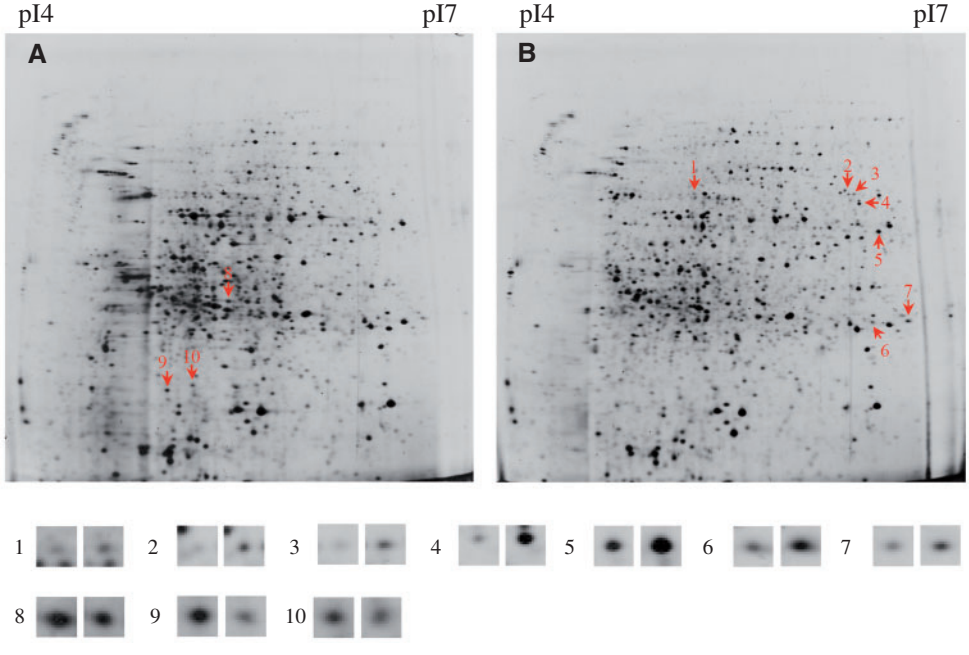

Fig. 1 2-D gel electrophoresis patterns of soluble proteins from Arabidopsis roots. Each protein sample (150 μg) was separated using an IPG gel (pI 4–7) as the first dimension and 12.5% SDS–PAGE as the second dimension. The 2-D gel patterns of the protein samples from roots grown on MS plates (A) and on MS plates containing 300 μM $ZnSO_4$ (B). The up-regulated and down-regulated proteins in response to excess Zn are indicated by arrows in A and B, respectively. The lower panel shows magnified spots of each protein affected by excess Zn.

(**Supplementary Fig. S2A**). Based on this result, we suggest that filter paper plays a critical role in the absorption of impurities at the early stages of IEF analysis. Uncharged substances did not enter the IPG gel (**Fig. 3B**). Consequently, the impurities included in the samples would not disturb the focusing of proteins in the filter paper method. On the other hand, if samples prepared without desalting steps were separated using the sample rehydration method, the focusing of the proteins should be disturbed by such impurities.

Although SDS is an effective reagent for extracting insoluble proteins such as membrane proteins, these proteins will not be focused at their isoelectric points in the presence

Table 1 Proteins whose levels were significantly increased or decreased in response to Zn

Spot No.	Protein name	AGI code	Mol. wt.	pI	Coverage (%)[a]	Change value[b]	STDEV	Transcriptomics[c]	Cd[d]	Na[e]
Increased proteins in the soluble fraction										
1	Monodehydroascorbate reductase	AT5G03630	47,508	5.24	58	1.64	0.48	*	*	*
2	Aminoacylase, putative	AT4G38220	47,768	5.93	58	1.28	0.14			
3	Isocitrate dehydrogenase, putative	AT1G65930	46,065	6.13	63	1.84	0.13	*	*	*
4	Cobalamin-independent methionine synthase	AT5G17920	84,652	6.09	28	2.53	1.32	*	*	*
5	Malate dehydrogenase, cytosolic, putative	AT1G04410	35,896	6.11	58	1.95	0.32	*	*	*
6	Glutathione S-transferase 9	AT2G30860	24,131	6.17	49	1.31	0.17			
7	Manganese superoxide dismutase 1	AT3G10920	25,486	8.47	52	1.34	0.11	*		*
Decreased proteins in the soluble fraction										
8	Dienelactone hydrolase family protein	AT2G32520	25,963	5.26	62	−1.66	0.63			
9	Dehydroascorbate reductase	AT1G19570	23,742	5.56	48	−1.45	0.37			
10	Glutathione S-transferase (class phi) 2	AT4G02520	24,114	5.92	56	−1.29	0.23			*
Increased proteins in the insoluble fraction										
11	Kelch repeat-containing protein	AT3G07720	36,011	5.07	58	1.66	0.63	*		
12	Jacalin lectin family protein	AT3G16450	32,003	5.06	70	1.54	0.45	*		
13	Jacalin lectin family protein	AT3G16450	32,003	5.06	62	2.29	0.93	*		
Decreased proteins in the insoluble fraction										
14	PDI-like 1-1	AT1G21750	55,857	4.81	66	−1.40	0.23	*		*
15	Elongation factor 1B-gamma, putative	AT1G57720	46,602	5.55	57	−1.57	0.31	*		
16	Chaperonin, putative	AT3G02530	59,435	5.83	65	−1.43	0.60	*	*	
17	Peroxidase 22	AT2G38380	38,664	5.66	18	−1.60	0.23			*
18	60S acidic ribosomal protein P0	AT3G09200	34,170	5.00	62	−1.62	0.86	*		*
19	Ferretin 1	AT5G01600	28,160	5.73	47	−1.47	0.36	*		
20	Cytosolic triose phosphate isomerase	AT3G55440	27,384	5.39	74	−1.27	0.27		*	*
21	Proteasome subunit PAB1	AT1G16470	25,685	5.53	82	−1.22	0.17			
22	20S proteasome alpha subunit A1	AT5G35590	27,393	5.60	69	−1.24	0.24			
23	Dehydroascorbate reductase	AT1G19570	23,742	5.56	92	−1.51	0.13			
24	20S proteasome beta subunit A 1	AT4G31300	25,194	5.31	72	−1.23	0.20	*		
25	Cold, circadian rhythm, and RNA binding 1	AT4G39260	16,627	5.58	60	−1.44	0.56			*
26	MLP-like protein 328	AT2G01520	17,618	5.42	65	−1.33	0.17	*		
27	Cold, circadian rhythm, and RNA binding 2	AT2G21660	16,938	5.85	70	−1.52	0.40			*

Protein spots, reproduced in three independent experiments, were identified by LC-MS analysis. The fold change values are based on data from three independent experiments. Protein spots with fold change values >1.2 were selected.

[a]The percentage of sequence coverage (%).

[b]The change value calculated by Progenesis PG-200 software ($n = 3$).

[c]Responsive proteins were compared with transcriptomic data (Mortel et al. 2008).

[d]Cadmium-responsive proteins, based on Sarry et al. (2006).

[e]Salt-responsive proteins, based on Palmgren et al. (2008).

of SDS owing to its anionic charge. This complicates the analysis of insoluble proteins by 2-D gel electrophoresis. Based on our above results and in order to examine whether samples containing SDS can be analyzed by the filter paper method, IEF was performed using insoluble proteins (**Fig. 2**). To evaluate the effect of SDS, equal amounts of Arabidopsis root extract were ultracentrifuged, and then the pellets were dissolved in SDS-free buffer A or in buffer B (**Fig. 2**). As a result, 340 and 1,306 spots were detected, respectively (**Fig. 2A, B**). Therefore, dissolving proteins in a buffer containing SDS may be useful not only for increasing the number of detectable spots but also for improving the resolution of 2-D images (compare **Fig. 2A and B**). This result suggests that filter paper may absorb free SDS as well as impurities from the IPG gel (**Supplementary Fig. S3B**). Based on these findings, we concluded that the filter paper method is suitable for IEF of SDS-containing samples.

Next, we investigated proteome changes in the insoluble proteins using samples dissolved in lysis buffer B. Three proteins were found to be up-regulated and 14 proteins were down-regulated in response to excess Zn (**Fig. 2B, C**; **Table 1**).

Here we propose a model to explain why insoluble protein samples containing SDS are well resolved using the filter paper method (**Fig. 3D**). Upon applying a voltage to the gel, SDS detaches from the proteins and is replaced by Triton, which has a neutral electric charge. Free SDS together with ionic impurities then reaches the filter paper. Indeed, when protein samples dissolved in lysis buffer containing 5.0% SDS were separated by 2-D gel electrophoresis, the proteins were focused from pI 5 to 7, but were not focused around pI 4 (**Supplementary Fig. S3**). Excess SDS within the gel may confine the migration of proteins to the acidic side of the IPG gel.

To evaluate our results, we compared the Zn-responsive proteins obtained in this study with previously reported

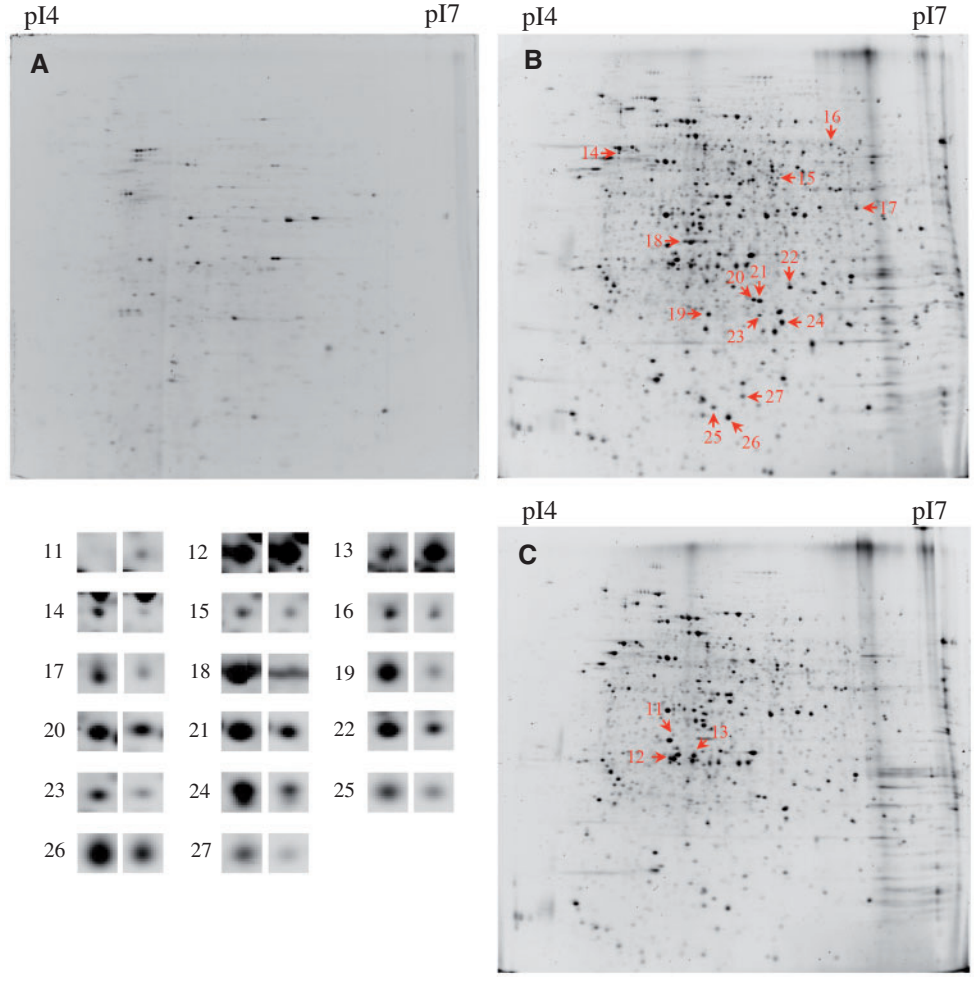

Fig. 2 2-D gel electrophoresis patterns of insoluble proteins from Arabidopsis roots. Each protein sample (250 μg) was separated using an IPG gel (pI 4–7) as the first dimension and 12.5% SDS–PAGE as the second dimension. The 2-D gel pattern of insoluble proteins from roots grown on MS plates (A, B) and on MS plates containing 300 μM $ZnSO_4$ (C). The insoluble proteins were dissolved in buffer A (A) or buffer B (B, C). Proteins up-regulated in response to excess Zn between B and C are shown by arrows in C. The proteins down-regulated in response to excess Zn are shown by arrows in B. The spot corresponding to each Zn-responsive protein was enlarged and is shown in the bottom left panel.

transcriptomic data. Mortel et al. (2008) used roots of Arabidopsis plants that were grown for 3 weeks on Hoagland's nutrient solution and then transferred for 7 d to solutions with different Zn concentrations. Fifteen of the 27 Zn-responsive proteins identified in the present study were either up-regulated or down-regulated in the same manner as those in the study by Mortel et al., even though the growth and Zn treatment conditions differed between that study and ours (**Table 1**). This confirmed that our results and those obtained in a previous transcriptomic analysis were highly reproducible. Furthermore, six proteins were responsive to cadmium, a Zn analog (Sarry et al. 2006, Palmgren et al. 2008), and 13 were identified as NaCl-responsive proteins (Jiang et al. 2007) (**Table 1**). In addition, the amounts of several Zn-responsive proteins changed under various stress conditions.

Recently, liquid chromatography–mass spectrometry (LC-MS) shotgun analysis has been used to quantify proteome changes, because it is quick, easy and applicable to small amounts of proteins. However, shotgun analysis has a low dynamic range, as liquid chromatography has a lower separating ability than 2-D gel electrophoresis. Therefore, 2-D gel electrophoresis remains the method of choice for quantitative analysis in the proteomics field. Here, we reported an improved method of IEF analysis in which protein samples are directly applied after extraction, without any desalting steps. The results obtained with this method show higher reproducibility than conventional IEF results.

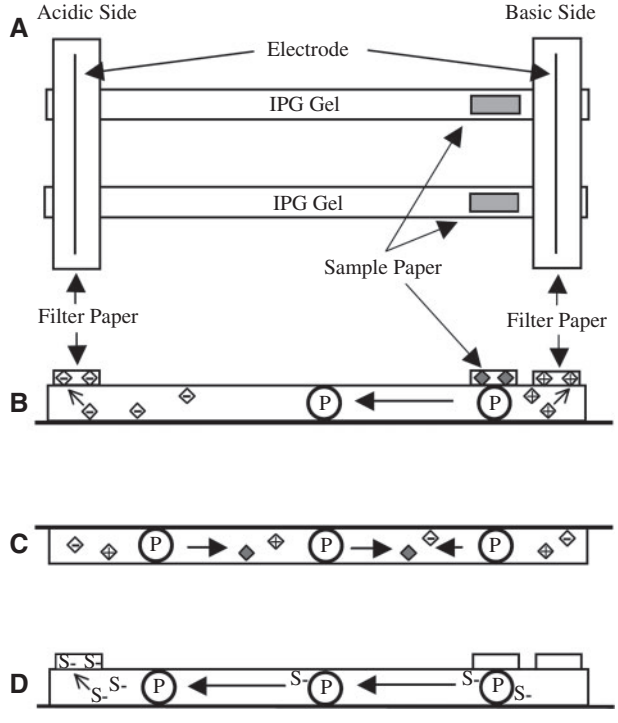

Fig. 3 The principle of the filter paper method. (A) Set-up of the CoolPhoreStar IPG-IEF system for the filter paper method. The rehydrated IPG gel was placed with the gel side up in a tray. Filter papers were placed on both the acidic and basic sides. The sample was loaded on a sample paper and then placed on the IPG gel at the basic side. (B) Model of IEF using the filter paper method. Proteins and impurities with an electric charge migrate in one direction, from the sample paper at the basic side to the acidic side. Diamonds with (+) or (−) indicate impurities with an electric charge. The black diamonds indicate impurities with no net electric charge. (C) Model of IEF using the sample rehydration method. Proteins and impurities with an electric charge migrate in two directions. The impurities block the migration of proteins. (D) Model of IEF using insoluble proteins with SDS. The insoluble proteins extracted by SDS, which are indicated as S^-, form micelles with SDS and Triton. The SDS gradually detaches from the proteins when voltage is applied to the IPG gel and is replaced by Triton, which has no electric charge. Owing to its negative charge, free SDS is pulled to the acidic side until it reaches the filter paper.

Materials and Methods

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments. Plants were germinated on sterile plates of MS medium containing 2.3 mM MES-KOH, pH 5.7, 1.0% (w/v) sucrose and 1.5% agar (MS plate) or MS plates supplemented with 300 μM $ZnSO_4$, and were grown for 10 d at 22°C under 16 h light/8 h dark conditions. Roots of Arabidopsis were homogenized with buffer A (50 mM HEPES-KOH, pH 7.5, 5 mM EDTA, 400 mM sucrose, protease inhibitor cocktail). The homogenates were centrifuged twice at 15,000 r.p.m. at 4°C for 20 min. The supernatants were centrifuged at 100,000 × *g* at 4°C for 60 min, and the resultant supernatants were concentrated using ultrafiltration (Millipore, Billerica, MA, USA). For microsomal fractions, the pellets were dissolved in buffer A or buffer B [5 M urea, 2 M thiourea, 2.0% CHAPS, 1.5% SDS, 60 mM dithiothreitol (DTT) and 2.0% Pharmalyte 3–10 (GE Healthcare UK, Buckinghamshire, UK)].

Chlorophyll content was measured as described by Arnon (1949).

2-D gel electrophoresis was performed as described by Toda and Kimura (1997) with minor modifications. The IPG gel (GE Healthcare UK) was rehydrated overnight in 5 ml of rehydration buffer containing 7 M urea, 2 M thiourea, 2.0% Triton X-100, 10 mM DTT, 1.0% Pharmalyte 3–10, 2.5 mM acetic acid and Orange G (Merck, Darmstadt, Germany). IEF was performed using a CoolPhoreStar IPG-IEF system (Anatech, Tokyo, Japan) according to the manufacturer's instructions. The voltage was increased in a stepwise manner, with eight steps: 500 V for 2 h; 700 V for 700 Vh; 1000 V for 1,000 Vh; 1,500 V for 1,500 Vh; 2,000 V for 2,000 Vh; 2,500 V for 2,500 Vh; 3,000 V for 3,000 Vh; and 3,500 V for 35,000 Vh. Filter papers were placed on the acidic side with dH_2O and on the basic side with DTT (5 mg ml^{-1}). After IEF, the gels were incubated for 30 min with shaking at room temperature in equilibration buffer (6 M urea, 25 mM Tris–HCl,

pH 6.8, 2.0% SDS, 30% glycerol and a trace of bromophenol blue) containing 30 mM DTT, followed by 20 min in equilibration buffer without urea and with 240 mM iodoacetamide instead of DTT. The proteins in the equilibrated IPG gels were separated using 12.5% SDS–PAGE (DRC, Tokyo, Japan) as the second dimension. Proteins were detected using Flamingo (Invitrogen, Carlsbad CA, USA) and were scanned with a FluoroPhoreStar 3000 (Anatech). The spots were quantified by Progenesis PG-200 software (Shimadzu, Kyoto, Japan).

The gel spots were excised using a FluoroPhoreStar 3000 (Anatech), and peptides were prepared as described by Fujiwara et al. (2009). LC-MS analysis was performed using an LTQ-Orbitrap XL-HTC-PAL system (Thermo Fisher Scientific, Bremen, Germany). The MS/MS spectra were compared against TAIR8 (The Arabidopsis Information Resource) using the MASCOT server (version 2.2) with the following search parameters: threshold set-off at 0.05 in the ion score cut-off; peptide tolerance at 10 p.p.m.; MS/MS tolerance at ±0.8 Da; peptide charge of 2+ or 3+; trypsin as the enzyme and allowing up to one missed cleavage; carboxymethylation on cysteines as a fixed modification; and oxidation on methionine as a variable modification.

Supplementary data

Supplementary data are available at PCP online.

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