Label-free quantitative proteomic analysis revealed a positive effect of ectopic over-expression of *PeaT1* from *Alternaria tenuissima* on rice (*Oryza sativa*) response to drought

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Abstract

The protein elicitor *PeaT1* was found in *Alternaria tenuissima* and exerted broad spectrum resistance in wheat, cotton, and rice. Recently, we found that overexpressing *PeaT1* rice (OE) could enhance plant drought tolerance. Elucidating some elevated drought stress-related proteins and associated mechanisms is inevitable for improving drought tolerance in rice. In this study, combining a label-free quantitative proteomic method, multiple proteins were differentially accumulated in OE plants. Among these, a total of 57 significant changed proteins (including 32 up-regulated and 25 down-regulated) were mainly involved in metabolic, cellular, biological progress, and stress response. Using the RT-qPCR assay, 18 proteins' relative abundance was detected mostly consistent with the proteins abundance in proteomic data. Specially, proteins involved in abiotic stress, such as *OsSKIPa* and *OsPP2C*, which were significantly induced in early after dehydration treatment in transgenic rice, and the other stress response genes (prohibitin protein, PsbP protein, msrB Protein) also changed in *PeaT1* OE lines. Taken together, these results suggested that these differential proteins would be helpful for understanding the functional molecular mechanism of *PeaT1* in rice.

Keywords Proteomic analysis · Drought tolerance · *PeaT1* · Over-expression

Introduction

Drought is a critical abiotic stress that severely restricts crop production (Boyer 1982; Mahajan and Tuteja 2005; Xue et al. 2014). Deep understanding of plant responses to various stresses would be greatly crucial in improving the tolerance of plants to abiotic stresses through genetic engineering. When crops are exposed to drought conditions, several stress-related genes are induced such as transcription factors

(AREB, NAC, DREB1, and NF-YB), enzymes involved in osmotic compounds' biosynthesis (proline and mannitol), and reactive oxygen species (ROS) scavengers (Huang et al. 2009; Duan et al. 2012). Rice, as a staple food, feeds more than a half of the world's population (Sasaki and Burr 2000; Talbot 2003). When rice is exposed to water deficiency, the development and growth are seriously restricted. Mutation of *AM*, encoding a putative KEA (K⁺ efflux antiporter) in chloroplast, resulted in enhanced sensitivity to salinity in seed germination and increased tolerance to drought (Sheng et al. 2014). Overexpressing *OsSKIPa*, a rice homolog of human ski-interacting protein (SKIP), could significantly improve the drought resistance at seedling and reproductive stages in rice (Hou et al. 2009). Abscisic acid (ABA), a plant hormone, could mediate plant life cycle such as seed dormancy and affect flowering and fruit ripening. In addition, ABA also plays a crucial role in responses to a range of abiotic stresses such as drought, chilling, and salinity. It could regulate stomatal closure to adapt to water deficiency (Zhu 2002; Fan et al. 2004). When plants are exposed to adverse environment, ABA content is increased immediately to avoid the damage to plant.

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Elicitors, which generally consisted of proteins, peptides, carbohydrates, and lipids, could stimulate multiple of defense responses in plants (Hahn 1996). *PeaT1*, as a new plant activator from *Alternaria tenuissima*, contained 207 amino acids. *PeaT1*-treated plants exhibited enhanced systemic resistance with a significant reduction in the number and size of TMV lesions on wild tobacco leaves compared with control. Peroxidase (POD) activity and lignin increased significantly after *PeaT1* treatment (Zhang et al. 2011). Furthermore, we found that transgenic rice with overexpression of the *PeaT1* had better performance when exposed to drought stress (Shi et al. 2017). However, the function of *PeaT1* in rice remains largely unclear.

With advances in mass spectrometry, a label-free quantitative proteomic approach was developed for large-scale, high-throughput protein identification. Using this technology, each protein sample is digested, subsequently separated by liquid chromatography (LC), and finally analyzed by MS/MS. It is always combined with shotgun technology, multiple reaction monitoring, etc., and also used in studying the molecular mechanism and action target about pesticides (Chen et al. 2015; Zhao et al. 2018; Yu et al. 2018). Proteins could be quantified after MS/MS analysis by peptide peak intensity or based on spectral counting of identified peptides (Zhu et al. 2009). In this aspect, proteomic approach has the potential to illustrate proteins involved in transgenic elicitor *PeaT1* rice, making sense of the changes at protein level.

Although we found that the *PeaT1* overexpressing rice showed increased drought resistance, deeper knowledge of protein changes remain unclear. The purpose of this study was to understand the crucial role of *PeaT1* in rice. In addition, we obtained 57 differentially abundant proteins between the control and OE lines by label-free quantitative proteomic analysis, including the drought-related gene *OsSKIPa*. In all, these results would be helpful for exploration the molecular mechanism of *PeaT1* in rice.

Materials and methods

Plant materials and growth control

The Japonica rice (*O. sativa* L. ssp. *japonica*) cultivar Nipponbare, as the control, and homozygous transgenic lines OE1, OE40, and OE43 of overexpression, the elicitor *PeaT1* (using the vector pCAMBIA1305 contains 35S promoter) were obtained under the Nipponbare background (Shi et al. 2017). All plants were grown under climate chamber with 27 °C light for 16 h and 20 °C dark for 8 h. Additionally, *OsPPP2C* and *OsSKIPa* expression were detected by exposing 4-week-old transgenic seedlings to air (without water), and then collecting leaves 0.5, 1, 2, and 3 h later

for a subsequent RT-qPCR with three technical and three biological replicates.

Protein extraction

Soluble proteins were extracted from the leaves (500 mg) of plants for each independent biological control and OE lines (OE1, OE40, OE43). In detail, samples were ground into powder in liquid nitrogen using a mortar and pestle. The powder was transferred into extraction buffer [8 M urea, 1% DTT, 0.1 M Tris-HCl, pH 8.8, 1% complete protease inhibitors (Roche, Germany)]. Samples were sonicated and vortexed repeatedly, and the insoluble components were precipitated by centrifugation at 14,000 rpm for 0.5 h. Supernatants were precipitated overnight with 20% (v/v) trichloroacetic acid (TCA), washed three times with cold acetone, and solubilized in extraction buffer. All procedures were performed on ice. The protein concentration in the extracts was determined using the Bradford method. The soluble proteins were then frozen at −80 °C until subsequent treatment.

Protein digestion and analysis by nano-LC ESI-MS/MS

Samples were transferred into centrifuge tubes, and 50 mM NH_4HCO_3 was added until the concentration of urea reached 2 M. Modified trypsin (Thermo Fisher Scientific, USA) was added to each sample at an enzyme-to-substrate ratio of 1:100. Samples were then incubated overnight at 37 °C. Sample analysis was performed using an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, USA) coupled to an UltiMate 3000 RS nano-LC system (Thermo Fisher Scientific, USA). The chromatographic column was equilibrated with buffer A (98% water, 2% acetonitrile, and 0.1% formic acid). Each sample was automatically injected into the prepacked column (2 cm × 100 μm 3 μm-C₁₈). Liquid then entered an analytical column (12 cm × 150 μm 1.9 μm-C₁₈) at a speed of 600 nL/min. The next method consisted of an LC gradient with a linear ramp from 6% buffer B (80% acetonitrile, 0.08% formic acid) to 95% over 86 min (a linear gradient from 6 to 9% over 8 min, from 9 to 14% over 16 min, from 14 to 30% over 36 min, from 30 to 40% over 10 min, and from 40 to 95% over 10 min) followed by an equilibration of the column at 6% buffer B for 4 min.

Database search

Files obtained from the nano-LC-MS/MS analysis were converted to RAW format and searched against the UniProtRice.fasta (*Oryza sativa* subsp. *japonica*) database [Protein ID (UP000059680) and modified date in February, 2017] using Proteome Discoverer 2.0 software combined

with the Mascot search engine. The mass tolerance used for the database search was set to 20 ppm on full scans, and the fragment mass tolerance was set to 0.1 Da. The maximum missed cleavages were set to 1. Variable modifications checked oxidation (M) and acetyl (Protein N-term). For label-free quantitative methods, retention time matching between runs was performed within a time window of 1 min over a 20-min period. The peptide false discovery rate (FDR) and protein FDR cutoffs were set to 0.01. Protein biological functions were searched in Gene Ontology database.

RNA extraction and RT-qPCR

To complement the patterns of protein differential abundance, the corresponding transcripts often revealed differential patterns in the dynamics of their relative abundance levels. Total RNA was prepared from rice tissues using the TRIZOL reagent (TaKaRa, Japan) according to the manufacturer's instructions. cDNA (20 μ l) was synthesized from 1 μ g RNA using the QuantiTect Reverse Transcription Kit (Qiagen, China). RT-qPCR (20 μ l) was performed using 0.5 μ l of cDNA, 0.2 μ M of primer mix, and the SYBR Premix Ex Taq Kit (TaKaRa, Japan) in an ABI PRISM 7900HT

Fig. 1 Identification and label-free quantitative proteomic analysis of the wild-type (WT) and OE lines. **a** Volcano plot with different proteins, with $\log_{10}$ (a fold change of > 1.5) as the abscissa and $-\log_{10}(p < 0.05)$ as the ordinate. Proteins with significant variation are shown in red. **b** Pearson correlation coefficient analysis of the

WT and OE plants with values between -1 and 1 . Great similarity is shown in red and low similarity in green. **c** Up-regulated proteins were distributed among six groups. **d** Down-regulated proteins were distributed among six groups

sequence detection system (Applied Biosystems, <http://www.appliedbiosystems.com/>). The *UBQ* gene was used as an internal control. All primers used for RT-qPCR are listed in Table S1. Data were analyzed using the relative quantification method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analysis was performed using Student's *t* test to evaluate significant differences, which were expressed using a *p* value. *p* values lower than 0.05 were considered significantly different. The varying degrees of significance were $p < 0.05$ and $p < 0.01$ were labeled with one asterisk (*) and two asterisks (**), respectively.

Results and discussion

Label-free quantitative proteomic analysis

A label-free proteomic technique was used to analyze the function of the elicitor PeaT1 in OE transgenic rice. This technique was followed by protein extraction, protein digestion, LC-MS/MS, and data analysis. Samples of WT and OE lines were well separated in each group. A total of 4493 protein groups and 10,295 peptides were reproducibly identified from six rice leaf samples (OE1, OE40, OE43, and WT for three times) using Proteome Discoverer 2.0 software combined with Mascot. The following filter parameters were used: peptide FDR ≤ 0.01 and protein FDR ≤ 0.01 . Next, we

Table 1 Up-regulated proteins after label-free quantitative analysis

Gene ID	Description	Function	M_w (kD)	pI	Ratio (OE/control)
Os02g0759800	SKIP/SNW domain containing protein	Stress and defense	67	9.2	5.96
Os02g0686400	ATP binding	Metabolic process	61	6.3	3.15
Os03g0332400	Hydroxyacylglutathione hydrolase (<i>OsGLYH2</i>)	Metabolic process	29	5.6	3.06
Os10g0390500	Alanine aminotransferase (<i>OsAlaAT1</i>)	Metabolic process	53	6.7	2.52
Os04g0321700	OsSCP23—putative serine carboxypeptidase homologue	Protein metabolic process	56	6.6	2.02
Os01g0916400	Selenium-binding protein	Stress and defense	53	6.1	1.98
Os04g0504600	Carboxyvinyl-carboxyphosphonatephosphorylmutase	Metabolic process	53	7.3	1.96
Os03g0792400	Peptidase M50, mammalian sterol-regulatory element binding protein	Cellular	61	5.9	1.88
Os03g0249400	S10/S20 domain containing ribosomal protein	Translation	14	9.5	1.77
Os02g0803700	26S protease regulatory subunit 6A	Metabolic process	48	5.1	1.77
Os06g0116600	DAG protein, chloroplast precursor	Cellular	19	7.1	1.77
Os04g0479200	Dehydrogenase	Metabolic process	44	8.2	1.76
Os02g0757900	RNA recognition motif containing protein	Biological process	24	5.2	1.76
Os07g0684000	Stress responsive protein	Biological process	23	7.8	1.75
Os08g0109200	Dehydrogenase	Metabolic process	45	8.3	1.75
Os02g0170100	Glycine cleavage system H protein	Cellular	17	5	1.73
Os03g0356484	Tetratricopeptide repeat containing protein	Cellular	19	7.3	1.73
Os03g0286200	P-protein	Metabolic process	43	7	1.72
Os02g0720900	Aspartic proteinase nepenthesin-1 precursor	Protein metabolic process	34	7.9	1.71
Os06g0667600	Glycine cleavage system H protein	Cellular	17	4.9	1.71
Os08g0475400	Gibberellin receptor GID1L2	Metabolic process	35	5.3	1.71
Os04g0346100	40S ribosomal protein S27	Translation	10	8.7	1.7
Os02g0478700	40S ribosomal protein S27	Translation	10	8.7	1.7
Os10g0500700	OsGrx_S17—glutaredoxin subgroup II (<i>OsGRX22</i>)	Metabolic process	53	5.3	1.7
Os03g0841700	Prohibitin-2	Stress and defense	32	9.6	1.7
Os03g0814600	SPFH domain/band 7 family protein	Biological process	46	5.8	1.68
Os01g0256900	LSM domain containing protein	Translation	16	9.9	1.68
Os02g0510200	Thiamine pyrophosphate enzyme, C-terminal TPP binding domain (<i>OsALS</i>)	Metabolic process	69	7	1.65
Os04g0662800	RCC2 protein	Biological process	58	8.5	1.65
Os04g0661800	Mediator of RNA polymerase II transcription subunit 7	Metabolic process	56	8.6	1.63
Os05g0244600	DNA-binding protein-related	Biological process	15	8.9	1.6

analyzed the correlation between the detected proteins in different samples. Results indicated good relativity in the samples using the Pearson correlation coefficient (Fig. 1a). The transgenic lines OE1, OE40, and OE43 exhibited high similarity, as represented by red; green represents low correlation between WT and OE plants. Using these data, we selected proteins with > 1.5-fold changes as an additional standard; to screen for differential protein accumulation, a volcano plot was generated. And differentially abundant proteins (two standard deviations, SD) are shown as red spots (Fig. 1b).

According to the screening criteria, 57 protein groups were commonly identified as differentially expressed (OE1/WT, OE40/WT and OE43/WT), including 32 up-regulated (Table 1), and 25 down-regulated (Table 2). More information of proteins was shown in supplement table S2. Using Gene Ontology database, these proteins were divided into six groups related to metabolic processes: the response to stress, protein metabolic processes, cellular processes, translation, and biological processes (Fig. 1c, d).

In detail, among the up-regulated proteins, three genes were previously cloned including SKIP/SNW protein (*Os02g0759800*), hydroxyacylglutathione hydrolase (*OsGLYII2*), and Alanine aminotransferase (*AlaAT*). OE plants showed sixfold increased expression of SKIP/SNW protein, compared with the wild type. *OsSKIPa* is a regulator of drought stress-related genes and regulates cell activity and the response to ABA (Hou et al. 2009). Hydroxyacylglutathione hydrolase (*OsGLYII2*) (3.05 times increased in OE plant) is a glyoxalase II enzyme that functions in maintaining photosynthetic efficiency in rice and is involved in the adaptation to salt stress (Ghosh et al. 2014). Alanine aminotransferase (*AlaAT*) (2.52 times increased in OE plant) is an alanine amino transferase and plays an essential role in the regulation of starch storage in rice endosperm (Yang et al. 2015). Among the up-regulated proteins, 39% proteins are involved in metabolic processes (Fig. 1c), 19% in cellular processes, and 10% in translation. Three genes participated in the plant response to stress: SKIP/SNW protein (*OsSKIPa*), selenium-binding protein (*Os01g0916400*), and prohibitin-2 protein (*Os03g0841700*). Homologous

Table 2 Down-regulated proteins after label-free quantitative analysis

Gene ID	Description	Function	M_w (kD)	pI	Ratio (control/OE)
Os08g0154700	Uncharacterized	Metabolic process	32	9.9	3.15
Os02g0149800	Protein phosphatase 2C	Stress and defense	38	5.2	3.06
Os01g0958100	Signal recognition particle receptor	Cellular	39	8	2.52
Os10g0377800	Pyridoxamine 5%27-phosphate oxidase family protein	Metabolic process	52	6.4	1.98
Os07g0141400	PsbP protein	Stress and defense	27	8.5	1.88
Os10g0321700	RNA recognition motif containing protein	Cellular	36	5.6	1.88
Os12g0589100	Phosphoribosyl transferase, putative	Metabolic process	25	9.1	1.88
Os06g0472000	Peptide methionine sulfoxide reductase msrB	Stress and defense	23	9.3	1.87
Os03g0586500	Chloroplast post-illumination chlorophyll fluorescence increase protein	Metabolic process	26	8.3	1.83
Os08g0154700	Expressed protein	Biological process	11	7.4	1.83
Os03g0129900	Expressed protein	Metabolic process	17	9.5	1.83
Os03g0128400	ESTs AU078175 (C51476)	Cellular	20	9.8	1.83
Os01g0662300	Ribosomal protein L7/L12 C-terminal domain containing protein	Stress and defense	19	5.4	1.82
Os02g0761900	Regulator of ribonuclease	Metabolic process	18	5.9	1.83
Os05g0101200	pex14 protein	Cellular	54	6.5	1.81
Os03g0170500	Putative ribonuclease P	Metabolic process	32	10.1	1.8
Os08g0535600	Mitochondrial import inner membrane translocase subunit Tim	Cellular	8	5.3	1.79
Os01g0881550	Expressed protein	Biological process	9	9	1.79
Os05g0584200	Late embryogenesis abundant protein	Translation	17	5.3	1.78
Os03g0832300	Huntingtin-interacting protein HYPK	Biological process	13	5.2	1.79
Os03g0859600	ATP binding protein	Protein metabolic process	19	10	1.78
Os01g0139200	PB1 domain containing protein	Cellular	70	5.9	1.78
Os01g0771900	Periplasmic beta-glucosidase precursor	Metabolic process	68	6.4	1.78
Os04g0644400	LTPL125—protease inhibitor/seed storage/LTP family protein precursor	Metabolic process	17	7.5	1.7
Os05g0566500	Eukaryotic translation initiation factor 3 subunit D	Translation	65	5.5	1.65

Fig. 2 RT-qPCR analysis of differentially abundant proteins. **a** Up-regulated proteins in the wild-type (WT) and OE plants. **b** Down-regulated proteins in the WT and OE plants. * $0.01 < p < 0.05$; ** $p < 0.01$, Student *t* test

selenium binding protein (SBP) in *Arabidopsis thaliana* functions in cadmium (Cd) stress resistance (Duttilleul et al. 2008). Prohibitin-2 protein function is unclear. 25 down-regulated proteins were categorized into six groups (Fig. 1d). Among them, 36% are involved in metabolic processes, 24% participate in cellular processes, 8% are involved in translation, and 4% play a role in metabolic processes. 16% of the up-regulated proteins participate in the stress response, including phosphatase 2C protein (*Os02g0149800*), PsbP protein (*Os07g0141400*), methionine sulfoxidereductase (*Os06g0472000*), and ribosomal protein (*Os01g0662300*). Taken together, these results showed that OE plants with high expression of *PeaT1* exhibited differential proteins' relative abundance, and these proteins functioned in various processes.

RT-qPCR analysis of different proteins

Following the proteomic analysis, RT-qPCR was used to analyze the expression patterns of coding genes. To complement these changes in protein abundance, 10 up-regulated

Fig. 3 Expression levels of *OsSKIPa* (a) and *OsPP2C* (b) genes related to drought stress in transgenic *PeaT1* rice plants (OE43) and WT during the drought conditions. The data represent the mean $\pm$ SD, $n=6$, * $0.01 < p < 0.05$; ** $p < 0.01$, Student's *t* test

Fig. 4 Model of proposed biological function of elicitor *PeaT1* gene in transgenic rice

(Fig. 2a) and 8 down-regulated proteins (Fig. 2b) were selected. We carried out the quantitative analysis of these selected genes. Results showed that 6 and 7 genes were significantly up-regulated and down-regulated in OE plants compared with WT, while others exerted little changes between them. The transcription of *OsSKIPa* mRNA was altered approximately twofold, while the protein content was increased approximately sixfold. *OsGLYII2*, *AlaAT*, *Os03g0332400*, and *Os02g0686400* also showed similar changed trends with *OsSKIPa*. However, the mRNA level of the down-regulated genes was found to correspond with the protein relative abundance levels.

Several proteins were identified in proteomics analysis which involved in drought and salt tolerance. *OsSKIPa* is involved in drought tolerance in rice (Hou et al. 2009). Overexpression of *OsSKIPa* in rice could enhance tolerance to drought and high salinity, and the *AtSKIP* in *Arabidopsis* could also mediate the responses to ABA, salinity and drought stress during germination and seedling development. Ectopic expression of the *AtSKIP* gene modulated the induction of salt tolerance, dehydration resistance, and insensitivity towards abscisic acid under stress conditions (Lim et al. 2010). Selenium binding protein with unknown function in rice and *Arabidopsis* is involved in decreasing metal toxicity (Agalou et al. 2005; Dutilleul et al. 2008). In this study, both SKIP/SNW protein and phosphatase 2C protein showed higher abundance in OE plants, indicating that the *PeaT1* transcripts could influence the two genes' mRNA levels and play a vital role in *PeaT1* OE rice to resist the drought. It has been reported that the homologous protein PP2Cs in *Arabidopsis* directly interacted with ABA-related components (Yoshida et al. 2006). Over-expression of the gene *ZmPP2C* augmented the damages caused by salt and drought stresses, suggesting *ZmPP2C* acted as a negative regulator in salt- and drought-induced adaptive response signaling pathways that protect plant from damage (Gosti et al. 1999; Liu et al. 2009). Expression of *Os02g0149800* (protein phosphatase 2C) (Meyer et al. 1994; Reyes et al. 2005) was decreased in OE plant, suggesting it might contribute to the increase of drought in OE plants.

In addition, prohibitin protein (1.7 times increased in OE plant) is involved in mitochondrial biogenesis and protects against stress and senescence in plant cells (Ahn et al. 2006). PsbP protein (1.88 times decreased in OE plant) is essential for the regulation and stabilization of photosystem II in plants. The PsbP protein is involved in the defense response; silencing of PsbP expression in rice increases the severity of stripe virus disease symptoms and virus accumulation (Kong et al. 2006). Methionine sulfoxidoreductase (*msrB*) (1.87 times decreased in OE plant) is involved in the response to abiotic stress (Guo et al. 2009). All these proteins involved in stress response and the abundance of these proteins have

changed in OE lines, indicating that the functions of the elicitor *PeaT1* are worthy for further elucidation.

The expression of *OsSKIPa* and *OsPP2C* during drought

In order to understand the function of the proteins related to stress, we detected the expression of *OsSKIPa* and *OsPP2c* in transgenic rice. Two genes were significantly induced after dehydration treatment in transgenic rice. The expression level of *OsSKIPa* was higher than that in wild type (Fig. 3). The expression level of *OsPP2C* was significantly decreased at time of 0.5 h and 1 h, although the expression level was very high at 0.5 h.

Conclusion

With the label-free proteomic approach, we identified the change of proteome level in overexpressing rice. Interestingly, proteins related to stress processes were mostly enriched in OE lines, indicating these proteins might contribute to plant's resistance on stress (Fig. 4). Combined with the proteomic analysis, we were more likely to understand better about the mechanism of elicitor *PeaT1* in OE lines. However, further studies should be performed on the molecular level in transgenic rice.

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Compliance with ethical standards

Conflict of interest This study is not related to any potentially competing financial or other interests.

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