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RESEARCH ARTICLE

Comparative proteomic analysis of autotetraploid and diploid *Paulownia tomentosa* reveals proteins associated with superior photosynthetic characteristics and stress adaptability in autotetraploid *Paulownia*

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Abstract To enlarge the germplasm resource of *Paulownia* plants, we used colchicine to induce autotetraploid *Paulownia tomentosa*, as reported previously. Compared with its diploid progenitor, autotetraploid *P. tomentosa* exhibits better photosynthetic characteristics and higher stress resistance. However, the underlying mechanism for its predominant characteristics has not been determined at the proteome level. In this study, isobaric tag for relative and absolute quantitation coupled with liquid chromatography-tandem mass spectrometry was employed to compare proteomic changes between autotetraploid and diploid *P. tomentosa*. A total of 1427 proteins were identified in our study, of which 130 proteins were differentially expressed between autotetraploid and diploid *P. tomentosa*. Functional analysis of differentially expressed proteins revealed that photosynthesis-related proteins and stress-responsive proteins were significantly enriched among the differentially expressed proteins, suggesting they may be responsible for the photosynthetic characteristics and stress adaptability of autotetraploid *P. tomentosa*. The correlation analysis between transcriptome and proteome data revealed that only 15 (11.5%) of the differentially expressed proteins had corresponding differentially expressed unigenes between diploid and autotetraploid *P. tomentosa*. These results indicated that there was a limited correlation between the differentially expressed proteins and the previously reported differentially expressed unigenes. This work provides new clues to better understand the superior traits in autotetraploid *P. tomentosa* and lays a theoretical foundation for developing *Paulownia* breeding strategies in the future.

Keywords *Paulownia tomentosa* · Autotetraploid · Superior traits · Proteomics · iTRAQ

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Introduction

Paulownia is a fast-growing deciduous ligneous plant indigenous to China where it is cultivated widely, and has also been introduced into many other countries (Zhu et al. 1986). Because of its good properties, such as short rotation, high-quality wood, high biomass, pollution tolerance and attractive flowers, *Paulownia* is versatile for fodder, paper industry, pencil manufacturing, house construction, furniture making, solid biofuel, forestation and ornamental plant (Zhu et al. 1986; Ates et al. 2008; López et al. 2012; Kaygin et al. 2015).

Polyploidy is a remarkably pervasive phenomenon in eukaryotes, especially in angiosperms (Leitch and Bennett 1997; Comai 2005). Most extant plants have undergone ancient polyploidization events or recent genome duplication events which has been regarded as a great force in the long-term diversification and evolution of flowering plants (Adams and Wendel 2005). According to the chromosomal composition and manner of formation, polyploids can be divided into three types: autopolyploids (duplications of one chromosome set), allopolyploids (mergers of two or more structurally divergent chromosome sets) and

segmental allopolyploids (an intermediate condition between auto- and allopolyploids) (Yoo et al. 2014). In addition to creating gene redundancy, polyploidization can also evoke nuclear enlargement, chromosomal rearrangement, epigenetic remodeling and organ-specific subfunctionalization of gene expression, resulting in restructuring of the transcriptome, metabolome, and proteome (Leitch and Leitch 2008). Compared with their diploid progenitors, polyploids are generally more vigorous and adaptive, and can survive better in harsh environments (Comai 2005). Polyploids often exhibit novel useful traits such as asexual reproduction, flowering phenology, increased adversity tolerance, increased disease and pest resistance, and increased organ size and biomass, making polyploids significant in plant breeding (Osborn et al. 2003; Chen 2007). To enrich the germplasm resources of *Paulownia* plants, autotetraploid *Paulownia tomentosa* was induced by colchicine using the leaves of diploid *P. tomentosa* (Fan et al. 2007). Besides differences in morphology and ultrastructure, autotetraploid *P. tomentosa* exhibits better photosynthetic characteristics and higher stress tolerance than its diploid parent (Zhang et al. 2012, 2013; Deng et al. 2013; Dong et al. 2014a). Recently, Fan et al. (2014, 2015a) revealed the differences in gene expression between diploid and autotetraploid *P. tomentosa* using high-throughput sequencing. However, information about changes in protein profiles between autotetraploid *P. tomentosa* and its diploid parent is still undetermined. Proteomics is necessarily complementary to transcriptome, because proteins are the final gene products and the direct executors of biological function (Koh et al. 2012). Besides, there is no strict linear relationship between mRNAs and proteins (Gygi et al. 1999), because post-transcriptional regulations and post-translational modifications (e.g. glycosylation, phosphorylation, ubiquitination etc.) have great influence on the proteome (Alam et al. 2010), making it difficult to predict protein expression through transcriptional level analysis. Therefore, investigation of proteome changes between diploid and autotetraploid *P. tomentosa* using proteomic approaches is imperative to better understand the superior characteristics observed in autotetraploid *P. tomentosa*. Hitherto, only a few studies have examined proteome changes in polyploids compared with their progenitors, including those of allohexaploid *Triticum aestivum* (Islam et al. 2003), autopolyploid *Brassica oleracea* (Albertin et al. 2005), allopolyploid *Tragopogon mirus* (Koh et al. 2012), Arabidopsis polyploids (Ng et al. 2012), tetraploid *Robinia pseudoacacia* (Wang et al. 2013) and autotetraploid *Manihot esculenta* (An et al. 2014). However, most of these studies used two-dimensional electrophoresis, which made it difficult to automate and resolve hydrophobic proteins and proteins with low abundance, extreme isoelectric point or molecular mass (Rabilloud 2002; Gilmore and Washburn 2010). Isobaric tag for relative and absolute quantitation (iTRAQ) combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS), which can identify and quantify proteins from multiple samples simultaneously (Wiese et al. 2007), has been widely applied to many proteomic researches recently (Yang et al. 2013; Fan et al. 2015b; Wang et al. 2015a, b).

In the present study, we carried out a comparative proteomic analysis of autotetraploid *P. tomentosa* and its diploid parent using iTRAQ-based proteomic methods to reveal the proteins associated with the superior characteristics of autotetraploid *P. tomentosa* (Fig. S1). In addition, we also investigate the correlation between transcriptome and proteome. Our results may help to refine the knowledge of the mechanisms associated with the superior features of autotetraploid *P. tomentosa* and provide some help for future paulownia breeding programs.

Materials and methods

Plant materials

Autotetraploid *P. tomentosa* (PT4) was induced by colchicine using the leaves of diploid *P. tomentosa* (PT2) in our previous study (Fan et al. 2007). Then, autotetraploid and diploid *P. tomentosa* were conserved by tissue culture in the Institute of Paulownia, Henan Agricultural University, Zhengzhou, Henan Province, China. The conservation conditions were as follows: 25 ± 2 °C, 70% humidity, 130 μmol m^{-2} s^{-1} illumination intensity and 16 h/8 h (day/night) photoperiod. Compared with diploid *P. tomentosa*, autotetraploid *P. tomentosa* exhibits better photosynthetic characteristics (Zhang et al. 2013) and higher stress tolerance (Deng et al. 2013; Dong et al. 2014a, b, c). In this study, leaves of PT2 and PT4 were used for callus induction and plantlets regeneration according to the method of Zhai et al. (2004) and Dong et al. (2009). All the materials were cultured under the condition mentioned above. After culturing, uniformly terminal buds were collected from different PT2 and PT4 plantlets, then mixed respectively, frozen immediately in liquid nitrogen, and stored at −80 °C until protein extraction. Two parallel samples were prepared for each genotype.

Morphological and physiological index measurements

30-day-old plantlets for each genotype were used to measure the physiological indexes. The lengths and widths of 10 leaves from three replicates of PT2 and PT4 were measured. The content of chlorophyll was determined with the method described previously (Wang et al. 2016).

Protein extraction and digestion

Leaf tissues from PT2 and PT4 were well ground in liquid nitrogen and treated with lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 40 mM Tris–HCl, pH 8.5) consisting of 1 mM PMSF and 2 mM EDTA (final concentration). DTT was added to the samples at a final concentration of 10 mM after 5 min. The protein was extracted, reduced and alkylated according to the method of Fan et al. (2015b) and quantified using the Bradford assay with BSA as the standard (Bradford 1976). For each sample, 100 µg of total protein extract was digested using Trypsin Gold (Promega, Madison, WI, USA) with a protein:trypsin ratio of 30:1 for 16 h at 37 °C.

iTRAQ analysis

The digested peptides were dried, reconstituted with 0.5 M TEAB and labeled using iTRAQ 8-plex kits (Applied Biosystems, Foster City, CA, USA) according to the manufacture's manual. PT2 and PT4 peptide samples were labeled with 113- and 115-iTRAQ tags separately. The iTRAQ experiment was performed on two independent biological replicates. Subsequently, the labeled peptides were pooled, lyophilized and redissolved in 4 ml buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7) for fractionation by strong cation exchange (SCX) chromatography using LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) with a Ultremex SCX column (4.6 × 250 mm, 5 µm particles) (Phenomenex, Torrance, CA, USA). The peptides were eluted with a gradient of buffer B (25 mM NaH_2PO_4, 1 M KCl in 25% ACN, pH 2.7) according to the method of Ge et al. (2014). Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted fractions were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex, Torrance, CA, USA), vacuum-dried, and then analyzed by LC–MS/MS using LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) and TripleTOF 5600 systems (AB SCIEX, Concord, ON) as described previously (Ge et al. 2014). Briefly, each fraction was resuspended in buffer A (5% ACN, 0.1% FA) and centrifuged. Then, 5 µl supernatant was loaded on a LC-20AD nanoHPLC by the autosampler onto a 2 cm C18 trap column. Data acquisition was performed with a TripleTOF 5600 system fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA). The process of LC–MS/MS analysis was detailedly described in the Supplemental protocol. The raw mass spectra data were processed using the Proteome Discoverer 1.2 software (Thermo Fisher Scientific, San Jose, CA, USA). Proteins identification was performed by using the Mascot software version 2.3.02 (Matrix Science, London, UK) against the recently annotated *P. tomentosa* transcriptome dataset containing 26,059 entries. The search parameters were used as described earlier (Yang et al. 2013). These parameters were set as follows: MS/MS ion search; trypsin enzyme; the fragment mass tolerance of ±0.1 Da; the intact peptide mass tolerance of ±0.05 Da; monoisotopic mass values; one max missed cleavage site; fixed modifications of carbamidomethyl (C), iTRAQ 8-plex (N-term) and iTRAQ 8-plex (K); variable modifications of Gln->pyro-Glu (N-term Q), Oxidation (M) and iTRAQ 8-plex (Y). iTRAQ 8-plex was chosen for quantification during the search simultaneously.

For protein identification, only peptides with significant hits (99% confidence) were counted as identified, and each confident protein identification involved at least one unique peptide (Ge et al. 2014; Peng et al. 2015; Zhong et al. 2017; Chen et al. 2017). For protein quantification, a protein had to contain at least two unique peptides and the quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Proteins with fold changes >1.2 or <0.83 and *p* values <0.05 in at least one of the repeated analysis and having the same trend (1 < fold changes < 1.2 or 0.83 < fold changes < 1) in the other repeated analysis were defined as differentially expressed proteins (DEPs) (Shen et al. 2015).

Bioinformatics analysis of proteins

All the identified proteins were annotated and categorized based on the Gene Ontology (GO) (Blake et al. 2013) and Clusters of Orthologous Groups of proteins (COG) databases (Tatusov et al. 2003). Additionally, the identified proteins were also mapped to the Kyoto Encyclopedia of Genes and Genome (KEGG) database (Kanehisa and Goto 2000) for metabolic pathway analysis.

Quantitative real-time PCR (qRT-PCR) confirmation

To validate the DEPs identified by iTRAQ, 10 proteins were selected randomly based on the proteomic data and the corresponding unigenes sequences were used for the qRT-PCR analysis. The gene-specific primers were designed using Beacon Designer 7.7 software (http://www.premierbiosoft.com/index.html), and their sequences and other details are listed in Table 1. Total RNA was isolated from the leaves of PT2 and PT4 using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using a iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf). qRT-PCR was performed on

Table 1 Primer sequences of the unigenes used for qRT-PCR

Unigene ID	Protein name	Expression trends[a]	Primer sequence
m.48523	8-Hydroxygeraniol dehydrogenase	Up	GATGTTGGTGCTCCTCTC TGCTTCCTTCTTCTTACTAATAG
m.37390	Proteasome subunit alpha type-7-like	Down	CGATATGACAGAGCGATTACG GGCGGCATTTCCTTTACG
m.1174	33 kDa ribonucleoprotein	Down	TGAATGGAGTGGAGGTGGAG CTGGTGGCGGAGGAGATG
m.20714	Phosphoglycerate kinase	Up	TTCCAAGCCAAGCAGTAG GTCAAGGTCCACATCTCC
m.22866	Light dependent NADH:protochlorophyllide oxidoreductase 2	Up	GGGCATTTCCTCCTTTCAAG TCAATCCTCCAGCAAGTCC
m.49884	Catalase	Up	CCTGCTGTTATTGTTCCTG ATGGTGATTGTTGTGATGG
m.6239	Ascorbate peroxidase	Down	GGAAGATGCCACAAGGAAC CAGGGTCAGAAAGAAGAGC
m.13987	Chloroplast oxygen-evolving protein	Up	AACACTGATTGACAAGAAGG TTGCTGATGGTCTGGAAG
m.52984	Unnamed protein product	Up	GGAGGTGGTGGAGGCTATG CAGATTGTGAGGTCGGATTGG
m.10949	PHB1	Down	GGGCAGCGATTATTAGGG AGGCAGATATACCACATTCC
Internal control	18s rRNA		ACATAGTAAGGATTGACAGA TAACGGAATTAACCAGACA

[a] Expression trends indicated the expression trends of the selected proteins in PT4 compared with PT2

CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction system (20 µl) and amplification procedure referred to the method described by Fan et al. (2015a). 18S rRNA was used as an internal control to normalize the gene expression, and the relative gene expression level was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results and discussion

Morphological and physiological index measurements

30-day-old plantlets for each genotype were used for morphological and physiological index measurements. The phenotypic differences in plantlets and leaves of PT2 and PT4 are presented in Fig. 1. Compared with PT2 plantlets, PT4 plantlets were more robust with denser bristles (Fig. 1a, b). The leaves of PT4 were larger and thicker than those of PT2 (Fig. 1c, d). As is shown in Fig. 1e, the leaf length and leaf width of PT4 showed increase in contrast with those of PT2. When examining the chlorophyll content, the level of chlorophyll in PT4 was higher than that in PT2 (Fig. 1f).

Protein identification, functional annotation and classification

A total of 386,933 spectra were generated from the iTRAQ-based experiments using the proteins extracted from the PT2 and PT4 samples as the materials. The data collected from these samples were analyzed using Mascot 2.3.02 software. Among them, a total of 16,406 spectra matched to known spectra and 13,815 spectra matched to unique spectra (Table S1). Finally, 1427 proteins were identified in *P. tomentosa* (Table S2). The repeatability of the proteomic analysis is shown in Fig. S2, indicating that the results in our study were reliable. To understand the functions of the identified proteins, GO analysis was conducted under the three main categories: biological process, cellular component and molecular function (Fig. 2). Under the category of biological process, 17.57% of the proteins were related to "metabolic process", followed by "cellular process" (16.46%); under the category of cellular component, "cell" (21.92%) and "cell part" (21.92%) were the

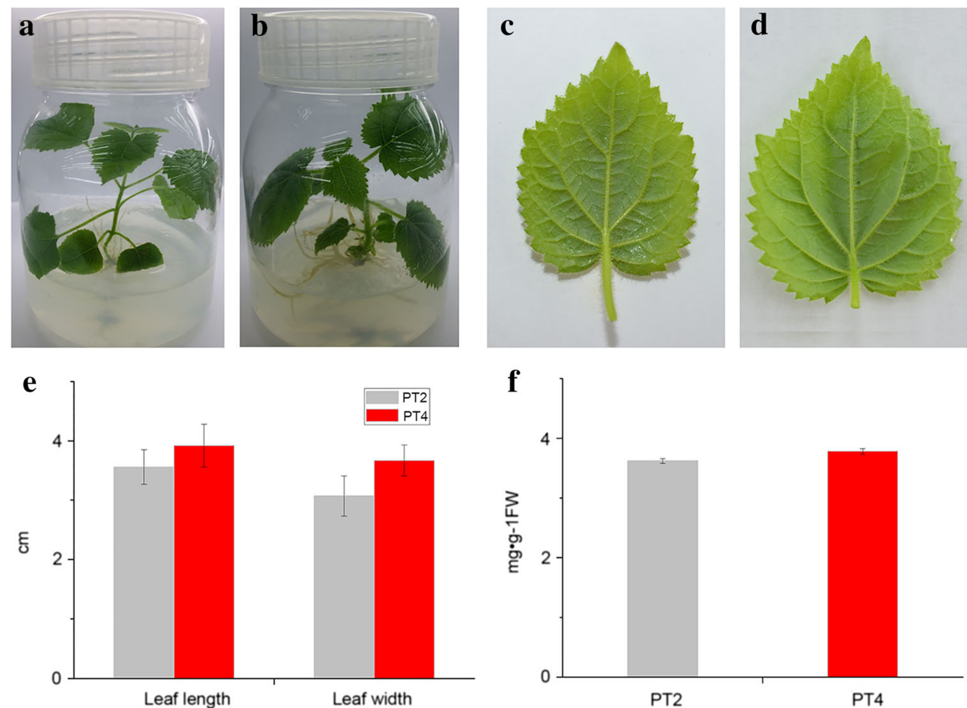

Fig. 1 Comparison of morphological and physiological indexes of PT2 and PT4. **a** Plantlet of PT2 genotype; **b** plantlet of PT4 genotype; **c** leaf of PT2; **d** leaf of PT4; **e** the leaf length and width of PT2 and PT4; **f** the chlorophyll content of PT2 and PT4

most highly represented; while under the category of molecular function, the highest number of proteins were assigned to "catalytic activity" (44.5%), followed by "binding" (41.58%). In addition, 1241 of the identified proteins were assigned to 23 functional groups in the COG database (Fig. 3). The largest category was "General function prediction only", followed by "Posttranslational modification, protein turnover, chaperones". Many of the identified proteins were involved in "Energy production and conversion", "Carbohydrate transport and metabolism" and "Translation, ribosomal structure and biogenesis". The KEGG analysis showed that the identified proteins participated in 112 pathways (Table S3). These results indicated that the identified proteins were involved in almost every aspect of *P. tomentosa* metabolism.

Analysis of differentially expressed proteins

Of the 1427 identified proteins, 130 DEPs with fold changes >1.2 and p values <0.05 were screened between PT4 and PT2. Compared with their abundance in PT2, 78 (60%) proteins were increased and 52 (40%) were decreased in PT4 (Table S4). To better understand the differences in the biological processes between PT2 and PT4, we performed a GO enrichment analysis of the DEPs. It was suggested that multiple biological process associated with stress resistance and photosynthesis were significantly enriched at the 5% significant level (Table S5), including defense response to bacterium (GO:0042742, $p = 1.29 \times 10^{-4}$), response to bacterium (GO:0009617, $p = 1.70 \times 10^{-4}$), defense response (GO:0006952, $p = 1.63 \times 10^{-2}$), response to biotic stimulus (GO:0009607, $p = 1.88 \times 10^{-2}$), photosynthesis (GO:0015979, $p = 3.78 \times 10^{-4}$), thylakoid membrane organization (GO:0010027, $p = 5.96 \times 10^{-3}$), and photosystem II assembly (GO:0010207, $p = 8.92 \times 10^{-3}$). Moreover, to further uncover the metabolic pathways in which these proteins may be involved, KEGG enrichment analysis was also conducted. Our results demonstrated that 103 DEPs were mapped to 60 KEGG pathways (Table S6). Among these pathways, DEPs were significantly enriched in ribosome (ko03010, $p = 2.97 \times 10^{-3}$), photosynthesis (ko00195, $p = 5.33 \times 10^{-3}$) and proteasome (ko03050, $p = 3.16 \times 10^{-2}$) at the 5% significant level.

Proteins related to superior photosynthetic characteristics in autotetraploid P. tomentosa

Photosynthesis is the basis of plant growth and development, and improved photosynthetic characteristics have been reported in tetraploid black locust (Meng et al. 2014), hexaploid *Miscanthus* × *giganteus* (Ghimire et al. 2016), triploid rice (Wang et al. 2016) and triploid Populus (Liao et al. 2016). The photosynthetic rate is mainly determined by light-dependent reaction, carbon fixation and CO_2 entry into the plant through stomata. Zhang et al. (2013) reported that the net photosynthesis rate, stomatal conductance, intercellular CO_2 concentration and chlorophyll content all increased in PT4 compared with PT2, which may partly explain the superior photosynthetic characteristics in PT4. Furthermore, in a

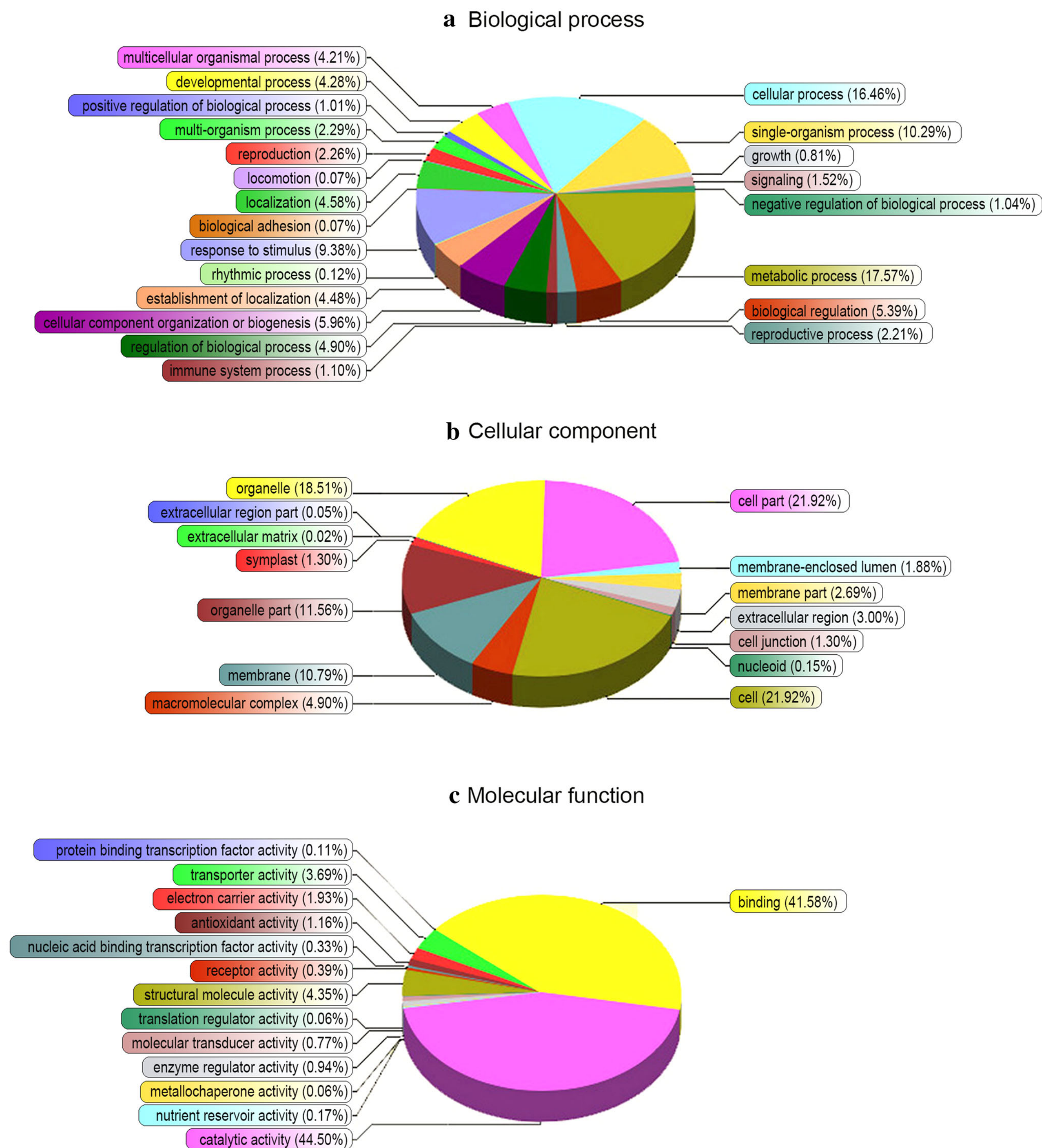

Fig. 2 GO analysis of proteins identified in *P. Tomentosa.* 1341 proteins (93.97% of total) were divided into 50 function groups. **a** Biological process; **b** cellular component; **c** molecular function

comparative transcriptome analysis that we reported previously, genes associated with photosynthesis were found to be up-regulated in the PT4 versus PT2 comparison, and it was suggested that the improved photosynthesis in PT4 may mainly attribute to the increased enzyme activity and photosynthetic electron transfer efficiency in the autotetraploid (Fan et al. 2015a). In this study, 14 DEPs with known functions in the non-redundant protein sequences (Nr) database were found to be related to photosynthesis in *P. tomentosa* (Table 2). Among these DEPs, 7 displayed increased abundances in PT4 compared with PT2 and were annotated as chloroplast rubisco activase (ABK55669.1),

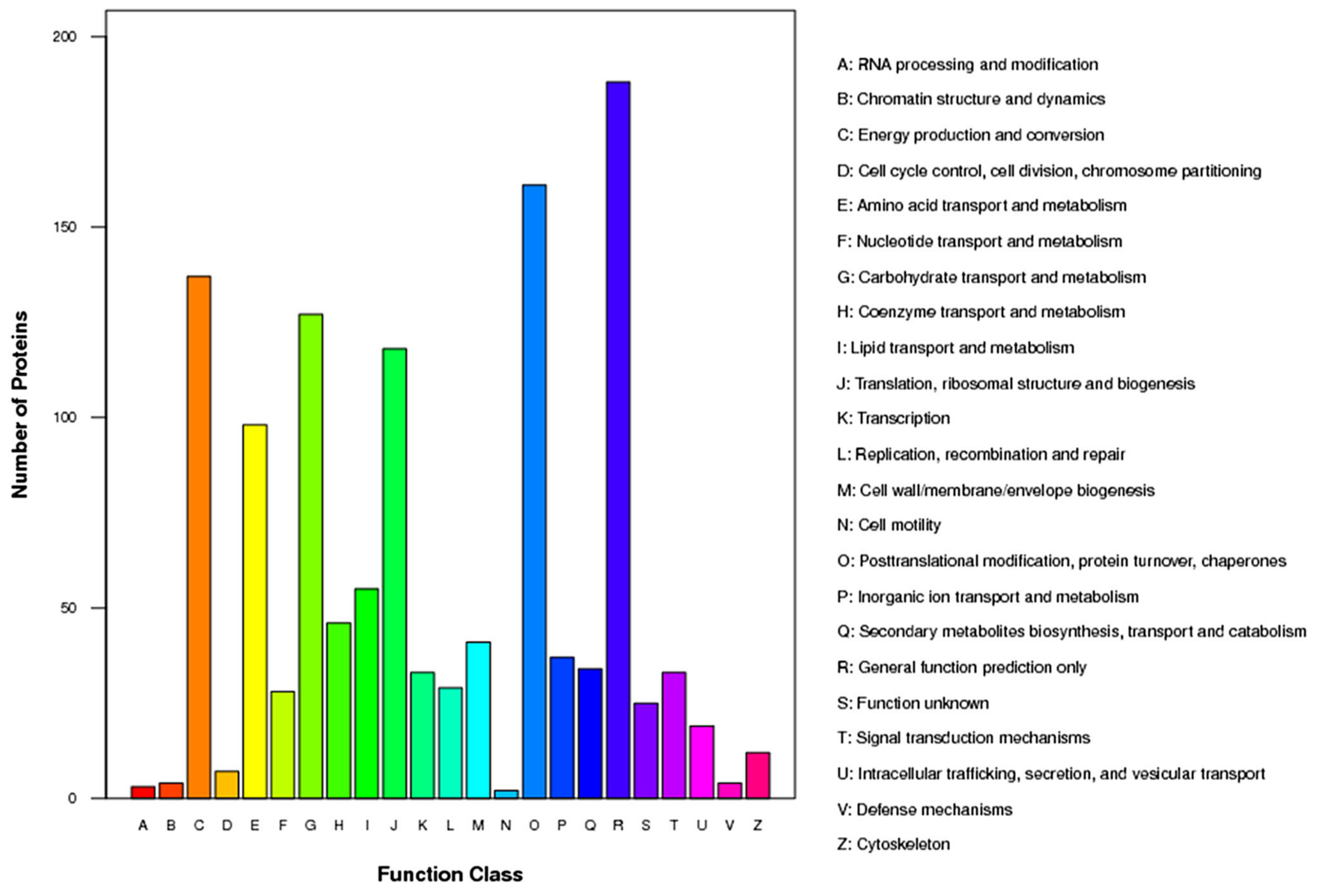

Fig. 3 COG analysis of proteins identified in *P. Tomentosa.* 969 proteins (67.90% of total) were categorized into 23 function groups

Table 2 DEPs related to photosynthesis between PT2 and PT4

Unigene	Accession no.[a]	Protein name	FC[b]
m.40546	ABK55669.1	Chloroplast rubisco activase	1.839
m.20714	AAA79705.1	Phosphoglycerate kinase	1.311
m.22866	AAF82475.1	Protochlorophyllide oxidoreductase 2	1.299
m.13987	ACA58355.1	Chloroplast oxygen-evolving protein	1.280
m.13986	ACA58355.1	Chloroplast oxygen-evolving protein	1.266
m.22039	XP_011080143.1	Photosystem I reaction center subunit IV B	1.246
m.10882	ACS44643.1	Putative cytochrome b6f Rieske iron-sulfur subunit	1.229
m.17700	P20121.1	Photosystem I reaction center subunit VI	1.219
m.48581	XP_002531690.1	Chlorophyll A/B binding protein	0.778
m.21971	BAH84857.1	Putative porphobilinogen deaminase	0.764
m.27093	CAM59940.1	Putative Mg-protoporphyrin IX monomethyl ester cyclase	0.750
m.13940	AAF19787.1	Photosystem I subunit III	0.638
m.43677	P83504.1	Oxygen-evolving enhancer protein 1	0.561
m.52524	ABW89104.1	Glyceraldehyde-3-phosphate dehydrogenase	0.553
m.19368	AEC11062.1	Photosystem I reaction center subunit XI	0.505

[a] NCBI accession number

[b] FC: Fold changes of proteins in PT4 compared with PT2

phosphoglycerate kinase (AAA79705.1), chloroplast oxygen-evolving protein (ACA58355.1), photosystem I reaction center subunit IV B (PsaE-2) (XP_011080143.1), putative cytochrome b6f Rieske iron-sulfur subunit (ACS44643.1), photosystem I reaction center subunit VI (P20121.1), and protochlorophyllide oxidoreductase 2 (AAF82475.1). The

light-dependent reaction involves light induced charge separation, electron transport and synthesis of ATP, and is mainly driven by four intrinsic multi-subunit membrane-protein complexes named photosystem I (PSI), photosystem II (PSII), cytochrome (cyt) b_6f complex and ATP synthase (Nelson and Yocum 2006). PSI is located in the stroma lamellae, and is composed of a reaction center consisting of different subunits and a light-harvesting complex (LHC). PsaE, a small hydrophilic subunit of PSI that is very accessible to the surrounding medium, plays an indispensable role in optimizing electron transport to ferredoxin and flavodoxin. Besides, PsaE is likely to be important for the stability of PSI (Scheller et al. 2001). Chloroplast oxygen-evolving protein is a nuclear-encoded extrinsic protein of PSII which is required for O_2 evolution activity under physiological conditions and it may constitute a benefit to stabilize the PSII system. Cytochrome b6f Rieske iron-sulfur subunit combining with other subunits of cytochrome (cyt) b_6f complex resides in the thylakoid membrane and provides the electronic connection between the PSI and PSII reaction centers, which generates a trans-membrane electrochemical proton gradient for ATP synthesis (Kurisu et al. 2003). These proteins are likely to contribute to the improved photochemical reaction efficiency of PT4. Carbon fixation catalyzed by a series of enzymes can assimilate CO_2 into organic matter and may contribute to the accumulation of biomass. Rubisco is a key enzyme involved in the incorporation of atmospheric CO_2 into ribulose bisphosphate, and this process generates the necessary building blocks for carbohydrate biosynthesis. Chloroplast rubisco activase (RCA) is a chaperone-like protein of the AAA + ATPase family, which utilizes ATP hydrolysis to remove tight-binding inhibitors and activate the catalytic activity of Rubisco (Henderson et al. 2013). It was reported that photosynthesis in rice can be improved by inducing RCA gene expression (Chen et al. 2014). Hence, the increased RCA may regulate the initial Rubisco activity, which will be beneficial to carbon fixation. Phosphoglycerate kinase catalyzes the phosphorylation of 3-phosphoglycerate to 1,3-diphosphoglycerate within the Calvin cycle using ATP (Tsukamoto et al. 2013), and it is also associated with glycolysis/gluconeogenesis. These findings indicate that changes in the protein expression profiles in PT4 compared with PT2 may help to explain the photosynthetic superiority observed in autopolyploids.

Proteins associated with the potentially enhanced stress adaptability of autotetraploid P. tomentosa

It is generally accepted that polyploidization always confers higher stress tolerance to polyploids compared with their corresponding parents (Podda et al. 2013; Wang et al. 2013), and enhanced adaptability to various stresses has been observed in autotetraploid paulownia (Deng et al. 2013; Dong et al. 2014a, b, c; Xu et al. 2014; Fan et al. 2016). Our previous anatomy research showed that the thickness of leaves, palisade tissue, cell tense ratio and thickness ratio of palisade tissue to sponge tissue of tetraploid paulownia were larger than those of the diploids (Zhang et al. 2012), which may provide a better protection mechanism for autotetraploid paulownia than for its diploid. In a previous study, we found that the constitutive expression levels of stress-responsive genes and miRNAs that mediated defense response were significantly up-regulated and down-regulated, respectively, in autotetraploid paulownia (Fan et al. 2014, 2015a). In the present study, 27 constitutively defense-responsive proteins with known functions were found to be differentially expressed in PT4 compared to its diploid progenitor (Table 3). Among these proteins, 2-hydroxyisoflavanone dehydratase-like (XP_011088284.1), calmodulin (XP_010645766.1), 8-hydroxygeraniol dehydrogenase (Q6V4H0.1), catalase (AFC01205.1), formate dehydrogenase (XP_002278444.1) and pyruvate dehydrogenase E1-beta subunit (ADK70385.1), which play vital roles in the adaptability of PT4 to stresses tended to be up-regulated in the comparison of PT4 versus PT2. Isoflavonoids are a large group of plant secondary metabolites, including pisatin, maackiain, genistin, daidzin and glyceollin, which play important roles as antimicrobial phytoalexins in plant defense systems against microorganisms and herbivores (Wang 2011). In soybean roots infected with *Fusarium solani,* glyceollin was rapidly produced to resist the fungus (Lozovaya et al. 2004). The abundance of 2-hydroxyisoflavanone dehydratase, which is the key enzyme in isoflavonoid biosynthesis, was increased 2.44-fold in PT4 compared with PT2. The Ca^{2+} signal is essential for the activation of plant defense responses and calmodulin acting as a Ca^{2+} signal transducer is reported to participate in Ca^{2+}-mediated induction of plant disease resistance responses (Heo et al. 1999; Ali et al. 2003; Takabatake et al. 2007). Catalase is an important antioxidant enzyme that plays a major role in the defense against oxidative stress by converting hydrogen peroxide to oxygen and water at an extremely rapid rate, thereby protecting the plants from oxidative damage (Michiels et al. 1994; Willekens et al. 1997). Transgenic tobacco plants expressing the maize *Cat2* gene had altered catalase levels that resulted in increased catalase activity and enhanced resistance to oxidative stress in the plants (Polidoros et al. 2001). The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, linking glycolysis to the tricarboxylic acid (TCA) cycle. In our iTRAQ data, a subunit of pyruvate dehydrogenase complex was found to be up-regulated in PT4, suggesting that the TCA cycle was enhanced, which would ensure sufficient energy for PT4 to resist stress. The alterations in abundances of these

Table 3 DEPs associated with stress response between PT2 and PT4

Unigene	Accession no.[a]	Protein name	FC[b]
m.6249	XP_002266488.1	Proteasome subunit beta type-6	6.8315
m.4321	ABE66404.1	DREPP4 protein	2.447
m.13643	XP_004230814.1	Probable carboxylesterase 7-like	2.4395
m.10909	XP_002263538.1	Calmodulin isoform 2	2.2845
m.54721	ACD88869.1	Translation initiation factor eIF(iso)4G	2.1205
m.49884	AFC01205.1	Catalase	1.8045
m.50291	AFP49334.1	Pathogenesis-related protein 10.4	1.734
m.13367	ADM67773.1	40S ribosomal subunit associated protein	1.5375
m.64528	ADK70385.1	Pyruvate dehydrogenase E1-beta subunit	1.477
m.48523	Q6V4H0.1	8-Hydroxygeraniol dehydrogenase	1.471
m.11708	NP_001234515.1	Temperature-induced lipocalin	1.316
m.27962	XP_002278444.1	Formate dehydrogenase	1.297
m.42262	XP_002514263.1	Elongation factor ts	1.292
m.64561	XP_004235848.1	Glycine–tRNA ligase 1	1.2565
m.26523	ABF46822.1	Putative nitrite reductase	1.24
m.63348	AFD50424.1	Cobalamine-independent methionine synthase	0.802
m.8635	ACB72462.1	Elongation factor 1 gamma-like protein	0.794
m.6239	AAL38027.1	Ascorbate peroxidase	0.771
m.10038	Q9XG77.1	Proteasome subunit alpha type-6	0.757
m.10949	AAZ30376.1	PHB1	0.7275
m.5417	Q05046.1	Chaperonin CPN60-2	0.712
m.37390	XP_004134855.1	Proteasome subunit alpha type-7-like	0.6895
m.64208	XP_003635036.1	Heat shock cognate protein 80-like	0.6875
m.8250	CAH58634.1	Thioredoxin-dependent peroxidase	0.66
m.1613	ABR92334.1	Putative dienelactone hydrolase family protein	0.6485
m.60046	CAA05280.1	Loxc homologue	0.624
m.29947	XP_002312539.1	MLP-like protein 28-like	0.504

[a] NCBI accession number

[b] FC: Fold changes of proteins in PT4 compared with PT2

constitutively expressed proteins in PT4 may contribute to its potential stress adaptability.

Verification of differentially expressed proteins by qRT-PCR

We further performed qRT-PCR to assess the expression of unigenes coding 10 DEPs selected randomly. The results are showed in Fig. 4. Among the unigenes corresponding to the selected proteins, five showed similar change trends in their expression levels with the DEPs based on the iTRAQ data. Three of these unigenes encoding proteasome subunit alpha type-7-like, 33 kDa ribonucleoprotein and PHB1 showed relatively decreased abundances in PT4, and two encoding catalase and NADP-dependent malic enzyme displayed relatively increased abundances in PT4. This result indicated that these proteins were regulated by transcription. However, the other five unigenes encoding 8-hydroxygeraniol dehydrogenase, phosphoglycerate kinase, light dependent NADH: protochlorophyllide oxidoreductase 2, ascorbate peroxidase and chloroplast oxygen-evolving protein, displayed discrepancy with the results of iTRAQ. This may be due to the post-transcriptional, translational, and post-translational regulation processes.

Correlation analysis of proteome with transcriptome data

To further determine whether transcript-level changes between PT4 and PT2 were correlated with changes in protein accumulation, the proteome data produced in this research and transcriptome data generated previously were compared (Fan et al. 2015a).

A subset of 2830 unigenes was differentially expressed between PT4 and PT2 with the absolute value of $\log_2$ ratio > 2 and FDR < 0.001. To compare the proteome with the transcriptome, we matched the DEPs against all

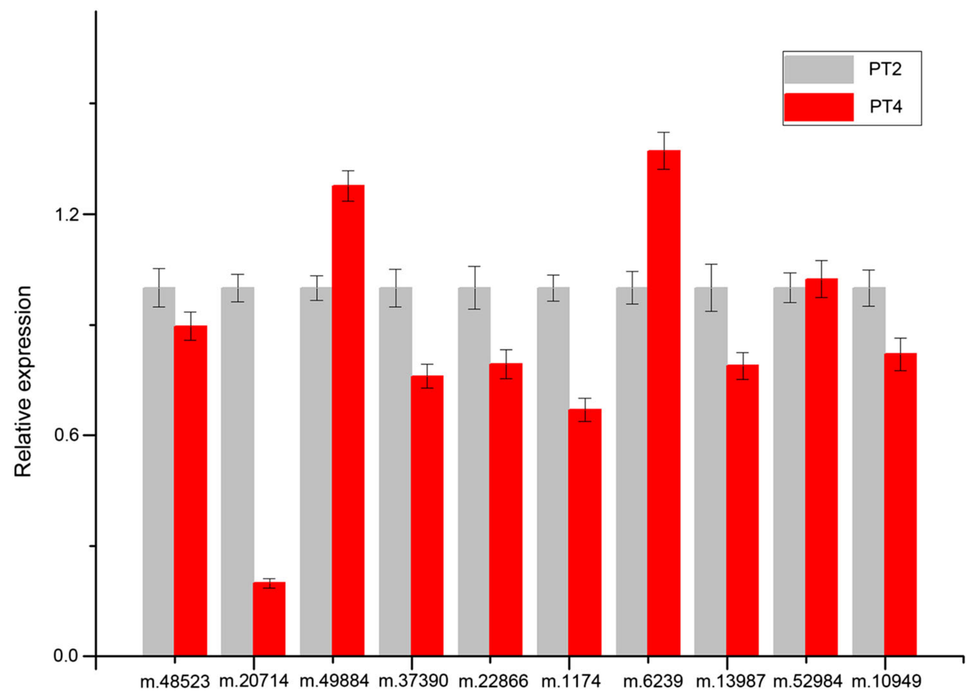

Fig. 4 qRT-PCR analysis of 10 differentially expressed proteins selected randomly between diploid *P. tomentosa* and its autotetraploid. *m.48523* 8-hydroxygeraniol dehydrogenase, *m.20714* phosphoglycerate kinase, *m.49884* catalase, *m.37390* proteasome subunit alpha type-7-like, *m.22866* protochlorophyllide oxidoreductase 2, *m.1174* 33 kDa ribonucleoprotein, *m.6239* ascorbate peroxidase, *m.13987* chloroplast oxygen-evolving protein, *m.52984* NADP-dependent malic enzyme, *m.10949* PHB1. *Bars* represent the mean (±SD)

differentially expressed unigenes. As a result, only 15 (11.5%) DEPs detected in the present study had corresponding differentially expressed unigenes between PT4 and PT2 datasets, indicating there was a poor correlation between mRNA expression and protein abundance (Table S7). Similar up-regulation or down-regulation of transcript and protein levels suggests transcriptional regulation of gene expression. In the PT4 and PT2 comparisons, only four and one proteins displayed the same trend of up-regulation and down-regulation in PT4 at transcript level, respectively. These five proteins were chloroplast rubisco activase (ABK55669.1), Chain A (2DCQ|A), protein S (AAU95203.1), catalase (AFC01205.1) and putative porphobilinogen deaminase (BAH84857.1). In addition, 113 proteins displayed significant expression differences between PT4 and PT2, while the corresponding mRNAs displayed no expression changes (Table S8). Futher, 58 proteins showed no significant expression differences between PT4 and PT2, although the corresponding mRNA showed significant expression differences (Table S9). These data indicated that gene expression was also regulated by post-transcription, translation, and post-translation modifications.

Limited correlation between mRNA expression and protein abundance levels has been also reported in *Citrus sinensis* (Fan et al. 2011), Arabidopsis polyploids (Ng et al. 2012), *T. mirus* (Koh et al. 2012) and *Brassica* hexaploid (Shen et al. 2015). There are several possible explanations for the discordance between transcriptome and proteome data. First, gene expression is a complicated process. In addition to transcriptional regulations, gene expression is also governed by post-transcriptional regulations, translational processes, and post-translational modifications. For instance, transposable elements have been found to participate in regulating the transcription of neighboring genes (Kashkush et al. 2003; Madlung et al. 2005); small RNAs (i.e. microRNAs and small interfering RNAs) may silence mRNAs and repress protein translation (Marmagne et al. 2010); and many mRNAs can give rise to more than one protein due to alternative splicing (Black 2003); moreover, the stability of mRNAs and proteins can also affect protein content. Second, several experimental factors and different detection methods could contribute to the poor correlation between mRNAs and proteins. High-throughput RNA sequencing can provide a precise analysis of RNA transcripts to detect transcriptome changes (Ansorge 2009), while iTRAQ may underestimate protein abundance ratios because of its imperfect accuracy (Ow et al. 2009; Karp et al. 2010). Furthermore, the possibility that the discordance was caused by the different sets of plant materials used in transcriptome and proteome comparative researches cannot be excluded.

Conclusions

iTRAQ-based quantitative proteomic approach was used for the comparative analysis of protein abundances in PT4 and PT2. A total of 1427 proteins were identified, of which

130 proteins were differentially expressed between PT2 and PT4. Photosynthesis-related proteins and stress-responsive proteins were significantly enriched between PT2 and PT4 based on GO and KEGG enrichment analysis. Among them, chloroplast oxygen-evolving protein, chloroplast rubisco activase, photosystem I reaction center subunit IV B, photosystem I reaction center subunit VI, cytochrome b6f Rieske iron-sulfur subunit, phosphoglycerate kinase, 2-hydroxyisoflavanone dehydratase-like, calmodulin, 8-hydroxygeraniol dehydrogenase, catalase and pyruvate dehydrogenase E1-beta subunit increased in PT4 which may be responsible for the superior photosynthetic characteristics and stress adaptability of PT4. Furthermore, the correlation analysis between proteome and transcriptome showed that the changes at protein level are not correlated well with the changes at transcripts level in PT4. This suggests that post-transcriptional, translational and post-translational regulations play important roles in protein expression. Complementary to previous transcriptome and small RNA profile, our proteomic analysis will provide new insights into better understanding the superior traits in PT4 and the candidate target genes identified in the present study could be used for genetic improvement of *Paulownia*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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