Plant Biotechnology 35, 31–37 (2018)
DOI: 10.5511/plantbiotechnology.18.0129a

Original Paper

Proteomic analysis of xylem vessel cell differentiation in VND7-inducible tobacco BY-2 cells by two-dimensional gel electrophoresis

Masahiro Noguchi[1], Masayuki Fujiwara[1,a], Ryosuke Sano[1], Yoshimi Nakano[1,b], Yoichiro Fukao[2], Misato Ohtani[1,3,*], Taku Demura[1,3,**]

[1] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan; [2] College of Life Sciences, Department of Bioinformatics, Ritsumeikan University, Shiga 525-8577, Japan; [3] RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan
* E-mail: misato@bs.naist.jp Tel: +81-743-72-5460 Fax: +81-743-72-5469
** E-mail: demura@bs.naist.jp Tel: +81-743-72-5460 Fax: +81-743-72-5469

Received December 22, 2017; accepted January 29, 2018 (Edited by K. Suzuki)

Abstract The xylem vessel is an essential structure for water conduction in vascular plants. Xylem vessel cells deposit thick secondary cell walls and undergo programmed cell death, to function as water-conducting elements. Since the discovery of the plant-specific NAC domain-type VASCULAR-RELATED NAC-DOMAIN (VND) transcription factors, which function as master switches of xylem vessel cell differentiation in Arabidopsis, much has been learned about the transcriptional regulatory network of xylem vessel cell differentiation. However, little is known about proteome dynamics during xylem vessel cell differentiation. Here, we performed two-dimensional electrophoresis-based proteomic analysis of xylem vessel cell differentiation using a transgenic tobacco BY-2 cell line carrying the VND7-inducible system (BY-2/*35S::VND7-VP16-GR*), in which synchronous *trans*-differentiation into xylem vessel cells can be induced by the application of a glucocorticoid. Of the 47 spots revealed by gel electrophoresis, we successfully identified 40 proteins. Seventeen proteins, including several well-characterized proteins such as a cysteine protease and serine carboxypeptidase (involved in programmed cell death), were upregulated after 24 h of induction. However, previous transcriptomic analysis showed that only eight of these proteins are upregulated at the transcriptional level during xylem vessel cell differentiation in BY-2/*35S::VND7-VP16-GR* cells. These findings suggest that post-transcriptional regulation strongly affects proteomic dynamics during xylem vessel cell differentiation.

Key words: post-transcriptional regulation, proteome, two-dimensional electrophoresis, VND7, xylem vessel cell differentiation.

Introduction

The xylem vessel is an essential tissue that transports water and minerals from roots to the aerial parts of vascular plants. Xylem vessel cells (tracheary elements in angiosperms) are distinctive cells characterized by lignified secondary cell walls (SCWs) with unique patterns. Xylem vessel cells undergo dynamic changes during differentiation, including SCW deposition, proteolysis, and programmed cell death (PCD) by tonoplast rupture. This differentiation process ends with the perforation of one side of the cell wall, allowing the cells to connect to each other to form a tubular vessel (Turner et al. 2007).

The molecular mechanism regulating xylem vessel cell differentiation has been investigated using various cell culture systems that can be induced to form xylem vessel cells in vitro (Fukuda 1997; Kubo et al. 2005; Oda et al. 2005; Yamaguchi et al. 2008). One such system involves *Zinnia elegans* mesophyll cells, which can be induced to form tracheary elements (Fukuda and Komamine 1980). Using this in vitro inducible system, many molecular aspects of xylem vessel cell differentiation have been revealed, such as the effects of phytohormones, dynamic cytological changes, and a novel signaling system involving peptide ligands that

Abbreviations: 2DE, two-dimensional electrophoresis; DEX, dexamethasone; HSP, heat shock protein; LC, liquid chromatography; MS, mass spectrometry; PCD, programmed cell death; SCW, secondary cell wall; VND, VASCULAR-RELATED NAC-DOMAIN.

[a] Present address: YANMAR Co., Ltd., 1-32 Chayamachi, Kita-ku, Osaka 530-8311, Japan.

[b] Present address: Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan

This article can be found at http://www.jspcmb.jp/
Published online March 28, 2018

functions in this process (Demura et al. 2002; Fukuda 1997, 2010; Fukuda and Kobayashi 1989; Groover et al. 1997; Ito et al. 2006; Oda et al. 2005; Tokunaga et al. 2006; Turner et al. 2007; Yamamoto et al. 1997). High-resolution analysis of transcriptomic changes associated with different stages of xylem vessel cell differentiation revealed novel factors involved in this process (Demura et al. 2002). In vitro inducible systems involving *Arabidopsis thaliana* suspension cells have become an increasingly powerful tool when combined with molecular genetics. This approach has led to the discovery of VASCULAR-RELATED NAC-DOMAIN (VND) proteins; these plant-specific NAC domain transcription factor are key regulators of xylem vessel cell differentiation (Kubo et al. 2005; Yamaguchi et al. 2008; Yamaguchi and Demura 2010). Overexpression analysis of *VND* genes revealed that VND7 and VND6 initiate the differentiation of protoxylem-type and metaxylem-type vessel cells, respectively (Kubo et al. 2005; Yamaguchi et al. 2010). Transcriptomic analyses of Arabidopsis *VND6* and *VND7* overexpressors have uncovered a set of direct target genes of VND6 and VND7, including genes required for deposition of the SCW and progression of PCD (Ohashi-Ito et al. 2010; Yamaguchi et al. 2010, 2011; Zhong et al. 2010). Based on the results of transcriptomic analysis, the evolutionarily conserved VND-based transcriptional network is currently thought to form the basis of xylem vessel cell differentiation (Endo et al. 2015; Kubo et al. 2005; Nakano et al. 2015; Yamaguchi and Demura 2010; Yamaguchi et al. 2010; Zhong et al. 2010).

Since these important transcriptomic analyses, other types of omics analyses have been performed using inducible in vitro xylem vessel cell differentiation systems, including proteome analysis targeting microtubule-interacting proteins in an inducible Arabidopsis suspension cell culture system (Derbyshire et al. 2015) and metabolome analysis using post-translationally activated VND7 (*35S::VND7-VP16-GR*, Yamaguchi et al. 2010) in Arabidopsis and tobacco BY-2 cells (Li et al. 2016; Ohtani et al. 2016). These studies suggested that a multilayered regulatory system orchestrates these cell differentiation processes. However, our understanding of proteomic dynamics remains limited. Fukuda and Komamine (1983) performed two-dimensional electrophoresis (2DE) analysis of differentially expressed proteins in tracheary elements induced from *Zinnia elegans* mesophyll cells, but they detected only two polypeptides that were newly synthesized preceding morphological changes in the cells. Also, when performing proteome analysis, Derbyshire et al. (2015) only investigated the dynamics of microtubule-associated proteins during xylem vessel differentiation.

To gain novel insight into the contribution of proteomic dynamics to xylem vessel cell differentiation, we performed comparative 2DE-based proteomic analysis of xylem vessel cell differentiation. We used transgenic tobacco (*Nicotiana tabacum*) BY-2 culture cells carrying the VND7 inducible system (BY-2/*35S::VND7-VP16-GR*) (Goué et al. 2013; Ishii et al. 2017; Ohtani et al. 2016; Yamaguchi et al. 2010). In this inducible system, the differentiation of BY-2 cells into xylem vessel cells can be induced with ca. 80% efficiency by applying a glucocorticoid analog of dexamethasone (DEX). We selected 47 spots from the electrophoretograms and identified 40 proteins: the expression of 17, 8, and 15 proteins increased, decreased, and remained stable, respectively, after 24 h of induction. The list of upregulated proteins includes PCD-related proteins (cysteine protease and serine carboxypeptidase) and cell wall-related proteins (pectate lyase-like proteins), whereas the downregulated proteins were heat shock proteins. Interestingly, previous transcriptomic analysis indicated that only half of these upregulated or downregulated proteins are up- or downregulated at the transcriptional level during xylem vessel cell differentiation. Our proteomic analysis also revealed spots of identical proteins whose positions shifted after the induction of xylem vessel cell differentiation, suggesting that post-translational modification of specific proteins occurs during xylem vessel cell differentiation. These findings suggest that post-transcriptional regulation strongly contributes to proteome dynamics during xylem vessel cell differentiation.

Materials and methods

Plant materials and growth conditions

A *Nicotiana tabacum* L. cv. BY-2 suspension culture cell line carrying the *VND7-VP16-GR* system (BY-2/*35S::VND7-VP16-GR*) (Goué et al. 2013; Ohtani et al. 2016; Yamaguchi et al. 2010) was used in this study. The BY-2/*35S::VND7-VP16-GR* suspension culture cells were grown in 100 ml liquid medium (Murashige and Skoog salt mixture [Wako], 0.2 mg ml^{-1} KH_2PO_4, 0.1 mg ml^{-1} myoinositol, 1 mg l^{-1} thiamine-HCL, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, and 30 g l^{-1} sucrose [pH 5.8]) containing 100 mg l^{-1} kanamycin in a 300-ml conical flask in the dark at 27°C at 130 rpm rotation and subcultured weekly at 95-fold dilution.

Dexamethasone (DEX) treatment to induce xylem vessel cell differentiation

To induce xylem vessel cell differentiation, 1 mM dexamethasone (DEX) (final concentration) was applied to BY-2/*35S::VND7-VP16-GR* cells at 4 day after subculture. After DEX treatment, the cells were collected at 0 and 24 h after subculture by vacuum filtration and frozen in liquid nitrogen.

Protein extraction for 2DE analysis

The methods and buffer conditions used for protein extraction

were described by O'Farrell (1975), Molloy et al. (1998), and Zhu et al. (2006). Briefly, 1 mg of cells was ground with a pestle and mortar in liquid nitrogen, and 10 ml of trichloroacetic acid (TCA)/acetone (1 : 9) was added to the cell powder. The samples were incubated for 1 h at −20°C to precipitate proteins and remove lipids and ribonucleic acids. The samples were washed three times with 100% acetone, and the pellets were resuspended in extraction buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, 65 mM dithiothreitol [DTT], 0.01% [w/v] IPG buffer pH 4–7 [GE Healthcare Bioscience]). The protein samples were stored at −80°C until subsequent analysis.

2DE analysis

For the first dimension of isoelectric focusing (IEF), an 18-cm long IPG ReadyStrip gel (strip gel) with a pH range of 4–7 was used (GE Healthcare Bioscience). The dried strip gel was imbibed in imbibition buffer (6 M urea, 2 M thiourea, 2% [v/v] Triton X-100, 0.2% [w/v] DTT, 0.01% [w/v] IPG buffer pH 4–7, 2.5 mM acetic acid, 0.0025% [w/v] Orange G) with mild agitation for more than 10 h in a gel-swelling tube. The imbibed strip gel was placed in an IEF electrophoresis vessel filled with silicon oil (KF-96L-5CS; Shinetsu Chemical). Filter paper containing 15 μl of protein sample was placed on the side of the negative end of the strip gel, and IEF electrophoresis was carried out using the following steps: 500 V for 2 h, 700 V for 1 h, 1,000 V for 1 h, 1,500 V for 1 h, 2,000 V for 1 h, 2,500 V for 1 h, 3,000 V for 1 h, and 3,500 V for 10 h. The strip gel with protein samples was treated with sodium dodecyl sulfate (SDS) treatment buffer (6 M urea, 0.5% [w/v] DTT, 25 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 30% [v/v] glycerol, 0.0025% [w/v] bromophenol blue [BPB]) for 30 min with agitation. Subsequently, the strip gel was treated with alkylation buffer (25 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 30% [v/v] glycerol, 0.0025% [w/v] BPB, 4.5% [w/v] iodoacetamide [IAA]) for 20 min. The SDS-treated gel was then placed on a 16-cm precast 10% slab gel. After electrophoresis for 30 min at 10 mA, the strip gel was removed from the slab gel cassette, and electrophoresis was resumed at 40 mA until the electrophoresis marker reached the end of the gel (6–7 h). Fluorescence staining of the gels was performed with Flamingo reagent (Bio-Rad Laboratories). Fixation and staining were conducted according to the manufacturer's protocol. To detect the fluorescent signals, an LAS-3000NS imager (Fujifilm) was used, and the obtained signal intensities of each spot were compared manually between 0-h and 24-h samples. This analysis was repeated three times independently, and the spots detected repeatedly were subjected to a subsequent in-gel digestion procedure.

In-gel digestion of proteins

The target spots were sliced out of the gels. Each gel piece was collected in a 1.5-ml tube, immersed in 200 μl of 60% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3 solution, and agitated for 10 min at room temperature, followed by removal of the solution. After repeating this procedure twice, 200 μl of 10 mM DTT/50 mM NH_4HCO_3 solution was added to the sample, followed by incubation for 45 min at 56°C. After removing the DTT solution, 200 μl of 55 mM iodoacetamide (IAA)/50 mM NH_4HCO_3 was added to the sample, which was subsequently incubated for 30 min at room temperature in the dark. After removing the IAA solution, 300 μl of 60% (v/v) ACN/50 mM NH_4HCO_3 was added to the sample, followed by agitation for 10 min at room temperature. After repeating this procedure twice, 20 μl of trypsin solution (Promega; Trypsin Gold, Mass Spectrometry Grade; V5280) was added to the sample, followed by incubation for 5 min to allow it to be absorbed into the gel. Subsequently, 20 μl of 50 mM NH_4HCO_3 was added, and the sample was incubated at 37°C overnight with agitation. The supernatant was transferred to a new tube, and 20 μl of 50% (v/v) ACN/0.2% (v/v) formic acid (FA) was added to the remaining gel piece, which was then agitated for 10 min. The FA solution was collected and combined with the digestion solution to produce the peptide solution. The peptide solution was dried in a Refrigerated CentriVap Vacuum Concentrator (LABCONCO). The dried, concentrated peptides were re-dissolved in 20 μl of liquid chromatography (LC)/mass spectrometry (MS) buffer (5% ACN/0.1% FA) and filtered through a 0.45 μm filter (Millipore; Ultrafree-MC). The filtered sample was subjected to LC-MS/MS analysis.

LC-MS/MS analysis and database searching

Liquid chromatography was performed using a Paradigm MS4 HPLC pump (Michrom BioResources) and loaded with an HTC-PAL autosampler (CTC analytics) onto an L-column (100 mm internal diameter, 15 cm; CERI). The peptides were eluted from the L-column by applying a linear gradient from 5 to 45% of solvent B (0.1% [v/v] acetic acid and 90% [v/v] acetonitrile) with solvent A (0.1% [v/v] acetic acid and 2% [v/v] acetonitrile) for 26 min. The eluted peptides were directly introduced into an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer at a flow rate of 500 nl min^{-1} and a spray voltage of 2.0 kV. MS scanning and data acquisition were controlled with Xcalibur software version 2.0.7 (Thermo Fisher Scientific). The range of the MS scan was m/z 400–1,500, and the top three peaks were subjected to MS/MS analysis. The resulting spectra were compared with an in-house BY-2/*35S::VND7-VP16-GR* amino acid sequence database (Ohtani et al. 2016) using the MASCOT server (version 2.4). The MASCOT search parameters were as follows: threshold set at 0.05 in the ion-score cut-off mode, peptide tolerance at 10 ppm, MS/MS tolerance at ±0.8 Da, peptide charge of 2+ or 3+, trypsin as the enzyme, allowing up to one missed cleavage, carbamidomethylation on cysteine as a fixed modification, and oxidation on methionine as a variable modification.

Results and discussion

For our proteomic study, we used transgenic tobacco BY-2 cells carrying the chimeric *VND7-VP16-GR* construct driven by the *Cauliflower mosaic virus* 35S promoter (BY-2/*35S::VND7-VP16-GR*), whose cells can

be induced to differentiate into xylem vessel cells by application of DEX (Goué et al. 2013; Ishii et al. 2017; Ohtani et al. 2016; Yamaguchi et al. 2010). In this system, the first visible sign of SCW deposition was observed after 24 h of DEX treatment, and ultimately, up to 90% of cells transdifferentiated into xylem vessel cells with characteristic helical-patterned SCWs after 60 h of induction (Figure 1; Yamaguchi et al. 2010; Ohtani et al. 2016). To identify changes in protein accumulation before and after the induction of xylem vessel cell differentiation, we performed comparative 2DE-based proteomic analysis, which allowed us to detect changes in protein abundance and post-translational modifications (O'Farrell 1975) in BY-2/*35S::VND7-VP16-GR* cells at 0 and 24 h after induction.

Our 2DE analysis with extracted protein samples successfully detected more than 100 spots derived from protein extracts from BY-2/*35S::VND7-VP16-GR* cells (Figures 2 and 3). By comparing the two electrophoretograms, we identified 18 and 11 spots as upregulated and downregulated polypeptides, respectively, after induction (Figures 2 and 3). To identify these polypeptides, we excised gel regions containing these spots and subjected them to the mass spectrometric (MS) analysis with an LTQ-Orbitrap XL (Thermo Fisher Scientific). As controls, the 18 spots whose expression was stable in both electrophoretograms were also subjected to MS analysis. We analyzed the MS data using the MASCOT search engine (Matrix Science) with transgenic tobacco BY-2 cell contig information (Ohtani et al. 2016) to identify the protein corresponding to each spot. The IDs of the proteins with the top hits for each spot are listed in Table 1.

Among the 18 upregulated proteins, we successfully identified 17 proteins, including putative serine carboxypeptidase and cysteine peptidase, which are highly similar in sequence to Arabidopsis XYLEM CYSTEINE PEPTIDASE 1 (XCP1) and SERINE CARBOXYPEPTIDASE-LIKE 48 (SCPL48), the direct targets of VND7 in Arabidopsis (Table 1; Yamaguchi et al. 2010, 2011; Zhong et al. 2010); the transcriptome data for BY-2/*35S::VND7-VP16-GR* cells show that these genes are upregulated after DEX application (Table 1; Ohtani et al. 2016). Therefore, the accumulation of these proteins appears to depend on the transcriptional upregulation of the underlying genes

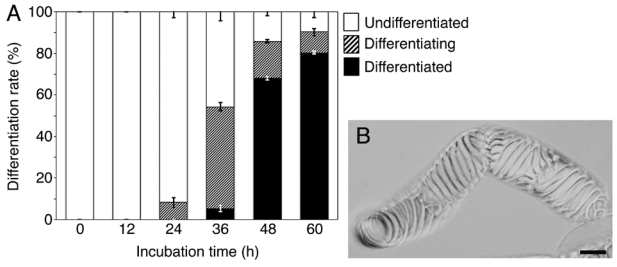

Figure 1. Induction of xylem vessel cell differentiation in BY-2/*35S::VND7-VP16-GR* cells. (A) Ratios of differentiated BY-2/*35S::VND7-VP16-GR* cells after 0, 12, 24, 36, 48, and 60 h of DEX treatment. Undifferentiated (with a clear nucleus and no SCW), differentiating (with a nucleus and a thin helical SCW), and differentiated (with thick SCW lacking nucleus and cytosolic structures) cells were counted for each time point. Data shown are means±SD. (B) Typical BY-2/*35S::VND7-VP16-GR* cells after DEX treatment. The cells had transdifferentiated into xylem vessel-like cells with patterned secondary cell walls. Bar=20 μm.

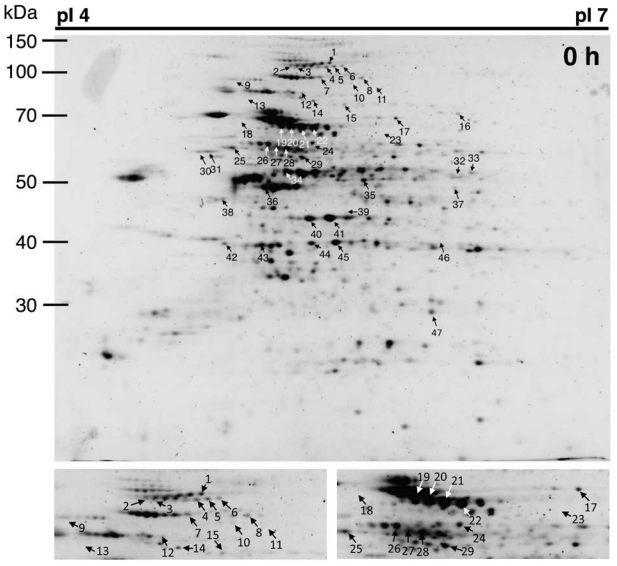

Figure 2. Images of representative 2-dimensional electrophoresis gels of extracted proteins from DEX-treated BY-2/*35S:VND7-VP16-GR* cells at 0 h. Samples were separated by 2DE and stained with fluorescent gel-staining reagent. The numbered spots were excised and subjected to LC-MS/MS analysis to identify the corresponding peptide sequences. Lower panels show magnified images of upper large panel. The numbers are identical to the spot numbers listed in Table 1.

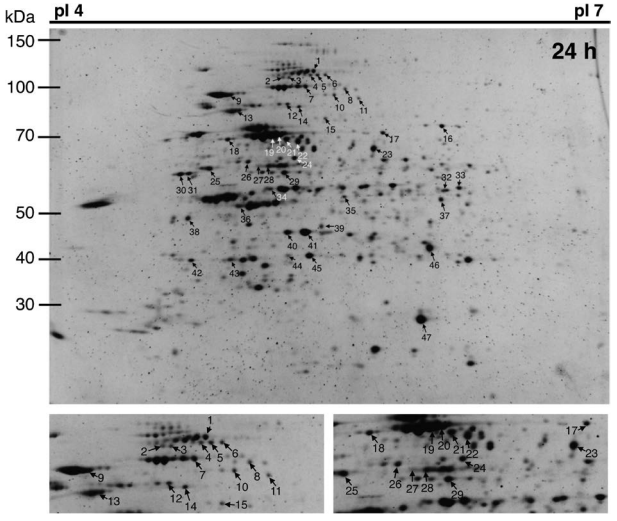

Figure 3. Images of representative 2-dimensional electrophoresis gels of extracted proteins from DEX-treated BY-2/*35S:VND7-VP16-GR* cells at 24 h. Lower panels show magnified images of upper large panel. The numbers are identical to the spot numbers listed in Table 1.

Table 1. Spots from 2DE gels selected for LC-MS/MS analysis.

Spot number	Contig ID[a]	Annotations based on Arabidopsis homologs	Estimated pI	Estimated molecular weight (Da)	Fold changes in transcript abundance (log_2)[b]	
					After 12 h	After 24 h
Upregulated						
2	BY2v7iAc38341	Heat shock protein 91	5.03	106644.29	0.399	0.041
3	BY2v7iAc34984	Glycosyl transferase, family 35	5.35	110347.28	**1.383**	0.837
7	BY2v7iAc10721	26S protease regulatory subunit 6B	5.17	89574.6	1.009	**0.929**
9	BY2v7iAc28974	Heat shock protein 90	4.85	93068.69	**2.016**	**2.330**
11	BY2v7iAc23908	Phospholipase D alpha 1	5.39	92042.17	**−0.561**	**−0.914**
13	BY2v7iAc66918	Heat shock protein 90	4.96	80269.19	−1.089	**−1.354**
16	BY2v7iAc06039	Transketolase	6.16	80101.58	0.243	0.609
18	BY2v7iAc67606	Rhamnogalacturonate lyase family protein	5.18	76158.72	**2.241**	**4.688**
23	BY2v7iAc31882	Polygalacturonase	5.24	49161.2	**0.113**	**−0.901**
30	BY2v7iAc53042	Protein disulfide isomerase	4.99	57072.76	**1.335**	**1.013**
31	BY2v7iAc68270	Protein disulfide isomerase	4.8	54593.03	**1.136**	**0.559**
25	BY2v7iAc47821	Chaperonin-60 alpha	5.3	62099.12	**−1.075**	−0.284
32	BY2v7iAc14230	UDP-glucose pyrophosphorylase 2	5.59	51906.6	0.898	−0.779
33	BY2v7iAc69582	ATP synthase subunit 1	5.84	55323.5	0.420	−0.020
37	BY2v7iAc11000	Translation elongation factor	5.73	47542.71	**−0.726**	**−1.359**
39	BY2v7iAc01254	Serine carboxypeptidase-like 48	5.33	56287.58	**3.782**	**5.240**
46	BY2v7iAc12517	Xylem cysteine peptidase 1	5.64	39861.12	**3.455**	**4.399**
47	BY2v7iAc12517	Xylem cysteine peptidase 1	5.64	39861.12	**3.455**	**4.399**
Downregulated						
19	BY2v7iAc15650	Mitochondrial heat shock protein 70	5.75	73131.94	**−2.981**	**−3.680**
20	BY2v7iAc15650	Mitochondrial heat shock protein 70	5.75	73131.94	**−2.981**	**−3.680**
21	BY2v7iAc15650	Mitochondrial heat shock protein 70	5.75	73131.94	**−2.981**	**−3.680**
22	BY2v7iAc15649	Mitochondrial heat shock protein 70	5.75	73131.94	**−1.238**	**−2.154**
26	BY2v7iAc12576	Heat shock protein 60	5.46	61305.53	**−2.241**	**−3.037**
27	BY2v7iAc12576	Heat shock protein 60	5.46	61305.53	**−2.241**	**−3.037**
34	BY2v7iAc14610	ATP synthase alpha/beta family protein	5.95	59695.07	0.151	−0.110
35	BY2v7iAc71412	Peptidase family M16 protein	5.99	54741.21	0.180	−0.110
36	BY2v7iAc02630	Tubulin 6	4.86	49753.08	0.092	0.194
44	BY2v7iAc19747	Glutamine synthase	5.5	39055.11	**−1.179**	**−1.522**
43	BY2v7iAc10935	Adenosine kinase	5.14	37553.86	**0.913**	**1.725**
Unchanged						
1	BY2v7iAc38341	Heat shock protein 91	5.18	94504.51	0.399	0.041
5	BY2v7iAc65648	Heat shock protein 91	5.27	93262.93	−0.277	−0.456
6	BY2v7iAc65648	Heat shock protein 91	5.27	93262.93	−0.277	−0.456
8	BY2v7iAc52936	Peptidase M1 family protein	5.97	110359.8	**0.871**	**0.705**
10	BY2v7iAc36993	ATP-dependent CLP protease	6.1	102277.11	0.350	0.244
12	BY2v7iAc38930	ENTH/VHS family protein	5.31	76226.48	**0.834**	**1.260**
14	BY2v7iAc38930	ENTH/VHS family protein	5.31	76226.48	**0.834**	**1.260**
15	BY2v7iAc30172	Metalloprotease family protein	6.63	91911.05	−0.166	**−1.123**
17	BY2v7iAc03329	Eukaryotic translation initiation factor	5.51	65503.89	**−0.095**	**−0.257**
24	BY2v7iAc24117	Dihydroxyacetone kinase	5.3	61826.21	0.391	0.160
29	BY2v7iAc03935	Phosphoglucose isomerase 1	5.54	68640.49	**0.476**	0.589
38	BY2v7iAc64626	Rad23 UV excision repair protein family	4.73	41645.78	**0.561**	0.353
40	BY2v7iAc13353	Actin 7	5.31	41697.75	**0.853**	**0.690**
41	BY2v7iAc05569	Actin 7	5.3	41728.78	**0.771**	**0.929**
42	BY2v7iAc04791	Late embryogenesis abundant protein, group 2	4.86	35810.74	**1.250**	**0.957**
45	BY2v7iAc19747	Glutamine synthase	5.5	39055.11	**−1.179**	**−1.522**
28	BY2v7iAc12576	Heat shock protein 60	5.46	61305.53	**−2.241**	**−3.037**
4	BY2v7iAc65648	Heat shock protein 91	5.27	93262.93	−0.277	−0.456

[a] Contigs were generated from RNA-seq data from BY-2/*35S::VND7-VP16-GR* reported in Ohtani et al. (2016). [b] Fold changes in transcript abundance between 0 and 12 or 24 h of DEX treatment were calculated based on the RNA-seq data for BY-2/*35S::VND7-VP16-GR* in Ohtani et al. (2016). Statistically significant changes are shown in bold.

by VND7. However, only eight of the 17 upregulated proteins were upregulated at the transcriptional level after induction (Table 1). A similar disconnect between protein and transcript levels was also observed for some downregulated proteins (Table 1). Of the 11 downregulated spots, we identified eight proteins, half of which previously exhibited no change or an increase in transcript level (Table 1). These findings suggest that post-transcriptional regulation greatly affects protein accumulation during xylem vessel cell differentiation.

We also found that some different but closely localized spots, i.e., spots 1 and 2, spots 19–21, spots 26 and 27, and spots 46 and 47, were derived from identical proteins (Figure 1; Table 1) but likely produced multiple spots due to changes in the molecular weight of each polypeptide. Indeed, Arabidopsis XCP1 is 36 kDa when it contains a Pro domain that suppresses its protease activity, and it is processed to the mature 23 kDa form when the Pro domain is removed (Funk et al. 2002). Perhaps the difference between spots 46 ($<$40 kDa) and 47 ($>$30 kDa), which correspond to a putative tobacco XCP1 homolog, also reflects this type of processing for maturation.

In addition, our list contains multiple heat shock proteins (HSPs) with several spots derived from the same proteins (Table 1). Notably, such spots sometimes exhibited different patterns of abundance after the induction of cell differentiation; for example, three different spots (spots 19–21) for same mitochondrial HSP70 (mtHSP70) protein were detected among downregulated polypeptides (Figures 2 and 3; Table 1). The degree of reduction in protein levels differed among spots; spots 20 and 21 were downregulated at a higher rate than spot 19 (Figures 2 and 3). Similarly, the expression patterns of spots 1 and 2, which were both derived from the same putative HSP91 protein, differed after the induction of xylem vessel cell differentiation (Table 1), as spot 2 appeared in a new position, whereas spot 1 did not (Figures 2 and 3). Moreover, mtHSP70 functions in the regulation of protein folding and intracellular trafficking of proteins (Mashaghi et al. 2016), and this protein can act as an antagonist of apoptosis in mammalian cells (Ravagnan et al. 2001), as well as heat- and H_2O_2-induced PCD in plants (Qi et al. 2011). Thus, the reduced intensity of the spot corresponding to mtHSP70 was not unexpected given that PCD is required for the progression of xylem vessel cell differentiation. The phosphorylation states of HSP family proteins are critical for determining the balance between protein folding and degradation (Muller et al. 2013). Therefore, the different behaviors among spots derived from the same HSP protein suggest that post-translational modification of these HSP proteins involved in the progression of xylem vessel cell differentiation might be actively regulated.

Recent comparative studies between proteomic and transcriptomic data revealed low correlations for a certain number of genes (Cheng et al. 2016; Liu and Aebersold 2016; Peng et al. 2015; Vogel and Marcotte 2012). The expression of functional proteins is tightly regulated at the post-transcriptional level, including translational regulation and post-translational modification (Cheng et al. 2016; Liu and Aebersold 2016; Peng et al. 2015; Vogel and Marcotte 2012). Likewise, our comparative 2DE analysis showed that post-transcriptional regulation has a large impact on the expression of functional proteins during xylem vessel cell differentiation. In-depth proteomic analysis, e.g. shotgun proteomics approach, should provide novel insight into the regulatory mechanisms that guide the various steps of the complex process of xylem vessel cell differentiation, including SCW deposition, proteolysis, PCD, and perforation of the cell wall.

Acknowledgements

We thank Dr. Chiaki Hori (Hokkaido University, Japan), Dr. Arata Yoneda, Dr. Ko Kato, and Dr. Minoru Kubo (Nara Institute of Science and Technology, Japan) for their fruitful discussions and Ms. Nobuko Shizawa and Ms. Rie Kurata (Nara Institute of Science and Technology, Japan) for the technical support. This work was supported in part by Japan Society for the Promotion of Science (KAKENHI Grant Number 25291062 to T.D.), the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grant-in-Aid for Scientific Research on Innovative Areas "The Plant Cell Wall as Information Processing System" Grant Number 25114520 and 15H01235 to M.O., 24114002 to T.D., and Grants-in-Aid from the NC-CARP project to T.D.), and the Exploratory Research for Advanced Technology (ERATO) from Japan Science and Technology Agency (JST) (Grant Number JPMJER1602 to M.O.) and Japan Advanced Plant Science Network.

References

Cheng Z, Teo G, Krueger S, Rock TM, Koh HWL, Choi H, Vogel C (2016) Differential dynamics of the mammalian mRNA and protein expression response to misfolding stress. *Mol Syst Biol* 12: 1–15

Derbyshire P, Ménard D, Green P, Saalbach G, Buschmann H, Lloyd CW, Pesquet E (2015) Proteomic analysis of microtubule interacting proteins over the course of xylem tracheary element formation in Arabidopsis. *Plant Cell* 27: 2709–2726

Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, Minami A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, et al. (2002) Visualization by comprehends microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proc Natl Acad Sci USA* 99: 15794–15799

Endo H, Yamaguchi M, Tamura T, Nakano Y, Nishikubo N, Yoneda A, Kato K, Kubo M, Kajita S, Katayama Y, et al. (2015) Multiple classes of transcriptional factors regulate the expression of *VASCULAR-RELATED NAC DOMAIN7*, a master switch of xylem vessel differentiation. *Plant Cell Physiol* 56: 242–254

Fukuda H (1997) Tracheary element differentiation. *Plant Cell* 9: 1147–1156

Fukuda H (2010) *Plant Tracheary Elements*. John Wiley & Sons. DOI: 10.1002/9780470015902.a0001814.pub2

Fukuda H, Kobayashi H (1989) Dynamic organization of the cytoskeleton during tracheary-element differentiation. *Dev Growth Differ* 31: 9–16

Fukuda H, Komamine A (1980) Establishment of an experimental system for the tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol* 65: 57–60

Fukuda H, Komamine A (1983) Changes in the synthesis of RNA and protein during tracheary element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Cell Physiol* 24: 603–614

Funk V, Kositsup B, Zhao C, Beers EP (2002) The Arabidopsis xylem peptidase XCP1 is a tracheary element vacuolar protein that may be a papain ortholog. *Plant Physiol* 128: 84–94

Goué N, Mortimer JC, Nakano Y, Zhang Z, Josserand M, Ohtani M, Dupree P, Kakegawa K, Demura T (2013) Secondary cell wall characterization in a BY-2 inductive system. *Plant Cell Tiss Org* 115: 223–232

Groover A, Dewitt N, Heidel A, Jones A (1997) Programmed cell death of plant tracheary elements differentiating in vitro. *Protoplasma* 196: 197–211

Ishii T, Matsuoka K, Ono H, Ohnishi-Kameyama M, Yaoi K, Nakano Y, Ohtani M, Demura T, Iwai H, Satoh S (2017) Characterization of xylan in the early stages of secondary cell wall formation in tobacco bright yellow-2 cells. *Carbohydr Polym* 176: 381–391

Ito Y, Nakanomyo I, Motose H, Iwamoto K, Sawa S, Dohmae N, Fukuda H (2006) Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 313: 842–845

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T (2005) Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev* 19: 1855–1860

Li Z, Omranian N, Neumetzler L, Wang T, Herter T, Usadel B, Demura T, Giavalisco P, Nikoloski Z, Persson S (2016) A transcriptional and metabolic framework for secondary wall formation in Arabidopsis. *Plant Physiol* 172: 1334–1351

Liu Y, Aebersold R (2016) The interdependence of transcript and protein abundance: New data-new complexities. *Mol Syst Biol* 12: 856

Mashaghi A, Bezrukavnikov S, Minde DP, Wentink AS, Kityk R, Zachmann-Brand B, Mayer MP, Kramer G, Bukau B, Tans SJ (2016) Alternative modes of client binding enable functional plasticity of Hsp70. *Nature* 539: 448–451

Molloy MP, Herbert B, Walsh BJ, Tyler MI, Traini M, Sanchez JC, Hochstrasser DF, Williams KL, Gooley AA (1998) Extraction of membrane proteins by differential solubilization for separation using two-dimensional electrophoresis. *Electrophoresis* 19: 837–844

Muller P, Ruckova E, Halada P, Coates PJ, Hrstka R, Lane DP, Vojtesek B (2013) C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. *Oncogene* 32: 3101–3110

Nakano Y, Yamaguchi M, Endo H, Rejab NA, Ohtani M (2015) NAC-MYB-based transcriptional regulation of secondary cell wall biosynthesis in land plants. *Front Plant Sci* 6: 228

Oda Y, Mimura T, Hasezawa S (2005) Regulation of secondary cell wall development by cortical microtubules during tracheary element differentiation in Arabidopsis cell suspensions. *Plant Physiol* 137: 1027–1036

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007–4021

Ohashi-Ito K, Oda Y, Fukuda H (2010) *Arabidopsis* VASCULAR-RELATED NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. *Plant Cell* 22: 3461–3473

Ohtani M, Morisaki K, Sawada Y, Sano R, Uy AL, Yamamoto A, Kurata T, Nakano Y, Suzuki S, Matsuda M, et al. (2016) Primary metabolism during biosynthesis of secondary wall polymers of protoxylem vessel elements. *Plant Physiol* 172: 1612–1624

Peng X, Qin Z, Zhang G, Guo Y, Huang J (2015) Integration of the proteome and transcriptome reveals multiple levels of gene regulation in the rice *dl2* mutant. *Front Plant Sci* 6: 351

Qi Y, Wang H, Zou Y, Liu C, Liu Y, Wang Y, Zhang W (2011) Over-expression of mitochondrial heat shock protein 70 suppresses programmed cell death in rice. *FEBS Lett* 585: 231–239

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jäättelä M, Penninger JM, Garrido C, et al. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 3: 839–843

Tokunaga N, Uchimura N, Sato Y (2006) Involvement of gibberellin in tracheary element differentiation and lignification in *Zinnia elegans* xylogenic culture. *Protoplasma* 228: 179–187

Turner S, Gallois P, Brown D (2007) Tracheary element differentiation. *Annu Rev Plant Biol* 58: 407–433

Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* 13: 227–232

Yamaguchi M, Demura T (2010) Transcriptional regulation of secondary wall formation controlled by NAC domain proteins. *Plant Biotechnol* 27: 237–242

Yamaguchi M, Goué N, Igarashi H, Ohtani M, Nakano Y, Mortimer JC, Nishikubo N, Kubo M, Katayama Y, Kakegawa K, et al. (2010) VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. *Plant Physiol* 153: 906–914

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) VASCULAR-RELATED NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *Plant J* 55: 652–664

Yamaguchi M, Mitsuda N, Ohtani M, Ohme-Takagi M, Kato K, Demura T (2011) VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. *Plant J* 66: 579–590

Yamamoto R, Demura T, Fukuda H (1997) Brassinosteroids induce entry into the final stage of tracheary element differentiation in cultured *Zinnia* cells. *Plant Cell Physiol* 38: 980–983

Zhong R, Lee C, Ye ZH (2010) Global analysis of direct targets of secondary wall NAC master switches in *Arabidopsis*. *Mol Plant* 3: 1087–1103

Zhu J, Chen S, Alvarez S, Asirvatham VS, Schachtman DP, Wu Y, Sharp RE (2006) Cell wall proteome in the Maize primary root elongation Zone. I. extraction and identification of water-soluble and lightly ionically bound proteins. *Plant Physiol* 140: 311–332