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Original Article

A proteomics study of auxin effects in *Arabidopsis thaliana*

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Many phytohormones regulate plant growth and development through modulating protein degradation. In this study, a proteome study based on multidimensional non-gel shotgun approach was performed to analyze the auxin-induced protein degradation via ubiquitin-proteasome pathway of *Arabidopsis thaliana*, with the emphasis to study the overall protein changes after auxin treatment (1 nM or 1 μM indole-3-acetic acid for 6, 12, or 24 h). More than a thousand proteins were detected by using label-free shotgun method, and 386 increased proteins and 370 decreased ones were identified after indole-3-acetic acid treatment. By using the auxin receptor-deficient mutant, *tir1-1*, as control, comparative analysis revealed that 69 and 79 proteins were significantly decreased and increased, respectively. Detailed analysis showed that among the altered proteins, some were previously reported to be associated with auxin regulation and others are potentially involved in mediating the auxin effects on specific cellular and physiological processes by regulating photosynthesis, chloroplast development, cytoskeleton, and intracellular signaling. Our results demonstrated that label-free shotgun proteomics is a powerful tool for large-scale protein identification and the analysis of the proteomic profiling of auxin-regulated biological processes will provide informative clues of underlying mechanisms of auxin effects. These results will help to expand the understanding of how auxin regulates plant growth and development *via* protein degradation.

Keywords proteomics; auxin; *tir1-1*; *Arabidopsis*; shotgun

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Introduction

Recent studies revealed the crucial roles of protein degradation in multiple processes of plant growth and development including the cell cycle, embryogenesis, senescence, environmental stimuli, and hormone signaling [1]. Proteins designed to be destroyed are tagged with a polyubiquitin chain by a cascade of reactions involving three enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). The ubiquitinated proteins are recognized and degraded by the 26S proteasome. Studies of the ubiquitin-proteasome-related regulation revealed a complex regulatory network controlling protein stability.

Proteomics, in combination with the availability of genome sequence data, have opened up enormous possibilities to identify the total expressed proteins, as well as expression changes during the growth and development and in response to biotic and abiotic stimuli [2]. Currently, various methods have been developed for analysis of the proteomics changes with mass spectrometry or 2D difference gel electrophoresis (2D-DIGE), which makes it possible to compare different protein samples in the same gel, circumventing the problems of gel-to-gel variation. However, the limitation of 2D-DIGE in sensitivity and separation capacity makes it inefficient when analyzing the insoluble or basic proteins or proteins with very high molecular mass.

Alternatively, direct multidimensional liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of total peptide digests overcomes many of the problems in detecting proteins by 2D gels. In addition, elimination of gel-based separation also increases the sensitivity in detecting proteins [3]. The shotgun approach has been used to identify the proteins in *Arabidopsis* leaf, mature pollen, and cotyledon. By combining two kinds of shotgun tandem MS proteomics approaches, MudPIT (multidimensional protein identification technology) and 1D gel-LC-MS/MS, a total of 2342 non-redundant proteins and protein groups were detected in *Arabidopsis* leaves [4], and by using a shotgun proteomics approach, ~3500 proteins were detected in *Arabidopsis* pollen, including 537 proteins that were not identified in genetic or transcriptomic studies [5], which is very helpful to study the functions of many previously uncharacterized proteins. To investigate the cell dedifferentiation process, shotgun proteomics coupled with spectral count quantification has been used to identify the proteins in *Arabidopsis*

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cotyledons, and among the 758 identified proteins, 358 proteins are shown to be differentially regulated, suggesting that cell dedifferentiation results in a change of all aspects of cellular processes [6].

As shotgun proteomics is not a quantitative technique, isotope-labeling techniques (e.g., isotope-coded affinity tags) have been integrated with LC-MS/MS for relative protein quantification [7]. Although this enables the direct comparison of relative peptide abundances, expenses of isotopic labeling and requirement for binate comparisons between samples prevent reviewed comparisons and complicate studies [8]. Recently, by direct comparison of peptide peak areas between LC-MS runs without isotope labeling, shotgun proteomics coupled with label-free quantification has been demonstrated with great potential for comparative proteomics studies [9,10]. In addition, it was shown that the intensities of peptide peak signals correspond nearly linearly to the concentrations in the samples [11], which is particularly effective for large-scale protein identification [12].

Protein degradation by ubiquitination is important for plant hormone effects in the regulation of plant growth and development. Auxin regulates the expression of downstream genes by stimulating the degradation of the Auxin/indole-3-acetic acid (AUX/IAA) proteins via the $SCF^{TIR1/AFBs}$-mediated proteolysis [13,14], and modification of CUL1 (CULLIN 1) by an ubiquitin-related protein RUB1 (related to ubiquitin 1) is essential for normal auxin response [15]. Signal transduction of jasmonate involves the degradation of JAZ (JA-ZIM domain) proteins *via* the SCF^{COI1}-dependent 26S proteasome pathway [16,17]. In addition, effects of cytokinin, gibberelic acid, and brassinosteroid action may also depend on protein degradation [18].

Auxin mainly regulates gene expression by TIR1-mediated degradation of AUX/IAAs; however, this is diversified and expanded by the presence of several TIR1/AFBs and the multitude of AUX/IAAs and ARFs (auxin response factors) in distinct tissues. Apart from the primary auxin-responsive genes, little is known about how downstream genes mediate the auxin effects. Complicated regulations are involved in mediating the diverse physiological responses regulated by auxin, which require signaling components other than the TIR1-dependent signaling pathway [19,20]. A F-box protein SKP2A (S-phase kinase-associated protein 2A) binds auxin and involves in auxin-regulated cell division. However, the auxin-dependent degradation of SKP2A is not mediated by TIR1 [21].

Although a large number of auxin-regulated genes have been identified by microarray studies [22–25], few proteomics studies have been performed to study the complexity of the auxin regulatory network. The observations that none of the genes whose encoded proteins involved in SCF^{TIR1} or RUB pathway are regulated at the transcriptional level by auxin [26] suggested that the auxin effects through SCF^{TIR1} or RUB pathway maybe achieved at the protein level, and thus the proteomics studies at the translational or post-translational level will provide new clues into the auxin effects.

Proteomics studies have provided novel insights into the BR (brassinosteroid) signaling [27,28] and by using an auxin autotrophic cell culture of *Arachis hypogea*, it was demonstrated that ubiquitin-mediated selective protein turnover in response to auxin is necessary for reversing the stress sensitivity of the *Arachis* cells [29]. Here we reported a large-scale analysis of proteins by using the label-free shotgun approach, and further proteomics analysis of *Arabidopsis* seedlings by treatment with auxin. Identification of a number of auxin-regulated proteins will be valuable for studying the mechanisms by which auxin regulates the plant growth and development.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (Col-0 and *tir1-1*) seeds were sterilized in 20% bleach and 0.04% Tween-20 for 10 min, rinsed several times with sterilized water, and held at 4°C for 2 days to enhance the germination. These seeds were dispersed on the growth media [1/2 Murashige and Skoog (MS) medium, 2% sucrose, pH 5.7, 0.8% agar], and grown in greenhouse with fluorescent lighting (16 h light/8 h dark) at 21–22°C. Seven-day-old seedlings were transferred to flakes containing 50 ml liquid medium (1/2 MS, 2% sucrose, pH 5.7), IAA (1 μM or 1 nM final concentration) or mock solvent for 6, 12, or 24 h. After IAA treatment, seedlings were rinsed twice with media and harvested. Three biological replicates were used for each treatment.

Proteins extraction

Plant material was ground in liquid nitrogen for protein extraction. About 1 g of the powder was mixed with 10 ml precipitation solution [10% trichloroacetic acid and 0.07% dithiothreitol (DTT) in acetone (w/v, [30]), 2% Triton X-100] and incubated at −20°C overnight. Precipitated material was collected by centrifugation (15,000 rpm, 4°C, 25 min). After washing three times with acetone containing 0.07% DTT, the precipitate was dried in a vacuum centrifuge.

Protein digestion and identification by mass spectrometry

In-solution tryptic digestion was performed according to the previous description [31]. A modified step was the resuspened proteins were incubated at 90°C for 20 min. The reaction mixture was transferred to a centricon filter

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(Millipore, Billerica, USA; 10 kDa) and centrifuged at 12,000 rpm for 40 min. BCA-100 protein quantitative analysis kit was used for protein quantification (Shenergy Biocolor, Shanghai, China) and the eluted peptides were vacuum-dried to a final volume of 100 μl.

Peptide mixtures separation by a RP column and LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, USA) was performed according to the previous description [6]. In addition, the peptides were loaded directly on the reversed-phase column, equilibrated with 0.1% formic acid and 2% acetonitrile. Mass spectra for all samples were measured with an overall *m*/*z* range of 600–3500.

Protein comparative quantification using peptides peak area

The peak area detection (% area) was generated by ICIS algorithm of TurboSEQUEST (BioWorks 3.3.1; Thermo Electron, Marietta, OH), a commercial new software used in MS data analysis. The algorithm works by reading the MH+ value from the *.dta* file and calculates the precursor mass from this value. BioWorks then generates a reconstructed ion chromatogram (RIC) using the precursor mass. The integrated area of the peaks was calculated after smoothness of the RIC. Proteins with over 2-fold increase and decrease at least at one time point were identified based on peak area comparison.

Statistical analysis

MS/MS spectra were extracted from the raw data with BioWorks Browser 3.3.1. Individual data files and a combined file of all spectra were searched against the IPI ARATH FASTA. 3.20 protein database (34,992 protein sequences; ftp://ftp.ebi.ac.uk/pub/database/IPI/current) by TurboSEQUEST program. Trypsin digestion was applied to generate peptides and the database included mass changes due to cysteine carboxyamidomethylation as fixed modification and methionine oxidation as variable modification. The precursor ions mass tolerance was 1.4 Da, and the fragment ions mass tolerance was 1.0 Da. The threshold score for accepting individual spectra was 50,000. Low-quality matches were filtered using the default charge versus Xcorr criterion (1.2, 1.75, and 2.0 for +1, +2, and +3, respectively, and delta correlation value ≥0.1) (**Supplementary Tables S1** and **S2**).

Results

Identification of differentially expressed proteins under auxin treatment through shotgun proteomics

Accurate and reliable protein quantification is essential in understanding basic biological processes. To obtain the optimal separation of *Arabidopsis* proteins for shotgun proteomics, two methods for protein digestion were first tested, including guanidine/iodoacetic acid [32] and 2,2,2-trifluoroethanol (TFE)/iodoacetamide (IAM) [31]. Protein quantification using protein samples prepared with above two methods showed that less amount of proteins was lost under the same initiation quantity protein by TFE/IAM method, which yielded more proteins for LC-MS/MS analysis. Normally, 2 mg protein pellet was used for sample preparation and ~600 μg final proteins were yielded after treatment with TFE/IAM protocol, while only 200 μg proteins could be obtained with guanidine/iodoacetic acid protocol; therefore, TFE/IAM method was used in this study. Protein concentration was adjusted to 5–6 μg/μl after trypsin digestion and 20 μl of peptide mixtures (~100 μg) was used for proteome identification (**Fig. 1**). Peaks in the LC-MS domain corresponding to peptide ion species are highly sensitive to differences in protein abundance. Identification of LC-MS peaks that correspond to detected peptides and measurement of quantitative attributes of these peaks (such as height, area, or volume) offer a promising alternative to spectral counting methods [33]. In this study, the label-free relative quantification was carried out using BioWorks 3.3.1 via peak areas (% area) calculation. Typically, a total of >1000 proteins were identified based on the peptide matches (**Table 1**) in a single LC-MS/MS run.

Based on the established system, *Arabidaopsis* seedling proteome was investigated. Seven-day-old seedlings were chosen as materials because seedlings at this stage were used in many microarray studies for analysis of the auxin-regulated transcriptional profiling [22,24]. The time point of auxin treatment in most microarray studies was 0.5–3 h [25]; however, considering the temporal difference between transcription and translation, 6-h treatment was selected to identify the candidate proteins other than AUX/IAAs and 12- and 24-h treatment was employed to identify components that may involve in TIR1-independent pathway and

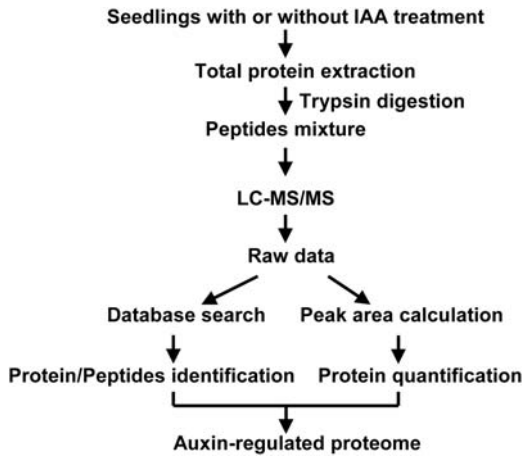

Figure 1 Strategy for label-free shotgun proteomics using LC-MS/MS.

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study the developmental and/or differentiation events by auxin regulation.

The identified proteins with differential expressions with or without auxin treatment were further analyzed (**Fig. 2**). The comparison of proteins with significant differences was based on that proteins can (or cannot) be detected before IAA treatment, but cannot (or can) be detected after IAA treatment. The peak area calculation method was used for proteins that can be detected both before and after IAA treatment, in this case, the threshold was with over 2-fold increase or decrease. Finally, 386 and 370 proteins displayed differential regulation (up- or down-regulation) under auxin treatment at least at one time point (6, 12, or 24 h) respectively (**Supplementary Tables S3** and **S4**). The significantly decreased proteins which were not detected in WT after auxin treatment were identified. After 6, 12, and 24 h of auxin treatment, the numbers of significantly decreased proteins were 65, 60, and 65 under 1 nM IAA-treatment, 97, 68, and 57 under 1 μM IAA-treatment, respectively. Further comparative analysis revealed that 51, 41, and 37 proteins were common under different IAA concentration (1 μM and 1 nM IAA) at 6, 12, or 24 h (**Supplementary Table S5**). The fact that a large number of differential proteins at the same time point were common under different IAA concentration suggests that the regulations of auxin effects at protein level may be more related to the treatment time. Functional classification of these identified proteins by Gene ontology analysis (http://www.arabidopsis.org/tools/bulk/go/) [34] including the cellular component (gene product), the molecular function (gene product activities at the molecular level) and the biological process (gene product participates) was performed. The results showed that the decreased proteins are involved in different biological process (**Table 2**).

Meanwhile, we also identified a number of increased/decreased proteins revealed by peak area calculation method after 6, 12, and 24 h of IAA treatment. Proteins with over

Table 1 Total number of detected proteins at different time points in the absence or presence of IAA

Group	Control	1 nM IAA	1 μM IAA
Col-0 6 h	1507	1937	763
Col-0 12 h	1391	751	878
Col-0 24 h	1497	1197	1368
tir1-1 6 h	1140	752	894
tir1-1 12 h	1124	994	949
tir1-1 24 h	1260	1474	566

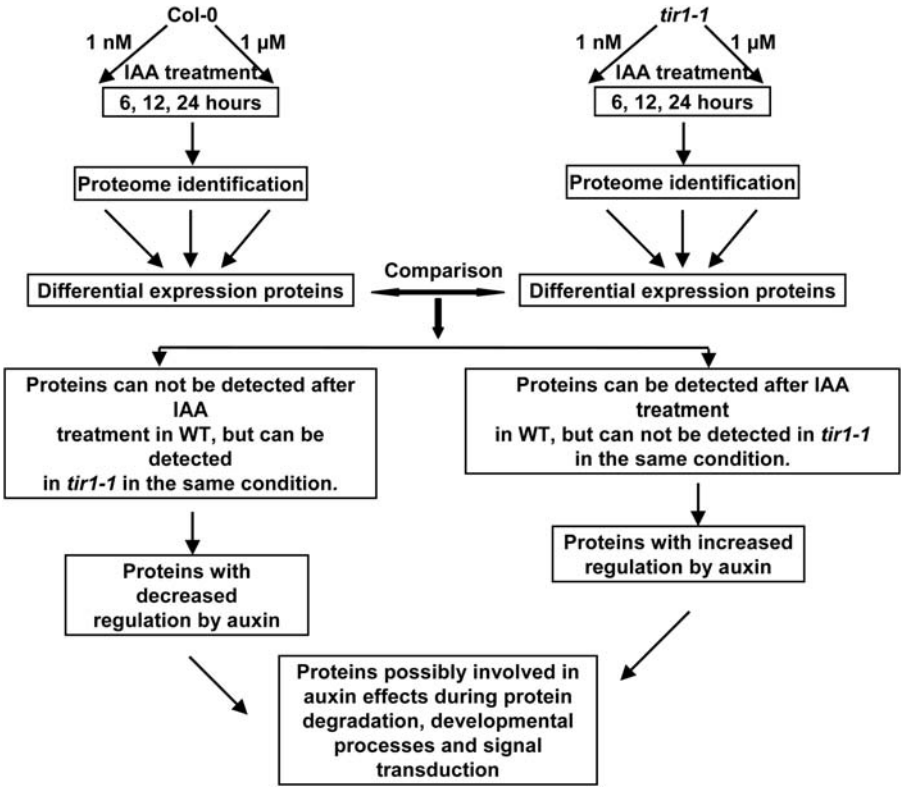

Figure 2 Identification of auxin-regulated proteins Significantly altered proteins of Col-0 and *tir1-1* seedlings treated with IAA (1 μM or 1 nM for 6, 12, and 24 h) were identified by comparison with the mock-treated samples by shotgun protein LC-MS/MS analysis. The proteins possibly involved in auxin signaling or effects were identified by comparison of differential proteins between Col-0 and *tir1-1*.

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Table 2 GO category of the biological process of significant decreased proteins in WT under IAA treatment ($P < 0.05$)

GO category (6-h treatment)	No.	GO category (12-h treatment)	No.	GO category (24-h treatment)	No.
Common decreased proteins under different IAA concentrations					
Response to blue light	6	Response to stimulus	16	Response to metal ion	7
Response to red light	6	Response to stress	12	Response to inorganic substance	7
Response to light stimulus	10	Response to cadmium ion	5	Response to cadmium ion	6
Response to radiation	10	Polar nucleus fusion	2	Response to light stimulus	8
Photosynthesis	7	Megagametogenesis	2	Response to radiation	5
Response to red or far red light	7	Defense response to bacterium	3	Brassinosteroid-mediated signaling pathway	2
Decreased proteins only under 1 nM IAA treatment					
Generation of precursor metabolites and energy	5	Response to abiotic stimulus	10	Nucleosome assembly	9
Photosynthesis, light harvesting	3	Response to light stimulus	6	Nucleosome organization	9
Photosynthesis	4	Root hair elongation	3	Chromatin assembly	9
Photosynthesis, light reaction	3	Regulation of dephosphorylation	2	Protein–DNA complex assembly	9
Energy-coupled proton transport, down electrochemical gradient	2	Cell maturation	3	Cellular response to nitrogen starvation	3
ATP synthesis-coupled proton transport	2	Regulation of protein modification process	3	Response to hydrogen peroxide	3
Decreased proteins only under 1 μM IAA treatment					
Nucleosome organization	9	Nucleosome assembly	9	*S*-adenosylmethionine biosynthetic process	4
Nucleosome assembly	9	Nucleosome organization	9	Purine ribonucleoside metabolic process	4
Chromatin assembly	9	Chromatin assembly	9	Sulfur metabolic process	4
Protein–DNA complex assembly	9	Protein-DNA complex assembly	9	Oxoacid metabolic process	6
DNA packaging	9	DNA packaging	9	Gluconeogenesis	2
DNA conformation change	9	DNA conformation change	9	Small molecule metabolic process	7

2-fold increase and decrease at least at one time point were 83/160, 84/52, and 117/79 under 1 nM IAA-treatment, and 113/70, 85/99, and 89/112 under 1 μM IAA-treatment, respectively. The accumulative number of increased/deceased unique proteins was 224/232 with 1 nM IAA-treatment and 248/234 with 1 μM IAA-treatment. Mutants of 34 obviously decreased proteins were obtained for further functional study and we hope the results will provide informative clues for understanding the auxin effects.

Gene ontotloy (GO) definition of the identified proteins showed that response to abiotic/biotic stimulus, response to stress, protein metabolism, electron transport or energy pathways, and cell organization and biogenesis are the five biological processes with the highest percentage of proteins that undergo differential expression after auxin treatment. A detailed analysis of the differentially regulated proteins showed that 112 proteins were decreased and 91 proteins were increased only under treatment of 1 μM IAA (**Supplementary Table S6**). Considering the different effects of 1 nM or 1 μM IAA (there is almost no negative effect on plant growth, for both roots and shoots, of 1 nM IAA) whether these differentially regulated proteins are targets of dose-specific and related to stress response is worth being studied in the future.

Identification of differentially expressed proteins between WT and *tir1-1* and potential degraded proteins via TIR1-dependent pathway

To study the auxin effects through regulating protein degradation, comparative analysis with the proteomes of WT (Col-0) and auxin-resistant mutant *tir1-1* (deficiency of auxin receptor TIR1) was performed and results showed that 130 proteins were detected only in Col-0, but not *tir1-1* (**Supplementary Table S7**), while 62 proteins were detected only in *tir1-1*, but not Col-0 (**Supplementary Table S8**) without IAA treatment (control condition). As TIR1 is a receptor mediating AUX/IAA degradation and auxin-regulated transcription [13], the identification of the differential proteins between Col-0 and *tir1-1* mutant would provide clues on the auxin effects through TIR1-dependent protein degradation and auxin signaling, and provide insights into the regulatory mechanisms or new components

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that possibly mediate protein degradation and subsequent regulation of development and differentiation by auxin.

Comparison of the proteins that were not detected after auxin treatment in Col-0 with the identified proteins of *tir1-1* under the same treatment condition (**Fig. 2**) resulted in that 69 proteins were identified (including the concentrations of 1 nM and 1 μM IAA, and at time point of 6, 12, and 24 h) (**Table 3**), which were supposed to be degraded *via* TIR1-dependent ubiquitin-proteasome pathway. Although the time point used may be relatively long and we did not detect any known targets of auxin effects like AUX/IAAs, these identified proteins maybe regulated *via* an indirect but TIR1-dependent pathway to involve in the regulation of development and differentiation by auxin.

Functional classification of the identified proteins by GO category analysis was performed (**Fig. 3**). The major cellular components and the corresponding number of identified proteins (in brackets) were related to chloroplast (29), plastid (23) and nucleus (16); the molecular functions category with the largest number of decreased proteins were protein binding (19), DNA or RNA binding (16) and hydrolase activity (9); and the protein distributions based on biological processes were response to abiotic or biotic stimulus (18), cell organization and biogenesis (14), response to stress (12), and protein metabolism (10). The identified proteins that were potentially degraded *via* TIR1-dependent pathway include several CABs (chlorophyll *a*–*b* binding protein), photosystem-related proteins, histone H2B, ribosomal protein, tubulin, actin, and other function known or unknown proteins.

Identification of potential degraded proteins via TIR1-independent pathway

One hundred and fifteen proteins were not detected after auxin treatment both in Col-0 and *tir1-1* under the same treatment condition (**Supplementary Table S9**). The decrease of these proteins may be through a TIR1-independent degradation pathway. These proteins include ribosome proteins, actin, chlorophyll *a*–*b* binding protein, and those involved in photosynthesis and protein metabolism, and respond to environmental stimuli, developmental processes, transcriptional regulation, and signal transduction.

Increased proteins by auxin treatment

Compared with Col-0 in the absence of auxin, 79 proteins were detected neither in Col-0 nor in auxin-treated *tir1-1* samples, but in auxin-treated Col-0 (**Supplementary Table S10**), including CAB, actin, histone, and ribosome proteins. Gene ontology analysis showed that these proteins were mainly localized to chloroplast (19) or plastid (15), and involved in a range of biological and molecular processes such as protein metabolism (12) or response to stress (13), or hydrolase activity (18) and protein binding (10) (**Fig. 4**, **Table 4**).

Previous studies on these increased proteins provided a link between their biological function and auxin regulation. ARF6 mediates auxin response through regulating the expression of auxin-regulated genes and acts redundantly with ARF8 to control stamen elongation and flower maturation [35].

Studies showed that ubiquitin-protein ligase 1 (UPL1) is one Homology to E6-AP C Terminus (HECT)-containing ubiquitin-protein ligases (UPL1-UPL7) and UPL3 is necessary to repress excess branching and endoreplication of trichomes [36,37]. Leucine-rich repeat/extensins (LRX) form a family of structural cell wall proteins containing a receptor-like domain and regulates root hair morphogenesis and elongation [38]. The repressor of *lrx1* (*rol1*) mutants of *Arabidopsis*, affected in rhamnose biosynthesis, has a modified flavonol glycosylation profile. A primary function of flavonols is thought to modify the auxin fluxes in plant [39]. Several heat shock proteins were identified being induced by auxin, including heat shock cognate 70 kDa protein 2, HSP70, and HSP100. Up-regulation of the heat shock proteins by auxin may contribute to increased thermal tolerance, being consistent with the previous studies [40].

Comparison of the auxin responses at protein and mRNA levels

Early auxin-regulated genes have been widely studied by microarray analysis [22–25] and a comparison of the identified auxin-regulated proteins to the 785 auxin-regulated genes (355 down-regulation and 430 up-regulation genes) showed that only two of the 370 decreased proteins, Annexin 3 (AT2G38760) and similar to TONSUKO-associating protein 1 (TSA1) (AT3G15950) were detected in down-regulated genes. In addition, none of the 386 auxin-stimulated proteins was detected in auxin up-regulated gene, but six of the increased proteins were shown in down-regulated genes under auxin treatment. In addition, a comparison of the significantly enriched GO terms of proteome and transcriptome of down-regulated and up-regulated proteins/genes revealed few overlap and obvious difference of them (**Supplementary Tables S11** and **S12**), suggesting that auxin may regulate the developmental processes on distinct translational or post-translational levels.

Discussion

Shotgun proteomics coupled with label-free relative quantification is suitable for the rapid identification of the components of a sample mixture and is a powerful approach for identifying altered proteins during a biological response. In addition, by using peak area calculation, it is possible to quantitatively identify a large number of

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Table 3 Identification of proteins with a significant decrease under IAA treatment by comparison with *tir1-1* mutant under same condition. The seedlings were treated with IAA (1 nM or 1 μM) for 6, 12, or 24 h and then collected for analysis. The IAA concentration and treated time of identified proteins were indicated

Gene locus	Annotation	IAA concentration	
		1 nM	1 μM
Components of chromatin, ribosome, and tubulin			
AT1G07790.1	Histone H2B.1		6 h, 12 h
AT2G28720.1	Histone H2B.3		6 h, 12 h
AT2G37470.1	Histone H2B.4		6 h, 12 h
AT3G45980.1	Histone H2B.6		6 h, 12 h
AT3G46030.1	Histone H2B.7		6 h, 12 h
AT5G22880.1	Histone H2B.10		6 h, 12 h
AT5G59910.1	Histone H2B.11		6 h, 12 h
AT3G53650.1	Putative histone H2B.8		6 h, 12 h
AT5G02570.1	Putative histone H2B.9		6 h, 12 h
AT2G39460.1	60S ribosomal protein L23a-1		6 h
AT3G45020.1	50S ribosomal protein-related	12 h, 24 h	
AT1G04820.1	Tubulin α-2/α-4 chain	12 h	
AT4G14960.1	Tubulin α-6 chain (TUA6)	12 h	
AT4G14960.2	Tubulin α-6 chain	12 h	
AT5G09810.1	Actin-7	12 h	
Proteins related to chloroplast or involved in photosynthesis			
AT2G05070.1	Chlorophyll *a*–*b* binding protein	6 h	6 h
AT2G05100.1	Chlorophyll *a*–*b* binding protein	6 h	6 h
AT2G28000.1	RuBisCO large subunit-binding protein subunit α, chloroplast precursor	6 h	
AT3G08940.1	Isoform 2 of Chlorophyll *a*–*b* binding protein CP29.2, chloroplast precursor		12 h
AT3G27690.1	Chlorophyll *a*–*b* binding protein (LHCB2:4)	6 h	6 h
AT3G47470.1	Chlorophyll *a*–*b* binding protein 4, chloroplast precursor	12 h	12 h
AT5G54270.1	Chlorophyll *a*–*b*-binding protein		6 h
ATCG00020.1	Photosystem Q(B) protein		6 h
ATCG00340.1	Photosystem I P700 chlorophyll *a* apoprotein A2		24 h
AT4G29670.1	Isoform 2 of Thioredoxin-like 6, chloroplast precursor	6 h	6 h
AT4G29670.2	Isoform 1 of Thioredoxin-like 6, chloroplast precursor		6 h
Unknown proteins			
AT1G54000.1	F15I1.8 protein	6 h	
AT1G56550.1	Expressed protein	12 h	12 h
AT3G01730.1	Expressed protein	24 h	24 h
AT3G19190.1	Expressed protein	6 h	6 h
AT3G50120.1	Expressed protein	12 h	12 h
Different functional proteins			
AT1G13980.1	Pattern formation protein EMB30	6 h	6 h
AT1G17480.1	Calmodulin-binding family protein	6 h	6 h
AT1G21750.2	Protein disulfide isomerase	6 h	
AT1G33350.1	Pentatricopeptide (PPR) repeat-containing protein	6 h	
AT1G35160.1	14-3-3-like protein GF14 phi		12 h, 24 h
AT1G35940.1	AT hook motif-containing protein-related	6 h	6 h
AT1G71120.1	GDSL-motif lipase/hydrolase family protein	6 h	6 h
AT1G77490.1	L-Ascorbate peroxidase, thylakoid-bound (tAPX)	6 h	6 h

Continued

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Table 3. *Continued*

Gene locus	Annotation	IAA concentration	
		1 nM	1 μM
AT1G78300.1	14-3-3-Like protein GF14 omega		12 h, 24 h
AT2G24610.1	Putative cyclic nucleotide-gated ion channel 14	6 h	6 h
AT2G36530.1	Enolase		24 h
AT2G38760.1	Annexin 3 (ANN3)	6 h	
AT2G39510.1	Nodulin MtN21 family protein	24 h	24 h
AT3G02260.1	Auxin transport protein (BIG)	6 h	6 h
AT3G03630.1	Probable cysteine synthase, chloroplast precursor	24 h	
AT3G15020.1	Malate dehydrogenase 2, mitochondrial precursor		6 h, 12 h
AT3G16000.1	MAR-binding filament-like protein 1		6 h, 12 h
AT3G16420.1	Jacalin lectin family protein		12 h
AT3G42670.1	SNF2 domain-containing protein	24 h	24 h
AT3G55800.1	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	12 h	12 h
AT3G56150.1	Eukaryotic translation initiation factor 3 subunit 8	12 h	12 h
AT3G62030.1	Peptidyl-prolyl cis-trans isomerase CYP20-3, chloroplast precursor	12 h	12 h
AT4G04620.1	Autophagy 8b (APG8b)	6 h	6 h
AT4G09000.1	14-3-3-like protein GF14 chi		12 h, 24 h
AT4G14880.1	Cytosolic O-acetylserine(Thiol)lyase	24 h	24 h
AT4G15960.1	Putative epoxide hydrolase	6 h	6 h
AT4G16680.1	RNA helicase	12 h	
AT4G23670.1	Major latex protein-related	6 h	
AT4G25880.1	Pumilio/Puf RNA-binding domain-containing protein	6 h	
AT4G32700.1	DNA-directed DNA polymerase family protein	12 h	12 h
AT4G33010.1	Putative glycine dehydrogenase [decarboxylating] 2, mitochondrial precursor	24 h	24 h
AT4G35130.1	Pentatricopeptide (PPR) repeat-containing protein		6 h
AT5G09930.1	ABC transporter family protein	12 h	12 u
AT5G37210.1	Similarity to CHP-rich zinc finger protein-like	12 h	12 h
AT5G42270.1	Cell division protease ftsH homolog 2, chloroplast precursor		6 h
AT5G56000.1	Heat shock protein 81-4 (HSP81-4)		12 h
AT5G67240.1	Similar to exonuclease family protein	12 h	12 h
ATCG00130.1	ATP synthase B chain	6 h	

proteins. Studies showed that 2D gel electrophoresis, a widely used proteome platform, is inappropriate for analysis of high-molecular-weight, hydrophobic, or highly acidic/basic proteins, thus shotgun proteomics that is based on peptides and avoiding gel-based separation will improve the sensitivity of analysis [3]. An in-depth study of rice proteins showed that 2363 proteins were identified from different tissues, while only 556 proteins were identified from the same samples by 2D gel analysis [41]. Similar results were obtained in our study when comparing these two methods.

In addition, complex mixtures of proteins are digested to peptides at first and multidimensional LC-MS/MS analysis can overcome the limitation of sample size for 2D gel separation, allowing more peptide mixture samples in a single LC-MS/MS run.

By using TFE/IAM method and increasing the sample size, we were able to obtain a highly yielded peptide mixture from *Arabidopsis* seedlings and separate over 1000 proteins in a single LC-MS/MS run, thus much more candidate proteins could be identified for further analysis. These results suggest that shotgun proteomics with label-free quantification method was helpful for special cellular and physiological processes study, especially to study the functions of previously uncharacterized proteins in addition to the transcriptomics studies.

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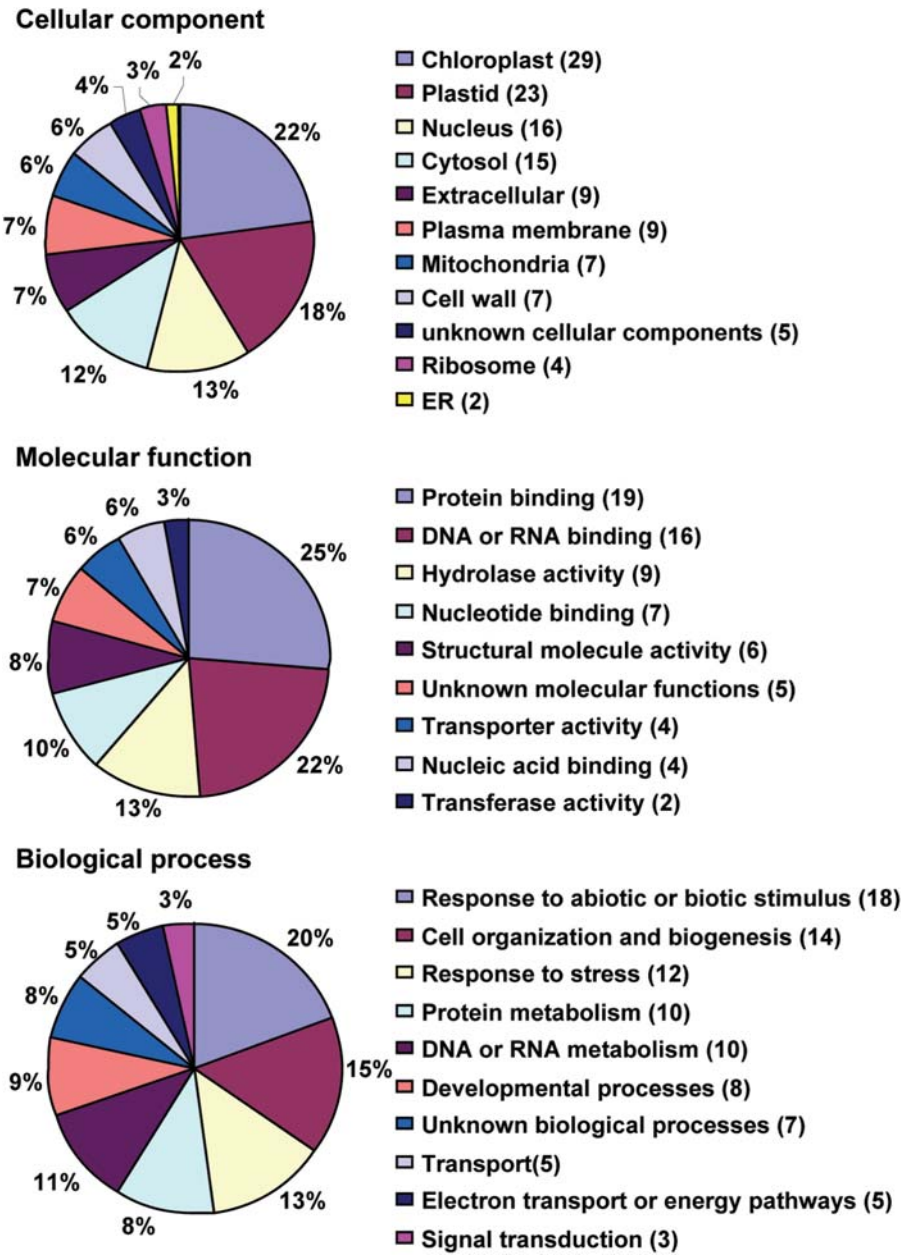

Figure 3 Distribution of the decreased proteins by auxin treatment among different GO categories The pie charts were generated using the program provided by The *Arabidopsis* Information Resource (TAIR). Gene distribution was grouped on the basis of cellular components, molecular functions, and biological processes. The corresponding number of identified proteins was included in brackets.

Studies of cell responses at protein levels using shotgun proteomics have been reported in yeast and human cells [8,42,43], while that of plant hormone effects is still uncommon. Our analysis by detecting the altered proteins following the time course of auxin treatment resulting in the identification of 756 differential proteins under IAA treatment within 24 h. In addition, 69 decreased and 79 increased proteins after auxin-treatment were identified compared with differential proteins of *tir1-1* at the same condition, suggesting that these proteins may involve in TIR1-signaling pathway. In addition, another 115 decreased proteins both in Col-0 and *tir1-1* were also identified as potential proteins which might involved in TIR1-independent degradation pathway. Interestingly, among the 69 decreased proteins, the down-regulation of tubulin, actin, and other cell structural proteins suggest that there is a substantial change in cell organization after auxin treatment. Although we still do not know whether these identified proteins are regulated by auxin-dependent degradation, further validation by functional studies may provide a clue for the auxin effects via protein degradation.

Previous studies indicated that some decreased proteins identified in this study including BIG (a calossin-like

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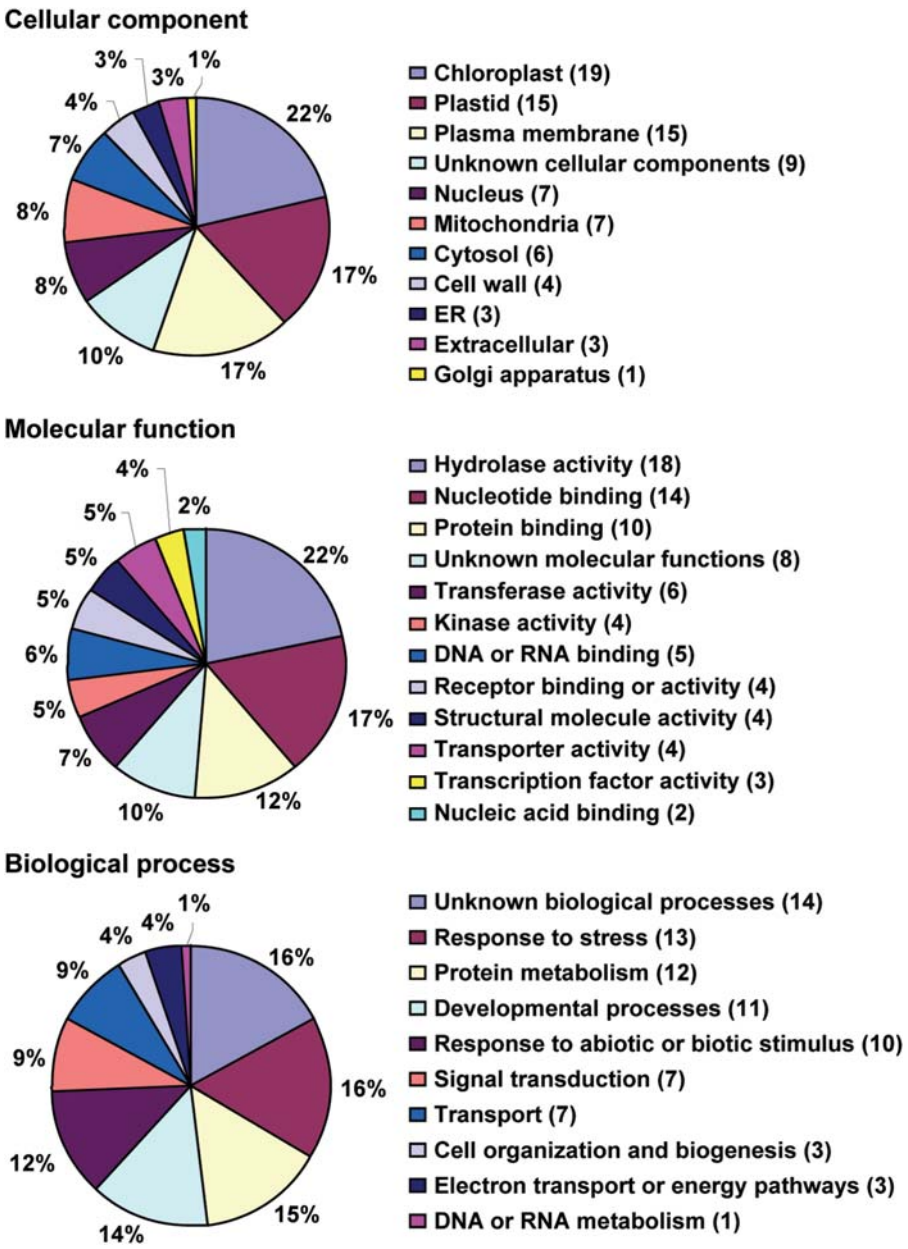

Figure 4 Distribution of the increased proteins by auxin treatment among different GO categories The pie charts were generated using the program provided by The *Arabidopsis* Information Resource (TAIR). Gene distribution was grouped on the basis of cellular components, molecular functions, and biological processes. The corresponding number of identified proteins was included in brackets.

protein), GNOM (a membrane-associated guanine-nucleotide exchange factor on ADP-ribosylation factor G protein), and 14-3-3 proteins potentially mediate auxin responses of specific cellular and developmental processes, by regulating the intracellular signaling, cytoskeleton, secretion, and vesicle trafficking, etc. In the presence of NPA, BIG is required for the proper positioning of the auxin efflux carrier at the plasma membrane, in a process that depends on the vesicle transport [44]. GNOM (pattern formation protein EMB30) is a GDP/GTP exchange factor for small G-proteins of the ADP ribosylation factor (ARF) class, and is a regulator of intracellular trafficking. Indeed, an ARF guanine nucleotide exchange factors (GEF)-dependent transcytosis-like mechanism is operational in plants and provides a credible mechanism that the change of PIN polarity mediated by GNOM effects auxin fluxes during embryogenesis and organogenesis [45]. 14-3-3 proteins bind specifically to phosphorylated proteins and modulate BR signaling by interacting with phosphorylated BZR1 to inhibit its nuclear localization [46]. Seedlings with altered expression of 14-3-3 isoforms through ethanol-inducible RNA interference-based gene silencing resulted in a disorganized root tip, defect in root hair as well as lateral root formation processes known to be regulated by auxin [47].

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Table 4 GO ontology analysis of the increased proteins under IAA treatment by comparison with *tir1-1* mutant under same condition ($P < 0.05$) The seedlings were treated with IAA (1 nM or 1 μM) for 6, 12, or 24 h and then collected for analysis. The IAA concentration and treated time of identified proteins were indicated

Gene locus	Annotation	IAA concentration	
Heat shock protein		1 nM	1 μM
AT5G02490.1	Heat shock cognate 70 kDa protein 2	6 h	
AT1G56410.1	Heat shock cognate 70 kDa protein	6 h	
AT2G25140.1	Heat shock protein 100		24 h
AT3G12580.1	Heat shock protein 70	6 h	
AT4G12400.1	Stress-inducible protein		24 h
Long-chain fatty acid metabolic process			
AT3G05970.1	Long-chain-fatty-acid–CoA ligase	24 h	
AT4G16760.1	Acyl-CoA oxidase (ACX1)	24 h	
Programmed cell death			
AT4G16940.1	Disease resistance protein (TIR-NBS-LRR class)		24 h
AT2G14080.1	Disease resistance protein (TIR-NBS-LRR class)	24 h	
AT5G47260.1	Putative disease resistance protein		24 h
AT4G19520.1	Resistance protein RPP5-like	6 h	
Photosynthesis, light reaction			
ATCG00540.1	Apocytochrome F precursor	6 h	
ATCG00580.1	Cytochrome b559 α subunit		24 h
AT4G04640.1	ATP synthase gamma chain 1, chloroplast precursor	6 h	
Response to cadmium ion			
AT3G12580.1	Heat shock protein 70	6 h	
AT4G16760.1	Acyl-CoA oxidase (ACX1)	24 h	
AT1G56410.1	Heat shock cognate 70 kDa protein	6 h	
AT5G53460.1	NADH-dependent glutamate synthase	24 h	
AT5G02490.1	Heat shock cognate 70 kDa protein 2	6 n	
Cell wall macromolecule catabolic process			
AT1G51940.1	Protein kinase family protein	24 h	
AT1G18310.1	Glycosyl hydrolase family 81 protein	6 h	
Small molecule metabolic process			
AT4G30210.2	NADPH-ferrihemoprotein reductase		12 h
AT4G04640.1	ATP synthase gamma chain 1, chloroplast precursor	6 h	
AT3G05970.1	Long-chain-fatty-acid-CoA ligase	24 h	
AT5G53460.1	NADH-dependent glutamate synthase	24 h	
AT2G16390.1	SNF2 domain-containing protein	6 h	
AT4G34135.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein		12 h
AT4G16760.1	Acyl-CoA oxidase (ACX1)	24 h	
AT3G01590.1	Aldose 1-epimerase family protein	6 h	
AT3G60330.1	ATPase 7, plasma membrane-type	6 h	
AT5G13710.1	Cycloartenol-C-24-methyltransferase	24 h	
Protein folding			
AT5G02490.1	Heat shock cognate 70 kDa protein 2	6 h	
AT2G26890.1	DNAJ heat shock N-terminal domain-containing protein	6 h	
AT1G56410.1	Heat shock cognate 70 kDa protein	6 h	
AT3G12580.1	Heat shock protein 70	6 h	
Cellular process			
AT4G31920.1	Two-component response regulator ARR10		24 h
AT3G17360.1	Kinesin motor protein-related		6 h

Continued

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Table 4. *Continued*

Gene locus	Annotation	IAA concentration	
AT1G51420.1	Sucrose-phosphatase	6 h	
AT3G14570.1	Glycosyl transferase family 48 protein	24 h	
AT1G17220.1	Translation initiation factor IF-2	6 h	
AT4G38780.1	Splicing factor-like protein		24 h
AT5G18500.1	Protein kinase family protein		24 h
AT1G12040.1	Leucine-rich repeat family protein	6 h	
AT5G61480.1	Leucine-rich repeat transmembrane protein kinase		24 h
AT2G28290.2	SPLAYED splice variant		24 h
AT3G48730.1	Glutamate-1-semialdehyde 2,1-aminomutase 2, chloroplast precursor	6 h	
AT1G03590.1	F21B7.20		12 h
AT5G65420.1	Cyclin		12 h
AT1G64740.1	Tubulin α-1 chain	6 h	
AT1G55860.1	Ubiquitin-protein ligase 1 (UPL1)	6 h	
Response to stimulus			
AT4G29940.1	Pathogenesis-related homeodomain protein	24 h	
AT1G30330.1	Auxin response factor 6		12 h
AT5G18370.1	Disease resistance protein (TIR-NBS-LRR class)	6 h	
AT1G71960.1	White-brown complex homolog protein 26		12 h

It is interesting to note that a large number of chloroplast proteins are decreased and the major cellular components and the corresponding number of identified decreased proteins were chloroplast and plastid under auxin treatment, which is consistent with the role of auxin in photosynthesis and nutrient mobilization. It was reported that biosynthesis of tryptophan (Trp), a presumed IAA precursor, occurs in the chloroplast as well as in the cytosol [48]. A chloroplastic site of IAA synthesis has also been suggested [49] and purified chloroplasts can convert L-Trp into IAA [50]. In addition, one-third of the free-IAA pool was present in chloroplasts and a partial free-IAA in chloroplasts is synthesized in the cytosol but rapidly transported in the chloroplast [51]. Although there is no report to emphasize the effects of auxin during plastid/chloroplast development, these results are great helpful to hint a link between auxin regulations and plastid/chloroplast development.

Another interesting observation is that a series of histone H2B were all down-regulated, which suggested that histones maybe directly related to chromatin decondensation and reorganization during cell dedifferentiation (which is known to be induced by auxin) [6]. H2B de-ubiquitination is required for heterochromatic histone H3 methylation and DNA methylation in *Arabidopsis*. In yeast and animals, methylation of H3K4 and H3K9 required H2B ubiquitination, leading to active transcription, the deubiquitination of H2B in *Arabidopsis* suggest its role in H3K9 dimethylation [52]. Indeed, the decreased histone H2B proteins which were not detected after auxin treatment implied that auxin may involve in epigenetic regulation in a direct or an indirect way to regulate plant growth. The detailed correlative between the regulation of auxin and the function of histone H2B is still a question to answer in the future.

Many auxin-regulated proteins identified in this study are very valuable for understanding auxin effects at the molecular levels. Auxin regulates the expressions of downstream genes through degradation of the AUX/IAA proteins; however, neither AUX/IAA nor ARFs proteins were detected after auxin treatment except ARF6. This may be because that AUX/IAA and ARFs proteins are low-abundant proteins, or most AUX/IAAs are extremely short-lived proteins (half-lives of the AUX/IAAs decrease further fast in the presence of auxin) [26]. It has been shown that auxin promotes the interaction between AUX/IAA proteins and SCF^{TIR1} and the auxin-induced interaction is evident as early as 5 min after auxin application [53], several hours application in this study may be actually too long for detecting the short-lived proteins.

Many studies of the auxin-responsive genes by microarray analysis showed the varied results upon the time of auxin exposure, auxin concentration and tissue examined [25]. Comparison of the auxin-regulated proteins identified in our study with the auxin-regulated genes identified in the microarray studies showed that only two of the 370

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decreased proteins were present in the previous identified down-regulated genes, while most of the auxin-regulated proteins were not reported. We thought that the different time of auxin treatments may be one reason for the small overlap between proteome and transcriptome analysis. Normally, 3 h or less time of auxin treatment was used for microarray studies, while 6, 12, and 24 h were used for auxin treatments in our study. In addition, microarray analysis showed that more genes were differentially expressed at high auxin concentrations and longer exposures to auxin [25], but this was not observed by the proteomics study. However, it is difficult to compare the proteomics profiling and transcriptome data due to the different sensitivities of the methods, different biological samples analyzed, different time of auxin treatments. The similar discrepancies between RNA and protein profiling have also been observed in other reports [27,46,47]. Regarding the post-transcriptional and post-translational regulation, how RNA changes lead to changes in protein level is not very clear, the proteomics data may be more relevant to biological responses, because proteins are the functional products of genes.

How auxin-regulated proteins involve in the regulation of plant growth, through a direct or an indirect way, is still unclear. Further analysis with shorter time auxin treatment to find the direct targets of TIR1-dependent pathway is necessary and will be of great help to illustrate as to how auxin regulates plant growth and development through protein degradation. In conclusion, our study demonstrated the capacity of a label-free shotgun approach in studying the cellular mechanisms at the level of proteins, which is a great help for identifying a large number of proteins in a complex proteome, despite the current technical limitations, and provide informative clues for understanding the complexity of auxin-regulated protein degradation.

Supplementary data

Supplementary data are available at *ABBS* online.

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