Journal of Molecular Catalysis B: Enzymatic 58 (2009) 84–92

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Release of proteins: Insights into oxidative response of *Taxus cuspidata* cells induced by shear stress

Jing-Sheng Cheng, Ying-Jin Yuan*

Key Laboratory of Systems Bioengineering (Tianjin University), Ministry of Education, Department of Pharmaceutical Engineering, School of Chemical Engineering & Technology, Tianjin University, P.O. Box 6888, Tianjin 300072, PR China

ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 17 November 2008
Accepted 17 November 2008
Available online 25 November 2008

Keywords:
Extracellular proteins
Shear stress
Taxus cuspidata cells
Oxidative response

ABSTRACT

The sensitivity of plant cells to shear stress has been regarded as one obstacle to the scale-up of plant cell suspension cultures. For better understanding on molecular levels of oxidative responses caused by laminar shear stress, extracellular proteins of the suspension cultured *Taxus cuspidata* cells were investigated by two-dimensional-liquid chromatography–tandem mass spectrometry (2D LC–MS/MS). Totally 35, 73, and 70 proteins in media were identified in shake flask (3 h) and a Couette-type shear device after laminar shear treatment (3 h and 6 h), respectively. These proteins were, respectively, categorized into different kinds of bioprocesses including stress responses, detoxification, transporter, cell wall catabolic and glycolysis and various metabolisms including carbohydrate, energy, and sulfur. Some cytoplasm proteins were released into the media after shear treatment. The glutathione S-transferase, aldo/keto reductase, peroxidase 3 precursor, oxidoreductase associated with oxidative stress and heat shock proteins, transport-associated proteins were only monitored in shear-treated cells. Particularly, shear treatment-induced accumulation of geranylgeranyl diphosphate synthase and isopentenyl diphosphate delta isomerase, which closely associated with Taxol biosynthesis. These findings provide new insights into better understanding of the molecular and signal basis of plant cell cultures in response of oxidative stress induced by shear stress.

1. Introduction

Plant cell cultures have been developed as a promising alternative for the production of secondary metabolites, Taxol, one of the most potent antitumor drugs [1]. The sensitivity of plant cells to shear stress as one of engineering issues has hampered the scale-up cultivation of plant cell suspension cultures [2,3]. Shear stress triggers a variety of biochemical and physical changes in cell structure and function [4]. Valda J. Vinson (Science Editor) said that cells exerted and responded to mechanical forces, but investigating how these signals were transduced was a challenge. Johnson et al. reported the co-localized signaling events such as phosphorylation with forced unfolding through monitoring cellular mechanical stress [5]. Some researches have manifested that some signaling molecules in plant cells, including intracellular calcium [6], reactive oxygen species (ROS) [7], mitogen-activated protein (MAP) kinase [8], are involved in response to mechanical stimulation. Our previous studies have also shown that shear stress induced burst of ROS [9] and nitric oxide [10], extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)-like mitogen-activated protein kinase (MAPK) phosphorylation [11], and RGD-dependent mechanotransduction [12]. Jeffers et al. [13] emphasized the importance of considering both oxidative and anti-oxidative capacities as part of a holistic approach to the determination of the oxidative burst in hydrodynamically stressed plant cell suspension cultures. Although the effects of hydrodynamic shear on plant cells have been widely investigated, the molecular and signal basis of oxidative responses induced by shear stress is still unclear. Therefore, identification of the oxidative stress response-related proteins will help us to understand the response of cultured plant cells to shear stress.

Proteomics provided a comprehensive assessment of stress response bioprocesses [14–16]. For example, in vascular endothelial cells, some proteins associated with cellular functions and many signaling pathways were up-/down-regulated by laminar shear stress [14]. Secretion of proteins into the growth environment plays an important role in the physiology and metabolism of prokaryotic and eukaryotic organisms [15,16]. Proteomic approaches were employed to identify changes in the extracellular proteome of *B. subtilis* in response to secretion stress [15]. Extracellular protein kinases have been implicated in extracellular signal perception and transduction during animal cell growth and development [17]. Gilchrist et al. [18] reported proteins of the secretory-pathway proteome and provided a comprehensive catalog of the ER and

* Corresponding author. Fax: +86 22 27403888.
E-mail addresses: yjyuan@tju.edu.cn, yjyuan@public.tpt.tj.cn (Y.-J. Yuan).

1381-1177/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2008.11.012

Golgi proteomes with insight into their identity and function by proteomic analysis. Accumulation of maize elicitor-induced secretion proteins also revealed the extracellular matrix plays a complex role in defense response [19]. It has been reported that fungal elicitors caused extracellular accumulation of xyloglucan endo-1,4-β-D-glucanase and peroxidase in *Arabidopsis* cell suspension cultures [20].

Based on these findings, some extracellular proteins might be associated with the oxidative responses of plant cells to shear stress. Here, we investigated the shear stress elicited the changes of extracellular proteins in media of the suspension cultured *Taxus cuspidata* cells by two-dimensional-liquid chromatography–tandem mass spectrometry (2D LC–MS/MS). We found some differentially expressed proteins in media while *T. cuspidata* cells were exposed to laminar shear stress in Couette-type shear device, which provides new insights that can help us to better understand the molecular basis and signal mechanism that mediates the oxidative responses of *T. cuspidata* cells to shear stress.

2. Materials and methods

2.1. Culture conditions

The cell line was derived from young stems of *T. cuspidata* and sub-cultured in modified B5 solid medium containing sucrose (25.0 g/L), α-naphthylacetic acid (2.0 mg/L), and 6-benzyladenine (0.15 mg/L). The medium pH was adjusted to 5.8 prior to autoclaving. Cells were cultured for 5 generations in liquid B5 medium at 25 °C with shaking at 110 rpm in the dark. Fresh cells (3.0 g) of the fifth generation were inoculated in 50 mL fresh modified B5 medium for freely suspended cultures at 25 °C in a 250 mL Erlenmeyer flask.

2.2. Cell cultured in the Couette-type shear reactor

The *T. cuspidata* cells were cultured in the Couette-type shear device as described previously [9,11]. The shear device was sterilized before inoculating. The 8-day-old suspension culture of *T. cuspidata* cells in the exponential growth phase (80 mL) was directly loaded into the shear device maintaining 25 °C. The shear rate corresponding to the rotation speed of 80 rev./min was calculated as 152 s^{-1}. The 15 mL suspension sample was collected for analysis at predetermined time.

2.3. Detection of H_2O_2 by 3,3′-diaminobenzidine (DAB) staining

H_2O_2 was visualized by staining with DAB [21]. Briefly, the suspension culture cells were pre-treated with a 1 mg/mL solution of DAB (pH 3.8) for 8 h in the dark at 25 °C. Alternatively, the DAB solution was supplemented with 20 mM ascorbic acid for certain studies of H_2O_2 in relation to cells. The DAB-treated cells were inoculated into the Couette-type shear device. The experiments were terminated by immersion of the treated cells in boiling ethanol (96%) for 10 min. After cooling, the cells were extracted at room temperature with fresh ethanol for 4 h and were preserved at room temperature in ethanol and photographed with a microscope (Eclipse E800; Nikon, Melville, NY).

2.4. Evans Blue staining

Evans blue staining was carried out following the procedure of Suzuki et al. with slight modification [22]. The cells was incubated for 5 min with a 1% (w/v) Evans Blue at room temperature and subsequently washed five times with the culture medium to remove excess stain. The stained cells were observed with a microscope (Eclipse E800; Nikon, Melville, NY). Images were captured with a Spot RT CCD camera (Diagnostic Instruments, Inc.) and Spot ver. 1.1 CE software.

2.5. Preparation of proteins in media and two-dimensional gel electrophoresis (2DE) analysis

Proteins were collected from media essentially as described by Antelmann et al. [15] with a slight modification. In brief, 1 mM phenylmethanesulfonyl fluoride (PMSF) was added when the cultures were harvested to prevent proteolysis during sample preparation. To remove cells, a 15 mL suspension sample was harvested by centrifugation at 3000 × *g* for 5 min. A further centrifugation step at 10,000 × *g* was performed for 30 min to gain cell-free supernatant. The cold trichloroacetic acid was then added to the 10 mL supernatant to a final concentration of 10%. The mixture was left on ice for 1 h and precipitated proteins subsequently collected by centrifugation (15,000 × *g* for 30 min at 4 °C). The supernatant was removed and the protein pellet washed at least four times with ice-cold acetone. The precipitate was freeze-dried and dissolved in 100 μL of rehydration buffer (8.0 M urea, 2.0% 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate, 20 mM dithiothreitol, 0.5% (v/v) IPG Buffer pH 4–7 (Amersham Pharmacia Biotech, Piscataway, USA), and protein content was determined according to the Bradford method [23].

2DE was carried out as described previously [24]. Briefly, the isoelectric focusing (IEF) was carried out using IPG strips (18-cm-long, pH 4–7, GE Healthcare). Proteins were loaded in 350 μL rehydration buffer and focused on an IPGphor apparatus (Amersham Biosciences). IEF was carried out under the following conditions: 30 V for 12–14 h, 500 V for 1 h, 1000 V for 1 h and 8000 V for 8 h. After IEF followed by equilibration in a sodium dodecyl sulfate (SDS) equilibration buffer (50 mM Tris–HCl of pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (m/v) SDS, 1% (m/v) DTT and trace amount of bromophenol blue), the proteins were separated on 12.5% SDS-PAGE gels and electrophoresis was run at 10 °C until the dye front reached the bottom of the gel. Each experiment was repeated three times.

2.6. Analysis of 2D gel and protein identification by MALDI-TOF-MS

Analysis of two-dimensional (2D) gel and protein identification by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-TOF-MS) were according to our previous methods [24].

2.7. Proteins in media directly analyzed by 2D-LC–MS/MS

The proteins in media were analyzed with 2D-LC–MS/MS (LCQ DecaXP MAX, Thermo Finnigan, Palo Alto, CA) as described previously [25].

2.7.1. Analysis of MS/MS data

Protein identification was performed with Bioworks version 3.1 (Thermo Finnigan) and SEQUEST algorithm. The raw MS/MS data were searched against the nonredundant protein database (updated 14 July 2007) that were download as FASTA formatted sequences from the National Center for Biotechnology Information (http://www.ncbi.nih.gov). DTA files were generated from the MS/MS threshold of 100,000, peptide mass tolerance 2.5 Da, fragment ion tolerance 0.5 Da, and minimum ion count of 35%. A tryptic enzyme restriction with maximum of two internal missed cleavage sites was used. A molecular mass of 57 Da was added to the static search of all cysteins to account for carboxamidomethylation. The lowest Xcorr values of the peptide were respectively set as 1.5 (+1 charge), 2.0 (+2 charge), and 2.5 (+3 charge), and DeltCn must larger

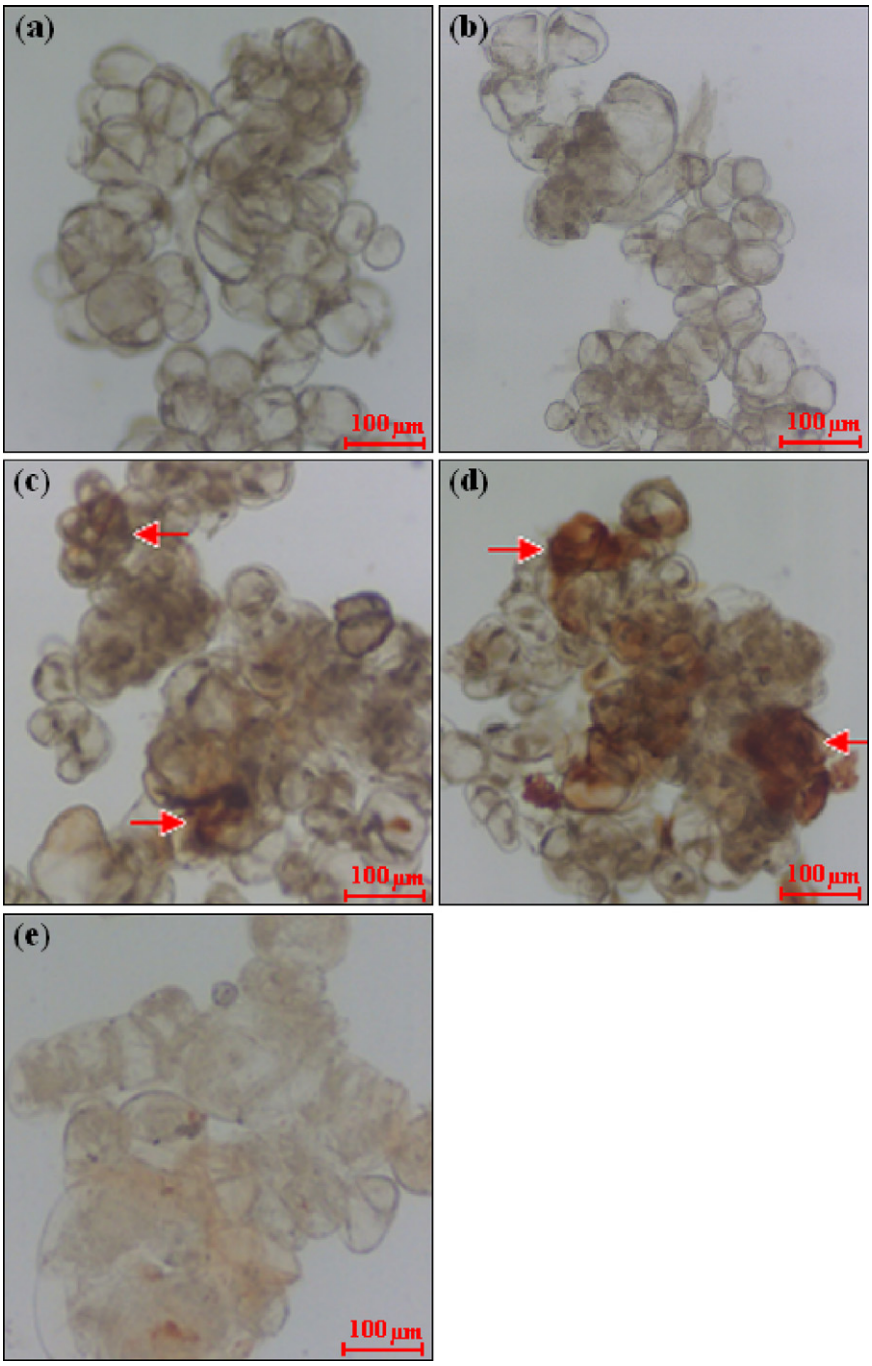

Fig. 1. Histochemical detection of H_2O_2 in *T. cuspidata* cells by DAB staining: (a and b) cells in shake flask for 3 h and 6 h, respectively; (c and d) cells treated with shear for 3 h and 6 h; (e) cells in the presence of ascorbic acid before treatment with shear for 6 h, as described in Section 2.

than 0.08. The MS/MS spectrum must be of good quality with fragment ions clearly above the baseline noise and there must be a continuous y-type and b-type ion series.

2.7.2. Bioinformatics annotation and protein classification

The theoretical p*I* and molecular weight based on the primary amino acid sequence of the identified proteins were calculated using the Expert proteins Analysis System (Expasy) web server (http://us.expasy.org/tools/pi_tool.html). The protein sub-cellular localization prediction was carried out using the program WolfPSORT (http://wolfpsort.seq.cbrc.jp/). The functional categories of identified proteins were elucidated by UniProtKB (http://beta.uniprot.org/uniprot/).

3. Results and discussion

3.1. Effects of shear stress on H_2O_2 generation, membrane permeability, and extracellular protein contents in T. cuspidata cells

The oxidative burst was taken as markers for induction of defense response. Recently, more researchers focused on the oxidative burst under shear stresses [9,10,13]. The production of reactive oxygen and nitrogen species were accompanied by alkalinization of the extracellular medium [9,10]. H_2O_2 generation in cells can be detected as a strong reddish-brown color with DAB staining [21]. As illustrated in Fig. 1, a strong H_2O_2-dependent DAB staining was

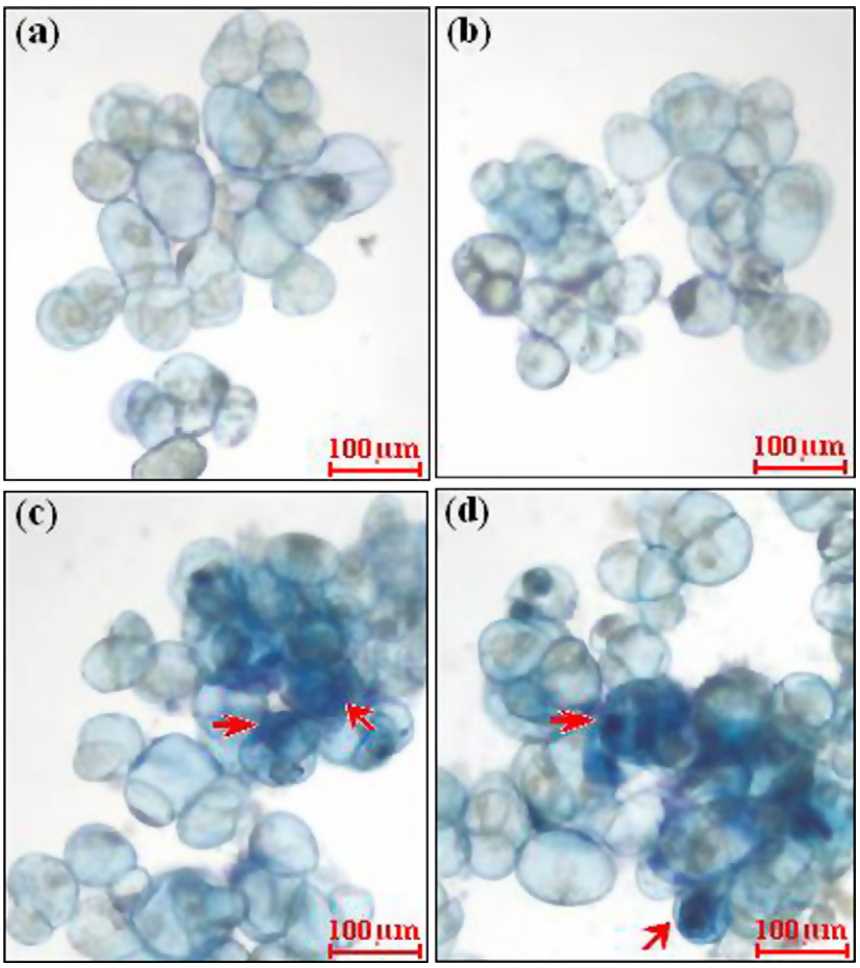

Fig. 2. Cell death was evaluated by Evans Blue staining: (a and b) cells in shake flask for 3 h and 6 h, respectively; (c and d) cells treated with shear for 3 h and 6 h.

observed in shear-treated *T. cuspidata* cells at 3 h and 6 h (Fig. 1c and d), while DAB staining of cells in shake flask was slightly monitored at 3 h and 6 h (Fig. 1a and b). Meanwhile, the presence of ascorbic acid, H_2O_2 scavenger, obviously decreased the degree of DAB staining (Fig. 1e).

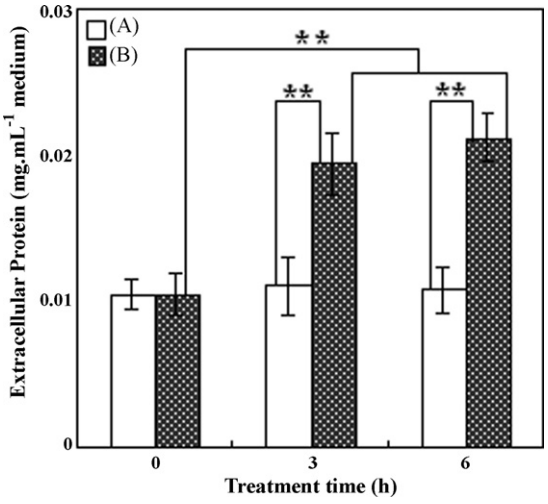

Fig. 3. Changes of protein content in media of the suspension cultured *Taxus cuspidata* cells: (A) in shake flask and (B) treated with shear. $^{**}p < 0.01$ (Student's *t*-test) with respect to the cells in shake flask.

As shown in Fig. 2 c and d, the Evans Blue-positive cells few detected in cells in shake flask 3 h and 6 h. However, shear stress increased the number of Evans Blue-positive cells, indicating that the cell membranes permeability were increased. Evans blue staining of tissue can show loss of membrane integrity [26]. Shear stress-induced H_2O_2 generation and enhanced the membrane permeability (Figs. 1 and 2), which likely led to some intercellular proteins release into media.

As shown in Fig. 3, concentration of proteins in shake flask is approximate 10 μg mL^{-1}, while it reached to 20 μg mL^{-1} after treatment with shear. The changes of extracellular proteins are likely related to increase of the membrane permeability in shear-treated cells. There was significant difference in extracellular protein contents between cells in the shake flask and in shear device after treatment 3 h and 6 h, while there was hardly any difference among 0 h, 3 h and 6 h in shake flask. Our previous works also showed that shaker flask with 110 rpm did not significantly impact on behaviors of *Taxus* cells in short term [9,10,12,43]. The following investigation was carried out in shake flask for 3 h and after treatment with shear for 3 h and 6 h in view of extracellular proteins.

3.2. Sub-cellular location of the proteins in media

Extracellular proteins analysis can provide a comprehensive assessment of various bioprocesses. To profile extracellular proteins, the 80 μg proteins were firstly analyzed using 2D-LC–MS/MS. Total 35, 73 and 70 proteins were identified for that in shake flask (3 h) and after treatment with shear (3 h and 6 h), respectively. We

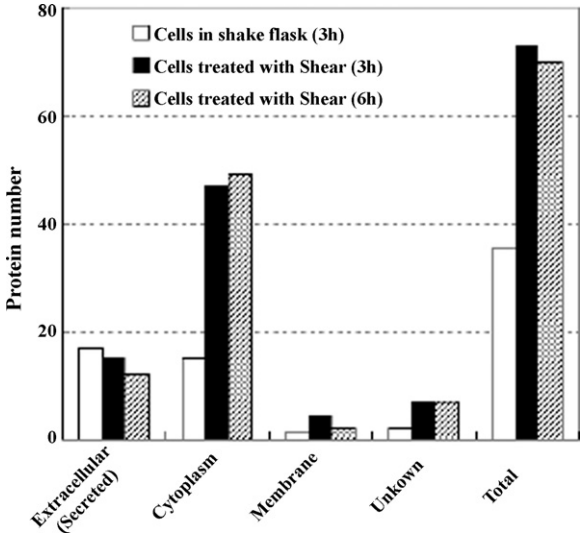

Fig. 4. Sub-cellular location of identified proteins in media of the suspension cultured *T. cuspidata* cells by 2D LC–MS/MS.

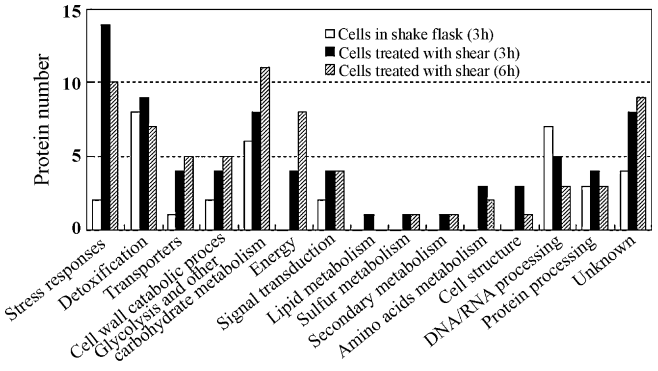

Fig. 5. Functional categories of identified proteins by 2D LC–MS/MS: (a) shake flask 3 h; (b) treatment with shear for 3 h; (c) treatment with shear for 6 h. In brackets are given the absolute numbers of identified proteins in each group.

applied the WolfPSORT to predict the sub-cellular location for the total identified proteins by 2D LC–MS/MS. Fig. 4 shows the predicative results of the located proteins in shake flask (3 h) and after treatment with shear (3 h and 6 h). Of the totally predicted proteins, 48.57% (17), 20.55% (15) and 17.14% (12) were extracellular/secreted proteins in shake flask (3 h) and after treatment with shear (3 h and 6 h), respectively. The 42.86% (15), 64.38% (47) and 70.00% (49) were, respectively, classified as cytoplasmic proteins in shake flask (3 h) and after treatment with shear (3 h and 6 h), indicating that shear stress resulted in the cytoplasmic proteins releasing into media. Some non-secreted proteins including enolase, triose phosphate isomerase, phosphoglucomutase, calmodulin, aconitase, and malate dehydrogenase were also detected in extracellular proteins with high-throughout MS/MS [27]. Vega et al. [28] also reported that the major stress-induced HSP70 was released into the extracellular environments in a membrane-associated form, sharing the characteristics of this protein in the plasma membrane. It is likely that shear stress induces oxidative burst and markedly increases cell membrane permeability.

3.3. Functional category distribution of proteins in media by LC–MS/MS analysis

As shown in Fig. 5, according to protein function or cellular location, the 73 identified proteins after treatment with shear (3 h) were divided into 15 different subgroups including stress response 19.18% (14), detoxification 12.33% (9), transporters 5.48% (4), cell wall catabolic process 5.48% (4), glycolysis and other carbohydrate metabolism 10.96% (8), energy 5.48% (4), signal transduction 5.48% (4), lipid metabolism 1.37% (1), secondary metabolism 1.37% (1), sulfur metabolism 1.37% (1), amino acids metabolism 4.11% (3), cell structure 4.11% (3), DNA/RNA processing 6.84% (5), protein processing 5.48% (4). Besides lipid metabolism, the 70 identified proteins after treatment with shear (6 h) were divided into above 13 subgroups (Fig. 5). However, the 35 proteins in shake flask were not detected to associate with cell structure, energy, sulfur metabolism, lipid metabolism and amino acids metabolism (Fig. 5). The numbers of the characterized extracellular proteins after treatment with shear (3 h and 6 h) were significant higher than that of cells in shake flask (Fig. 4), specially among the proteins closely associated with stress response, detoxification, transporter, cell wall catabolic process, glycolysis and other carbohydrate metabolism in shear-treatment *T. cuspidata* cells (Fig. 5). Additionally, unknown function proteins, including entries described as genes of no characterization and hypothetical or unnamed proteins, took part 11.42%, 10.96%, and 12.86% of the total identified proteins in shake flask (3 h) and after treatment with shear (3 h and 6 h).

3.4. Functional analysis of the identified proteins in media

3.4.1. Stress response and detoxification-related proteins

We detected several proteins associated with oxidative stress and defense responses, including thaumatin-like protein, heat

Table 1
List of proteins associated with stress response in media of the suspension cultured *Taxus cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
22830595	Thaumatin-like protein	22830595	Thaumatin-like protein	22830595	Thaumatin-like protein
60543122	Thaumatin-like protein	60543122	Thaumatin-like protein	60543122	Thaumatin-like protein
		227782	Heat shock protein 83	227782	Heat shock protein 83
		92893803	Heat shock protein Hsp70	92893803	Heat shock protein Hsp70
		109238641	Cystatin Hv-CPI3	109238641	Cystatin Hv-CPI3
		585076	Glucan endo-1,3-beta-glucosidase GIV	585076	Glucan endo-1,3-beta-glucosidase GIV
		92894943	Heat shock protein Hsp70	92872149	Heat shock protein Hsp70
		50947327	Heat shock protein 82	81074298	Heat shock protein 90-2-like
		86439735	Heat shock protein 90	15241102	Heat shock protein 81-4
		92868654	Heat shock protein Hsp70 oxidative stress proteomics extracellular	476003	Heat shock protein Hsp70
		7707812	Multicystatin		
		71794641	Putative cystatin		
		13661831	Disease resistance protein RPS2		
		84468288	Putative rubisco subunit binding-protein alpha subunit		

Table 2
List of proteins associated with detoxification in media of the suspension cultured *T. cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
15237190	Peroxidase	15237190	Peroxidase	15237190	Peroxidase, putative
53792939	Putative bacterial-induced peroxidase precursor	53792939	Putative bacterial-induced peroxidase precursor		
217997	Peroxidase	5002344	Peroxidase 3 precursor	5002344	Peroxidase 3 precursor
2944417	Peroxidase FLXPER4	62526573	Aldo/keto reductase	62526573	Aldo/keto reductase
577503	Cationic peroxidase	92894223	Glutathione peroxidase	538430	Superoxide dismutase
55057258	Peroxidase	78708648	Oxidoreductase, aldo/keto reductase family	11385459	Glutathione S-transferase GST 22
66840760	Peroxidase	87294807	Glutathione S-transferase	11385447	Glutathione S-transferase GST 16
77553822	L-Ascorbate peroxidase 5	10862871	Glutathione reductase	18398691	Disulfide oxidoreductase/oxidoreductase
		577503	Cationic peroxidase		

shock proteins (HSP70, HSP82, HSP83, HSP90), cystatins, glucan endo-1,3-beta-glucosidase GIV in media after shear treatment (Table 1). However, HSPs were not detected in media of shake flask. Lee et al. [29] demonstrated that HSPs played an essential part in protein folding and other cellular processes under stress conditions in plants. Wang et al. [14] also reported that shear stress dramatically increased HSP70 protein expression of vascular endothelial cells. Therefore, our results implicate that HSPs are involved in response of *T. cuspidata* cells to shear stress.

We herein also investigated the extracellular accumulation of various detoxification enzymes including superoxide dismutase (SOD), glutathione S-transferases (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and aldo/keto reductase AKR after shear treatment. However, these enzymes were not detected in extracellular proteins of cells in shake flask (Table 2). Han and Yuan reported that GST activation in *T. cuspidata* cells played a crucial role in oxidative response of shear stress [10]. Our previous works confirmed that GSTs were involved in the detoxification and limiting oxidative damage in two-liquid-phase suspension cultures (TLPSC) [30]. It was also demonstrated that the increases of GPx [31] and GR [32] activity may be important mechanisms whereby shear stress protects vascular or endothelial cells against oxidative stress. It was evidence that the peroxidases might play a role in the oxidative burst induced by the hydrodynamic stress which induced oxindole alkaloid production [33]. Extracellular peroxidases in suspended *T. cuspidata* cells were involved in regulation of oxidative responses induced by Ce^{4+} [34]. Takeshita et al. [31] reported that laminar shear stress up-regulated SOD expression in endothelial cells. Oleic acid in TLPSC enhanced the levels of antioxidant enzymes of SOD, ascorbate peroxidase (APX), and catalase and the anti-oxidant capacity of reduced ascorbate and glutathione [30]. As shown in Table 2, APX was only detected in shake flask. In *Arabidopsis*, the induction of heat shock proteins during light stress is mediated by H_2O_2 that is scavenged by APX [35]. It was likely that lack of this enzyme in media resulted in H_2O_2 accumulation after shear treatment. In addition, our results also displayed that multicystatin, putative cystatin and cystatin Hv-CPI3 were induced after shear treatment (Table 1). Plant cystatins took as one of defense strategies against various biotic and abiotic stresses [36]. These findings indicate that not only various detoxification-related proteins are involved in cellular redox homeostasis of *T. cuspidata* cells under shear conditions, but also that anti-oxidation and detoxification ultimately are related to sulfur metabolism, particularly to cysteine synthase, in response of *T. cuspidata* cells to shear stress. Therefore, changes of the oxidative response-related proteins could play important roles in shear-induced anti-oxidative stress.

3.4.2. Transport and signal transduction-related proteins

As shown in Table 3, the signal transduction-related proteins calmodulins were only monitored in media after treatment with shear (Table 3). In contrast, only protein kinases were detected in shake flask. Yang et al. [37] reported the presence of a calcium/calmodulin-regulated receptor-like cytoplasmic kinase and its possible role in stress signal transduction in plants. Recently, it was also found that calmodulin-like activities were necessary for the downstream signaling events that lead to cross-tolerance between wounding and salt stress [38]. We also confirmed that G-protein and Ca^{2+} channel were involved in the signal pathway for oxidative bursts induced by shear stress [9]. It have been reported that burst of ROS [9] and nitric oxide [10], JNK [11] and ERK [12], tyrosine [4] phosphorylation were involved in responses to shear stress. Therefore, these identified extracellular proteins might be integrated together into the shear response signal transduction pathways.

According to sub-cellular location and functional classification of identified proteins, Table 3 shows that four proteins (including metal ion binding protein, calcium ion binding protein, and copper chaperone) are detected after shear treatment (3 h), while five transporter-related proteins (including copper chaperone, protein transporter, GTP-binding nuclear protein Ran-A1, vacuolar proton

Table 3
List of proteins associated with transporters and signal transduction in media of the suspension cultured *T. cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
15227732	Calcium ion binding/peptidase	15227732	Calcium ion binding/peptidase		
		50942155	Putative copper chaperone	50942155	Putative copper chaperone
		15223663	Metal ion binding	79315642	Protein transporter
		53748477	Copper chaperone	1172835	GTP-binding nuclear protein Ran-A1
				12585490	Vacuolar proton pump subunit alpha
				3293271	Acyl carrier protein
92884722	Protein kinase	2388889	Calmodulin	2388889	Calmodulin
42563204	ATP binding/protein kinase (protein kinase family protein)	166655	Calmodulin-3	166655	Calmodulin-3
		15219675	ATP binding/protein kinase (protein kinase family protein)	49066035	Calmodulin
		92870245	Protein kinase	77554291	RuBisCO subunit binding-protein alpha subunit

Table 4
List of proteins associated with metabolisms (glycolysis and carbohydrate, secondary, and sulfur) in media of the suspension cultured *T. cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
		1087071	Enolase	1087071	Enolase
		78707886	Enolase	78707886	Enolase
		7650502	Triosephosphate isomerase	7650502	Triosephosphate isomerase
		6166505	Putative cytosolic phosphoglucomutase	6166505	Putative cytosolic phosphoglucomutase
86198284	Alpha-amylase	82623425	Enolase-like	609262	Triosephosphate isomerase
1220451	Alpha-amylase	90194338	Enolase	556171	Triosephosphate isomerase
32481073	Auxin-induced beta-glucosidase	61162203	Beta-D-galactosidase	92898538	Fructose-bisphosphate aldolase, class-I
54291174	Putative beta-galactosidase	57900303	Putative endo-1,3-beta-glucanase	15236768	Fructose-bisphosphate aldolase, putative
6573750	F20B24.8	2499710	Phospholipase D alpha 1 precursor	83283995	Fructose-bisphosphate aldolase-like protein
15230897	Glycosyl hydrolase family 3 protein			170757	Amylase (1 could be 2 or 3)
				599723	Aconitase
		45505274	Geranylgeranyl diphosphate synthase	45505274	Geranylgeranyl diphosphate synthase
		726032	S-adenosylmethionine synthetase	68655466	Putative S-adenosylhomocystein hydrolase 2

Table 5
List of proteins associated with cell wall catabolic process in media of the suspension cultured *T. cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
77551733	Barwin, putative, expressed	77551733	Barwin, putative, expressed	77551733	Barwin, putative, expressed
73991303	Putative class I chitinase	73991303	Putative class I chitinase	73991303	Putative class I chitinase
		21426919	Chitinase	21426919	Chitinase
		40806506	Basic class I chitinase	40806506	Basic class I chitinase
				6002766	Class II chitinase

Table 6
List of proteins associated with energy in media of the suspension cultured *T. cuspidata* cells by LC–MS/MS.

Shake flask (3 h)		Treatment with shear (3 h)		Treatment with shear (6 h)	
GI no.	Similar protein name	GI no.	Similar protein name	GI no.	Similar protein name
		39939491	Malate dehydrogenase	39939491	Malate dehydrogenase
		11991527	Mitochondrial formate dehydrogenase precursor	11991527	Mitochondrial formate dehydrogenase precursor
		15241592	Succinyl-CoA ligase (GDP-forming) alpha-chain	15241592	Catalytic/succinyl-CoA ligase (GDP-forming) alpha-chain
		92881487	Malate dehydrogenase, active site	24638017	Malate dehydrogenase, mitochondrial precursor
				30693852	6-Phosphogluconate dehydrogenase family protein
				21263612	Formate dehydrogenase, (NAD-dependent formate dehydrogenase) (FDH)
				4760483	Monodehydroascorbate reductase
				108706389	Cytochrome P450 family protein, expressed

pump subunit alpha, acyl carrier protein) were monitored after shear treatment (6 h). In contrast, we could only monitor calcium ion binding/peptidase in shake flask. Some cell structure-related proteins like K^+ efflux antiporter, profilin, and annexin were also detected in media after shear treatment 3 h and 6 h, respectively. It was reported that profilin had the ability to act as a regulator of actin-dependent events [39]. Therefore, it could affect the structure of the cytoskeleton by regulating the organization of actin filaments [40]. Plant annexins, Ca^{2+}- and membrane-binding proteins, were probably implicated in the cellular response to stress resulting from acidification of cytosol which also played a role in intracellular ion homeostasis [41]. In plants, regulation of ion homeostasis was fundamental to physiological activities. The plasma membrane Na^+/H^+ antiporter was the only key enzyme that extrudes cytosolic Na^+ and contributes to salt tolerance [42]. In our group, previous works found a pH increase in *Taxus* spp. cell cultures exposed to laminar shear stress [9,12,43]. Some signaling molecules for the stress resistance pathway and enzymes were required for cell wall modification [44]. These results possibly implicated shear directly damaged the cell wall and membrane, or activated the enzymes associated with the degradation of membrane and cell wall polysaccharides, which caused cell structure proteins release into media. Another reason was that shear changed the secrete system or activated transport protein, which resulted in cytoplasmic proteins release into media.

3.4.3. Cellular metabolism-related proteins

3.4.3.1. Sulfur metabolism-related proteins. As shown in Table 4, S-adenosylmethionine (SAM) synthetase and putative S-adenosylhomocystein hydrolase were only presented in media after shear treatment 3 h and 6 h, respectively. However, these proteins were absent in shake flask. It was confirmed that artificial wounding activated the transcription of SAM synthetase, which was also involved in the biosynthesis of polyamines in lima bean leaves [45]. The different expression of SAM synthetase responded to the changes of local microenvironments in immobilized cultures [24]. Proteomic analysis revealed that metal (Al) stresses up-regulated SAM synthetase, suggesting that anti-oxidation and detoxification ultimately related to sulfur metabolism [46]. Based on analysis of detoxification-related proteins, these findings together revealed the roles of sulfur metabolism in regulation of redox homeostasis of *T. cuspidata* cells after shear stress.

3.4.3.2. Glycolysis, energy, and cell wall catabolic and other carbohydrate metabolism-related proteins. As shown in Tables 4–6, some non-secreted proteins (triosephosphate isomerase, putative

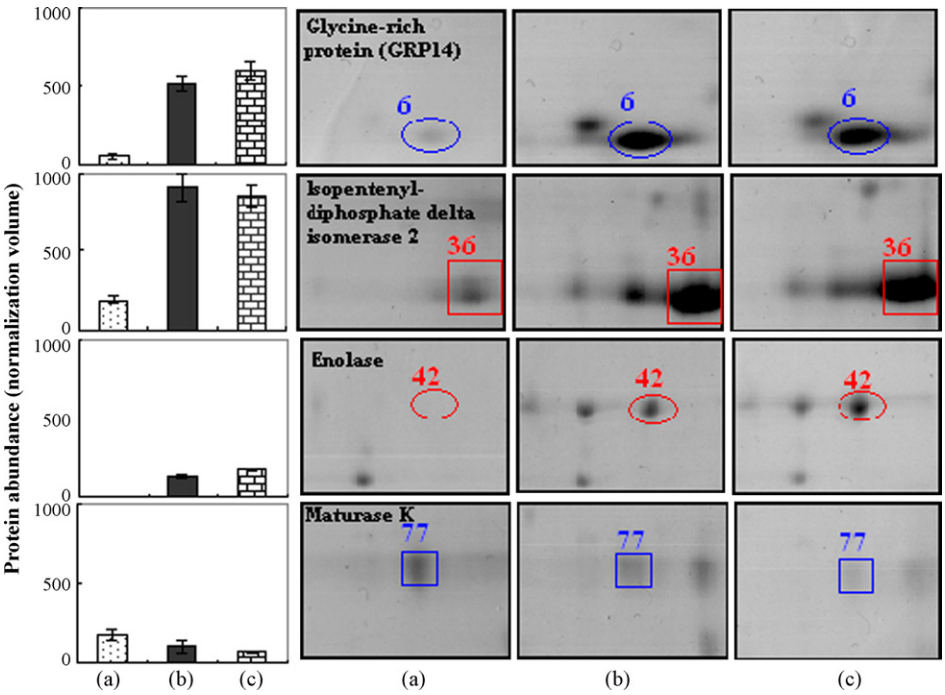

Fig. 6. Changes of four identified proteins in media based on 2DE: (a) shake flask 3 h; (b) treatment with shear for 3 h; (c) treatment with shear for 6 h. Each value represents the mean of three independent replicates ±S.D.

cytosolic phosphoglucomutase, enolase, malate dehydrogenase, mitochondrial formate dehydrogenase precursor, succinyl-CoA ligase (GDP-forming) alpha-chain, and basic class I chitinase were only observed in media after shear treatment (3 h and 6 h). They were involved in various bioprocesses including cell wall catabolic, tricarboxylic acid cycle, carbohydrate degradation, glycolysis and so on. However, these non-secreted proteins were not monitored in media of shake flask. Meanwhile, quantitative analysis using 2DE combined with MALDI-TOF-MS suggested that shear treatment up-regulated the abundance of enolase and glycine-rich protein GRP14, but down-regulated maturase K in media of the suspension cultured *Taxus cuspidata* cells (Fig. 6). Under biotic and abiotic stress, it was necessary that the primary metabolisms, including carbon, nitrogen, sulfur, and energy metabolism, were modulated to establish a new homeostasis. It was confirmed that glycolytic metabolism processes seem to be important targets in response to hormones and stresses [47]. Proteomic analysis revealed that the carbohydrate metabolism-related proteins (enolase, triosephosphate isomerase and glucan endo-1,3-beta-glucosidase) were involved in drought-stress response of poplar [48]. The plant cell wall depended on the secretory pathway to deliver its complex polysaccharides [49].

3.4.3.3. Secondary metabolism-related proteins. As shown in Table 4, the geranylgeranyl diphosphate synthase (GGPPs, EC: 2.5.1.29) were only detected in media after shear treatment. While the phospholipase D (PLD) alpha 1 precursor was only monitored in media after shear treatment (3 h). However, these proteins were absent in shake flask. Our group recently reported that continuous increase of PLD activity led to phosphatidic acid (PA) production accompanying with apoptosis in high-yield *T. chinensis* cells [50]. Further investigation illustrated that Ce^{4+} elicitation markedly increased PLD activity and high concentrations of PA production while it improved Taxol production [51]. Bargmann and Munnik reviewed that PLD regulated membrane degradation, organization of actin and microtubules, vesicular transport and signal transduction [52]. Involvement of phospholipase C was also demonstrated in the signal pathway for oxidative bursts induced by the shear [9]. Therefore, it is evidence that lipid metabolism is closely associated with shear response and Taxol production of *T. cuspidata* cells.

Meanwhile, we also observed that the shear treatment enhanced the abundance of isopentenyl diphosphate (IPP) delta isomerase by 2DE combined with MALDI-TOF-MS analysis (Fig. 6). The GGPPs catalyzes the biosynthesis of geranylgeranyl diphosphate, which is a key precursor for diterpenes including Taxol. IPP isomerase catalyzes the interconversion of isopentenyl pyrophosphate and dimethylallylpyrophosphate. The Taxol biosynthetic pathway arises from the primary isoprenoid precursors: isopentenyl diphosphate and dimethylallyl diphosphate in yew (*Taxus*) [53]. It was reported that Taxol production was induced by low-energy ultrasound in *Taxus chinensis* preceded by ROS [54]. Previous report showed that the activated non-mevalonate pathway should be responsible for the formation of IPP in Taxol biosynthesis in the presence of SA [55]. H_2O_2 burst induced by shear stress plays an important role in inducing secondary metabolites [9]. Trejo-Tapia recently also reported that hydrodynamic stress-induced monoterpenoid oxindole alkaloid accumulation [33]. Shuler's research group demonstrated that the cells within the culture cycle might generate chemical signals for secondary metabolite production through exchanging the media of different productive cell lines [56]. It had been demonstrated that arabinogalactan-proteins (AGPs), known as extracellular signal molecules, closely related to Taxol production [57]. The carbohydrate moieties of AGPs contain a reservoir of structural information that can serve as potential chemical signals consistent with oligosaccharide signaling in plants [58]. In this work, shear stress not only up-regulated IPPs and GGPPs associated with Taxol biosynthesis, but also increased the extracellular accumulation of proteins related to cell wall catabolic process, carbohydrate metabolic process, and carbohydrate degradation. We presumed that cell wall catabolic metabolism and polysaccharides degradation might produce oligosaccharide signaling, which elicited the Taxol production after long-term shear treatment. Work is going on to apply the global methods of comparative analysis for intracellular metabolomics (including lipidomic) and proteomics

to explore the specific mechanisms of long-term shear responses in *Taxus* cells.

4. Conclusion

Shear-induced oxidative burst might be closely associated with increase of membrane permeability, which consequently caused the release of proteins into media. The first systematic reference map for extracellular proteins of *T. cuspidata* cells under shear stress was illustrated. Moreover, some stress responses, detoxification, and sulfur metabolism-related proteins in media might play important roles in oxidative response and regulation of redox homeostasis of *T. cuspidata* cells after shear stress. HSPs as potential signal molecules were involved in shear response signal transduction pathways of *T. cuspidata* cells. The identification of extracellular proteins in media of the suspension cultured *Taxus cuspidata* cells will help us to obtain deeper insights into the poorly understood and complex cellular response to shear stress and consequently oxidative stress.

Acknowledgments

The authors are grateful for the financial support from the National Fund for Distinguished Young Scholars (20425620), from the National Major Basic research Program of China (973 Program: 2007CB714301), and from the National Natural Science Foundation of China (Nos.: 20736006, 20706044).

References

[1] Y. Yukimune, H. Tabata, Y. Higashi, Y. Hara, Nat. Biotechnol. 14 (1996) 1129–1132.
[2] P.M. Kieran, P.F. MacLoughlin, D.M. Malone, J. Biotechnol. 59 (1997) 39–52.
[3] B.M. Baumert, S.J. Muller, J. Non-Newtonian Fluid Mech. 83 (1999) 33–69.
[4] Z.G. Jin, C. Wong, J. Wu, B.C. Berk, J. Biol. Chem. 280 (2005) 12305–12309.
[5] C.P. Johnson, H.Y. Tang, C. Carag, D.W. Speicher, D.E. Discher, Science 317 (2007) 663–666.
[6] M.R. Knight, S.M. Smith, A.J. Trewavas, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 4967–4971.
[7] S.B. Gus-mayer, N.K. Hahlbrock, E. Schmelzer, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 8398–8403.
[8] L. Bögre, W. Ligterink, E. Heberle-Bors, H. Hirt, Nature 383 (1996) 489–490.
[9] R.B. Han, Y.J. Yuan, Biotechnol. Prog. 20 (2004) 507–513.
[10] Y.W. Gong, Y.J. Yuan, J. Biotechnol. 123 (2006) 185–192.
[11] J.S. Cheng, Y.J. Yuan, Biotechnol. Appl. Biochem. 44 (2006) 109–117.
[12] H. Gao, Y.W. Gong, Y.J. Yuan, Biotechnol. Prog. 23 (2007) 673–679.
[13] P. Jeffers, S. Kerins, C.J. Baker, P.M. Kieran, Biotechnol. Prog. 23 (2007) 138–145.
[14] X.L. Wang, A. Fu, S. Raghavakaimal, H.C. Lee, Proteomics 7 (2007) 588–596.
[15] H. Antelmann, E. Darmon, D. Noone, J.W. Veening, H. Westers, S. Bron, O.P. Kuipers, K.M. Devine, M. Hecker, J.M. van Dijl, Mol. Microbiol. 49 (2003) 143–156.
[16] M.P. Nandakumar, A. Cheung, M.R. Marten, J. Proteome Res. 5 (2006) 1155–1161.
[17] Y.H. Ehrlich, T.B. Davis, E. Bock, E. Kornecki, R.H. Lenox, Nature 320 (1986) 67–70.
[18] A. Gilchrist, C.E. Au, J. Hiding, A.W. Bell, J. Fernandez-Rodriguez, S. Lesimple, H. Nagaya, L. Roy, S.J. Gosline, M. Hallett, J. Paiement, R.E. Kearney, T. Nilsson, J.J. Bergeron, Cell 127 (2006) 1265–1281.
[19] S. Chivasa, W.J. Simon, X.L. Yu, N. Yalpani, A.R. Slabas, Proteomics 5 (2005) 4894–4904.
[20] B.K. Ndimba, S. Chivasa, J.M. Hamilton, W.J. Simon, A.R. Slabas, Proteomics 3 (2003) 1047–1059.
[21] H. Thordal-Christensen, Z. Zhang, Y. Wei, D.B. Collinge, Plant J. 11 (1997) 1187–1194.
[22] K. Suzuki, A. Yano, H. Shinshi, Plant Physiol. 119 (1999) 1465–1472.
[23] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[24] J.S. Cheng, Y.J. Yuan, Proteomics 6 (2006) 2199–2207.
[25] Y.X. Wang, Y.J. Yuan, J. Proteome Res. 4 (2005) 1999–2006.
[26] S. Chen, Z. Vaghchhipawala, W. Li, H. Asard, M.B. Dickman, Plant Physiol. 135 (2004) 1630–1641.
[27] J.M. Paper, J.S. Scott-Craig, N.D. Adhikari, C.A. Cuomo, J.D. Walton, Proteomics 7 (2007) 3171–3183.
[28] V.L. Vega, M. Rodríguez-Silva, T. Frey, M. Gehrmann, J.C. Diaz, C. Steinem, G. Multhoff, N. Arispe, A. De Maio, J. Immunol. 180 (2008) 4299–4307.
[29] G.J. Lee, E. Vierling, Plant Physiol. 122 (2000) 189–197.
[30] Q.M. Xu, J.S. Cheng, Z.Q. Ge, Y.J. Yuan, Appl. Biochem. Biotechnol. 125 (2005) 11–26.
[31] S. Takeshita, N. Inoue, T. Ueyama, S. Kawashima, M. Yokoyama, Biochem. Biophys. Res. Commun. 273 (2000) 66–71.
[32] Y. Hojo, Y. Saito, T. Tanimoto, R.J. Hoefen, C.P. Baines, K. Yamamoto, J. Haendeler, R. Asmis, B.C. Berk, Circ. Res. 91 (2002) 712–718.
[33] G. Trejo-Tapia, G. Sepúlveda-Jiménez, J.L. Trejo-Espino, C.M. Cerda-García-Rojas, M. de la Torre, M. Rodríguez-Monroy, A.C. Ramos-Valdivia, Biotechnol. Bioeng. 98 (2007) 230–238.
[34] Y.J. Yuan, J.C. Li, Z.Q. Ge, J.C. Wu, J. Mol. Catal. B: Enzym. 18 (2002) 251–260.
[35] L. Pnueli, H. Liang, M. Rozenberg, R. Mittler, Plant J. 34 (2003) 187–203.
[36] M. Pernas, R. Sánchez-Monge, G. Salcedo, FEBS Lett. 467 (2000) 206–210.
[37] T. Yang, S. Chaudhuri, L. Yang, Y. Chen, B.W. Poovaiah, J. Biol. Chem. 279 (2004) 42552–42559.
[38] D.A. Capiati, S.M. País, M.T. Téllez-Iñón, J. Exp. Bot. 57 (2006) 2391–2400.
[39] A.H. Valster, E.S. Pierson, R. Valenta, P.K. Hepler, A. Emons, Plant Cell 9 (1997) 1815–1824.
[40] D.R. Kovar, B.K. Drobak, C.J. Staiger, Plant Cell 12 (2000) 583–598.
[41] K.M. Gorecka, C. Thouverey, R. Buchet, S. Pikula, Plant Cell Physiol. 48 (2007) 792–803.
[42] H. Nakayama, K. Yoshida, A. Shinmyo, Biotechnol. Bioeng. 85 (2004) 776–789.
[43] Z.D. Shi, Y.J. Yuan, J.C. Wu, G.M. Shang, Appl. Biochem. Biotechnol. 110 (2003) 61–74.
[44] Y.H. Cheong, H.S. Chang, R. Gupta, X. Wang, T. Zhu, S. Luan, Plant Physiol. 129 (2002) 661–677.
[45] G. Arimura, R. Ozawa, T. Nishioka, W. Boland, T. Koch, F. Kühnemann, J. Takabayashi, Plant J. 29 (2002) 87–98.
[46] Q. Yang, Y. Wang, J. Zhang, W. Shi, C. Qian, X. Peng, Proteomics 7 (2007) 737–749.
[47] M. Khan, H. Takasaki, S. Komatsu, J. Proteome Res. 4 (2005) 1592–1599.
[48] C. Plomion, C. Lalanne, S. Claverol, H. Meddour, A. Kohler, M.B. Bogeat-Triboulot, A. Barre, G. Le Provost, H. Dumazet, D. Jacob, C. Bastien, E. Dreyer, A. de Daruvar, J.M. Guehl, J.M. Schmitter, F. Martin, M. Bonneu, Proteomics 6 (2006) 6509–6527.
[49] D.E. Shevell, T. Kunkel, N.H. Chua, Plant Cell 12 (2000) 2047–2060.
[50] S. Yang, B. Qiao, S.H. Lu, Y.J. Yuan, Biochim. Biophys. Acta 1771 (2007) 600–612.
[51] S. Yang, S.H. Lu, Y.J. Yuan, Biochim. Biophys. Acta 1781 (2008) 123–134.
[52] B.O. Bargmann, T. Munnik, Curr. Opin. Plant Biol. 9 (2006) 515–522.
[53] M. Chau, R. Croteau, Arch. Biochem. Biophys. 427 (2004) 48–57.
[54] J. Wu, X. Ge, Biotechnol. Bioeng. 85 (2004) 714–721.
[55] Y.D. Wang, J.C. Wu, Y.J. Yuan, Cell Biol. Int. 31 (2007) 1179–1183.
[56] B.J. Kim, D.M. Gibson, M.L. Shuler, Biotechnol. Prog. 20 (2004) 1666–1673.
[57] J.S. Cheng, C. Lei, J.C. Wu, Y.J. Yuan, J. Biotechnol. 133 (2008) 96–102.
[58] M.E. Etzler, J. Cell Biochem. 30–31 (1998) 123–128.