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ORIGINAL ARTICLE

Proteomic analysis of drought-responsive proteins in rice reveals photosynthesis-related adaptations to drought stress

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Abstract Drought stress inhibits rice growth and biomass accumulation. To identify novel regulators of drought-stress responses in rice, we conducted a proteome-level study of the stress-susceptible (SS) *Oryza sativa* L. cv. 'Leung Pratew 123' and its stress-resistant (SR) somaclonal mutant line. In response to osmotic-stress treatments, 117 proteins were differentially accumulated, with 62 and 49 of these proteins detected in the SS and SR rice lines, respectively. There were six proteins that accumulated in both lines. The proteins in the SS line were mainly related to metabolic processes, whereas the proteins identified in the SR line were primarily related to retrotransposons. These observations suggest that retrotransposons may influence the epigenetic regulation of gene expression in response to osmotic stress. To identify the biological processes associated with drought tolerance in rice, we conducted a co-expression network analysis of 55 proteins that were differentially accumulated in the SR line under osmotic-stress conditions. We identified a major hub gene; *LOC_Os04g38600* (encoding a glyceraldehyde-3-phosphate dehydrogenase), suggesting that photosynthetic adaptation via NADP(H) homeostasis contributes to drought tolerance in rice.

Keywords Rice · GeLC–MS/MS · Drought stress · Glyceraldehyde-3-phosphate dehydrogenase

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Abbreviations

AKT1	Inward-rectifier K^+ channel
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAPB	GAPDH β subunit
GeLC–MS/MS	Gel-based liquid chromatography–tandem mass spectrometry
GORK	Guard cell 'outward-rectifying' K^+ channel
NBS-LRR	Nucleotide-binding site–leucine-rich repeat
OSBPs	Oxysterol-binding proteins
PEG6000	Polyethylene glycol 6000
PP2C	Protein phosphatase 2C
PPR	Pentatricopeptide repeat
SKOR	Stelar K^+ 'outward-rectifying' channel
SR	Stress resistant
SS	Stress susceptible
TE	Transposable element

Introduction

Among constraining environmental conditions, drought is the most crucial factor that limits plant growth, development, and productivity (Boyer 1982). Osmotic stress

induces a series of molecular and physiological responses, including stomatal closure, osmotic adjustment, reduced photosynthetic activity, and abscisic acid (ABA) synthesis. Decreased water loss due to stomatal closure and/or reduced stomatal density is an example of a drought-avoidance mechanism (Chaves et al. 2003; Hadiarto and Tran 2010). However, a consequence of this strategy is often reduced photosynthesis and growth because of decreased carbon uptake.

Under stressful conditions, the earliest plant responses generally involve changes to gene expression (Yamaguchi-Shinozaki and Shinozaki 2006). These stress-induced changes in gene expression can alter plant protein profiles. However, there is not always a strong correlation between gene expression and protein abundance, often because of post-translational modifications (Park 2004; Urano et al. 2010; Webster and Thomas 2012). Therefore, to clarify plant responses to environmental stimuli, a proteome-level investigation may be a better option for revealing cellular adaptations (Kosová et al. 2015).

Proteomics-based studies have been used to identify drought-responsive proteins and genes in many crops, including rice (Ali and Komatsu 2006; Chamnanmanoontham et al. 2015; Ji et al. 2012), cotton (Deeba et al. 2012), grapevine (Lovisolo et al. 2010), soybean (Deshmukh et al. 2014; Oh and Komatsu 2015), wheat (Alvarez et al. 2014; Ford et al. 2011), and watermelon (Akashi et al. 2011). Different proteomics techniques, including two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with subsequent mass spectrophotometry identification (Ali and Komatsu 2006; Ji et al. 2012) or gel-free approaches (Oh and Komatsu 2015), have been used to identify important genes involved in abiotic stress responses. We herein describe the use of gel-based liquid chromatography–tandem mass spectrometry (GeLC–MS/MS) to overcome the limitations of 2D-PAGE, which is believed to be ill-suited for analyzing very high- and low-molecular-weight proteins, as well as hydrophobic, highly acidic, or alkaline proteins (Santoni et al. 2000). The accessibility of low-abundant proteins can be enhanced using sodium dodecyl sulfate (SDS)-PAGE/nano-LC–MS/MS rather than 2D-PAGE (Abere et al. 2012; Tefon et al. 2011; Wolff et al. 2006).

The increase of bioinformatics tools, which can be used for finding a novel gene or studying gene ontology, creates the extensive use of omics data. A gene co-expression network is one of the tools which can imply the complex gene interaction and provide us the understanding of plant process in particular hypothesis (Aoki et al. 2007; Usadel et al. 2009). The complexity of biological networks can classify into levels of genome-wide organization and individual molecular components such as node, degree, and module. Module is a word used to represent a group of highly co-express genes associated with biological processes which cannot be attributed to a single gene (Hartwell et al. 1999). A co-expression network has uncovered novel stress-responsive gene in many organisms such as rice (Chamnanmanoontham et al. 2015; Nounjan et al. 2016; Smita et al. 2015) *Escherichia coli* and yeast (Altaf-Ul-Amin et al. 2006).

Rice (*Oryza sativa* L. spp. *indica*) is staple food for more than one-third of the global population. However, rice production is limited by environmental stresses such as cold (Jia et al. 2015), drought (Haefele et al. 2016; Izanloo et al. 2008; Saikumar et al. 2016), and salinity (Javid et al. 2011). In this study, we conducted a proteome-level investigation of two Thai rice lines with contrasting stress tolerances. We used the stress-susceptible 'Leung Pratew 123' cultivar (LPT123; SS) and the stress-tolerant somaclonal line derived from LPT123 (i.e., LPT123–TC171; SR). The SR line exhibits greater tolerance to osmotic (Pongprayoon et al. 2013; Thikart et al. 2005) and salt (Thikart et al. 2005; Udomchalothorn et al. 2009) stresses. Some of the genes responsible for the increased salt-stress tolerance have been identified (Pongprayoon et al. 2013; Sripinyowanich et al. 2013; Thikart et al. 2005; Udomchalothorn et al. 2009, 2014; Vajrabhaya and Vajrabhaya 1991). To evaluate the potential mechanisms regulating osmotic-stress tolerance, we completed a GeLC–MS/MS proteome-level analysis of the SR line. A co-expression network, constructed based on the proteins that were significantly over- or under-accumulated in the SR line in response to osmotic stress, was analyzed to identify the hub gene(s), which are the genes with an extremely high connectivity to other genes in the module showing the high degree of co-expression with other genes, suggesting the involvement in osmotic adaptation. Differential transcription of these genes was confirmed, and their potential role in rice drought tolerance is discussed. This proteome-level investigation of closely related rice lines differing in osmotic-stress tolerance (Thikart et al. 2005) revealed a novel group of proteins and a mechanism regulating osmotic-stress adaptation in rice.

Materials and methods

Proteomic analysis of two rice lines

Plant materials and growth conditions

The SS and SR rice lines used in all experiments have been previously described (Chamnanmanoontham et al. 2015; Pongprayoon et al. 2013; Sripinyowanich et al. 2013; Thikart et al. 2005; Udomchalothorn et al. 2009, 2014; Vajrabhaya and Vajrabhaya 1991). The SS and SR lines

were grown using a completely randomized design with three biological replicates. Rice germination and growth conditions were similar to those described, previously (Chamnanmanoontham et al. 2015). Briefly, rice seeds were soaked in distilled water for 24 h and then germinated on sterilized sand flooded with distilled water under natural light. Two weeks after germination, modified WP solution (Vajrabhaya and Vajrabhaya 1991) was added and seedlings were grown in the greenhouse under natural light, which has the intensity of 93–99 μmol photon m^{-2} s^{-1} and a relative humidity of between 74 and 81%. The nutrient solution was refreshed every 7 days. After 4 weeks, seedlings of each line were separated into two groups. The plants of one group continued to grow in freshly prepared nutrient solutions, while plants of the other group were transferred to nutrient solution supplemented with 10% (w/v) polyethylene glycol 6000 (PEG6000) to simulate osmotic-stress conditions (Pongprayoon et al. 2013). We collected SS and SR leaves at 0, 2, 6, and 24 h after treatment, then, immediately froze them in liquid nitrogen and kept at −80 °C until analyzed. The proteins from SS and SR at each timepoint of collection were extracted separately for proteomics analysis.

Protein extraction and one-dimensional SDS-PAGE

Leaf tissue from three seedlings was pooled for each replicate, and proteins were extracted from ground tissue by incubating samples in 0.1% SDS at 37 °C for 3 h. Total protein concentrations were determined using an established procedure (Lowry et al. 1951), with bovine serum albumin serving as the protein standard. Equal amounts of extracted proteins were separated on a one-dimensional 12.5% (w/v) SDS-PAGE gel (Laemmli 1970), which was stained with Coomassie Blue R-250 to visualize proteins (Meyer and Lamberts 1965).

Trypsin digestion for mass spectrometry analysis

Proteins were manually excised from the SDS-PAGE gel using a previously described method (Jaresitthikunchai et al. 2009). The in-gel digestion procedure was conducted at the Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand. Gel lanes were divided into six groups according to molecular weight (with protein markers as standards) to reduce the complexity of the peptide analysis. The gel was cut into 1 mm^3 pieces that were individually washed with sterile milli-Q water. The gel pieces were then dehydrated with 100% acetonitrile (ACN) for 5 min and then dried for 5 min. Disulfide bonds were reduced by incubating gel pieces in a solution consisting of 10 mM dithiothreitol and 10 mM NH_4HCO_3 for 1 h. The gel pieces were then alkylated with 100 mM iodoacetamide and 10 mM NH_4HCO_3 for 1 h in the dark. Subsequently, the gel pieces were dehydrated three times for 5 min each in 100% ACN. Proteins were digested in a trypsin solution (10 ng μl^{-1} trypsin, 50% ACN, and 10 mM NH_4HCO_3) for 20 min and then incubated in 30% ACN overnight. The digested peptides were treated with a solution consisting of 50% ACN and 0.1% trifluoroacetic acid for 10 min. All procedures were completed at room temperature, except for the drying of extracted peptides, which occurred in an oven at 40 °C. Dried peptides were stored at −80 °C prior to MS analyses.

Liquid chromatography–electrospray ionization tandem mass spectrometry

The digested peptide solutions were injected into an Ultimate 3000 LC system (Dionex, CA, USA) equipped with an ESI-Ion Trap mass spectrometer (HCT Ultra PTM Discovery System, Bruker Daltonik GmbH, Germany). The electrospray flow rate to the μ-precolumn (Monolithic Trap Column; 200 μm internal diameter × 5 cm) was set at 20 μl min^{-1}. Peptides were separated on a nano column (Monolithic Column; 100 μm internal diameter × 5 cm) at a mobile phase flow rate of 1.0 μl min^{-1}. Three technical replications were performed.

The raw LC–MS/MS data were converted to mzXML format using the CompassXport 1.3.10 program (Bruker Daltonik GmbH, Germany). We used the DeCyder MS Differential Analysis software [GE Healthcare (Johansson et al. 2006; Thorsell et al. 2007)] to quantify proteins. The analyzed MS/MS data were checked against the National Center for Biotechnology Information non-redundant database 20170221 using Mascot software [Matrix Science, London, UK (Perkins et al. 1999)]. We used the following parameters: taxonomy: *O. sativa* (rice); enzyme: trypsin; allow up to: 1 missed cleavage; fixed modifications: carbamidomethyl; variable modifications: oxidation; peptide tolerance: ±1.2 Da; MS/MS tolerance: ±0.6 Da; peptide charge stage: 1+, 2+, and 3+ (monoisotopic); and instrument: ESI-TRAP.

Identification of genes/proteins and analysis of a co-expression network

The identified proteins were searched against the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu) (Kawahara et al. 2013) using BLASTP to annotate proteins and assign functions based on gene ontology. If more than one locus was predicted, the highest Mascot score or the lowest ANOVA *P* value (if the Mascot scores were the same) was used to determine the most likely locus according to the DeCyder MS Differential

Analysis software. The identified proteins in each set of treatments that matched the above criteria were visualized and analyzed with the MultiExperiment Viewer (MeV) program to identify the proteins with significantly differential expression between unstressed plants and osmotic-stress-treated plants at different timepoints with *t* test ($P < 0.05$) (Saeed et al. 2003). The hierarchical clustering was conducted using the Pearson correlation coefficient. The co-expression network analysis of proteins that were significantly affected by osmotic stress in the SR line was generated using a 'guide gene approach' by RiceFREND with hierarchy of 2 and mutual rank (MR) of 5 (Sato et al. 2013).

Detection of osmotic-stress-responsive genes in rice

Shoots of 7-day-old SS and SR rice seedlings were cut and air-dried for 2 h to simulate drought-stress conditions, similar to the method used by (Huang et al. 2010, 2011) used three replicates for each set. Total RNA was extracted with PureLink Plant RNA Reagent (Invitrogen, USA), treated with DNase I (Thermo Fisher Scientific, USA) to cleave genomic DNA, and purified by phenol–chloroform precipitation. The purified RNA (1 µg) was reverse-transcribed to generate first-strand cDNA using the iScript Reverse Transcription Supermix (Bio-Rad, USA). The major hub gene from the co-expression network, *LOC_Os04g38600,* was selected for transcriptional-level analysis. A semi-quantitative reverse transcription (RT) polymerase chain reaction (PCR) experiment was conducted in 50-µl samples containing Taq DNA Polymerase (Thermo Fisher Scientific). Primers specific for the selected genes (Supplementary Table 1) were designed based on coding sequences, which were retrieved from the Rice Genome Annotation Project database (Kawahara et al. 2013). *DREB2A* (Dubouzet et al. 2003) and *OsEF-1α* (Saeng-ngam et al. 2012) were used as the stress-responsive gene and internal controls, respectively.

Results and discussion

Drought stress induced by 10% PEG6000 treatment altered rice leaf protein profiles

Leaf proteins from unstressed and osmotic-stress-treated SS and SR rice lines were analyzed by GeLC–MS/MS. A total of 357 proteins (Supplementary Table 2) were detected in the SS and SR lines. Based on the comparison of proteins by the MeV program, 68 and 55 proteins from the SS and SR lines, respectively, were significantly up- or down-regulated in stressed plants relative to their levels in untreated control plants ($P \leq 0.05$) (Fig. 1a, b). It is worth mentioning that more than 1200 proteins were identified by the MascotTM software. Some had very short peptide sequences, containing only five amino acid residues. After the using Blastp in NCBI database (Coordinators 2016), only 357 proteins showed the highest similarity with rice proteins. Some of the proteins identified were not annotated by either MSU Rice Genome Annotation Project (Kawahara et al. 2013) or International Rice Genome Sequencing Project (Kawahara et al. 2013; Sakai et al. 2013). Therefore, the locus numbers of these proteins were not listed in the Supplementary Table 2. These data reveal the difference in annotation among various databases.

The SS and SR proteins were categorized into ten functional groups (Fig. 2; Supplementary Table 3). The number of proteins associated with metabolic process, defense, signaling, cellular process, transposon, and post-translation was higher in SS plants than in SR plants. Proteins associated with retrotransposon, transcription, post-transcription, and transport were more abundant in the SR rice line, suggesting the importance of changes in these functions for drought tolerance (Fig. 2).

Disregarding the proteins with unknown functions, retrotransposons were the main group of proteins affected by osmotic stress in SR plants, while proteins related to metabolic processes were the most commonly affected proteins in SS plants. The categories of post-translation were the group found only in SS line, while post-transcription was the category found only in SR plants. These differences suggest that SS and SR respond differently to osmotic stress.

The SR line was generated because of somaclonal variations in the SS line, and selected under salt-stress conditions for six generations (Sripinyowanich et al. 2013; Vajrabhaya and Vajrabhaya 1991). A large proportion of mutations in rice cell cultures are caused by the transposition of retroelements (Hirochika et al. 1996). Thus, the SR line may carry conserved retrotransposon genes within its genome. Retrotransposons are activated by multiple stresses (Alzohairy et al. 2012), including drought (Kapazoglou et al. 2013). The mechanism responsible for this activation under drought conditions is hypomethylation (Wang et al. 2011). In addition, drought stress induces epigenetic changes through DNA methylations, with 70% of methylated DNA being demethylated after stressed plants are returned to normal growth conditions (Wang et al. 2011). Grandbastien (2015) reviewed the up-regulation of the long terminal repeat (LTR) retrotransposons under various stress conditions. The 'EG4' rice cultivar contains more than 1000 *mPing* miniature inverted-repeat transposable elements (Naito et al. 2009). The expression of many genes containing the *mPing* element can be induced by exposure to salt or cold stress (Lisch 2013). In perennial ryegrass, drought stress leads to enhanced DNA

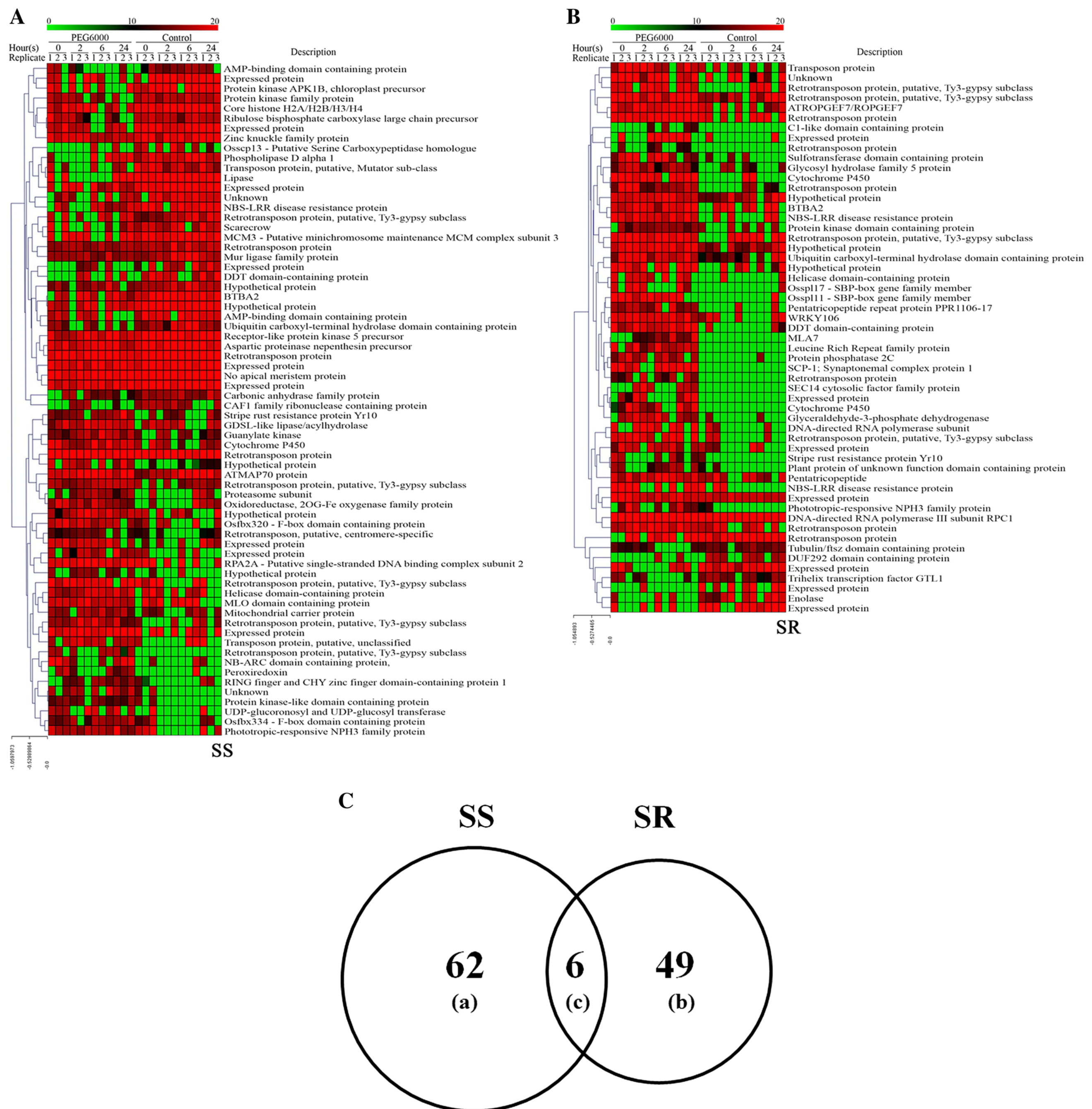

Fig. 1 Heat map of significantly up- or down-regulated leaf proteins in 4-week-old SS (**a**) and SR (**b**) rice plants at 0, 2, 6, and 24 h after osmotic-stress treatment with 10% PEG6000. The heat map was created using the MultiExperiment Viewer program. Columns represent treatments and harvest time after treatment. Rows correspond to individual proteins that were significantly up- or down-regulated by osmotic-stress treatment, relative to the levels in control plants. Light green-to-dark red bars indicate low-to-high protein abundances. Venn diagram of significant proteins (**c**). There were 62 (group a) and 49 (group b) identified proteins present only in the SS or SR lines, respectively. Six (group c) identified proteins were detected in both lines. The identified proteins are listed in Supplementary Tables 2 and 3

demethylation and up-regulated expression of demethylated genes (Tang et al. 2014). The drought-tolerant *Macrotyloma uniflorum* (Lam.) Verdc. genotype exhibits lower methylation levels than the drought-susceptible genotype (Bhardwaj et al. 2013). The demethylation of retroelements under drought-stress conditions is correlated with the expression of nearby genes, which may represent the mechanism regulating stress-induced or repressed gene expression.

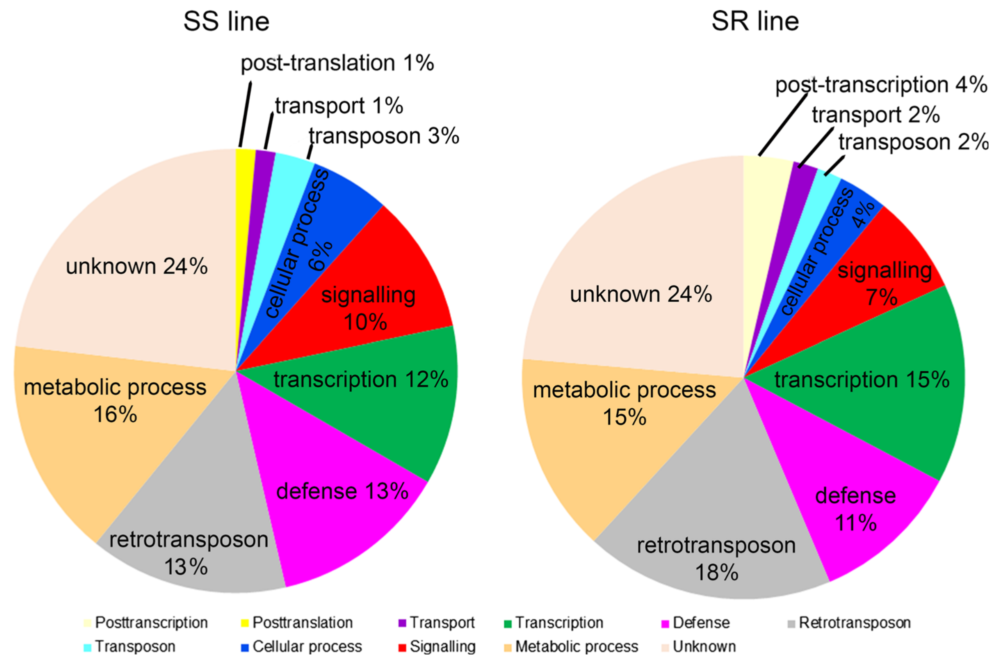

Fig. 2 Functional classification of drought-responsive proteins detected in LPT123 (SS) (**a**) and LPT123–TC171 (SR) (**b**) rice leaves. The functions were categorized according to gene ontology annotations from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu)

Drought-responsive genes in the SS and SR lines

According to osmotic-stress-induced differential expression of 68 proteins in SS line and 55 proteins in SR line, 6 of them were significantly affected by osmotic stress in both rice lines (Fig. 1c). The proteins up-regulated in both rice lines in response to osmotic stress included a helicase domain-containing protein, cytochrome P450, and stripe rust-resistance protein Yr10 (Table 1). The families of these three proteins are known to be involved in stress responses (Baldoni et al. 2015; Barak et al. 2014; Gao et al. 2014; Himmelbach et al. 2002; Kant et al. 2007).

The other three proteins (i.e., LOC_Os01g56200, LOC_Os04g35864, and LOC_Os07g29820) were affected differently in drought-stressed SS and SR lines. All of them were up-regulated in the SR line, but down-regulated in the SS line.

RNA helicase activity involves an ATP-driven unwinding of an RNA duplex, and has been observed in several organisms such as viruses, bacteria, humans, and plants. It plays an important role during cell growth and in responses to abiotic stresses. The DEAD-box genes, including *STRS1*, *STRS2*, *TaRH1*, *SlDEAD31*, and *OsBAT1*, form the largest group of RNA helicase genes (Barak et al. 2014; Chen et al. 2014; Kant et al. 2007; Zhang et al. 2014; Zhu et al. 2015). In *Triticum aestivum*, low temperature, dehydration, and salt stresses lead to an accumulation of an RNA helicase (TaRH1) (Zhang et al. 2014). In tomato, *SlDEAD31* expression is promoted by heat, cold, and dehydration stresses, and *SlDEAD31* overexpression results in increased salt tolerance and drought resistance (Zhu et al. 2015). Transgenic rice overexpressing *OsBAT1* can germinate and grow on Murashige and Skoog medium supplemented with 200 mM NaCl (Tuteja et al. 2015). OsSUV3, encoding DNA/RNA helicase and belonging to the Ski2 family of DExH/D-box helicases, was shown to function in salt tolerance in rice by maintaining photosynthesis and antioxidant machinery (Tuteja et al. 2014). Therefore, the helicase domain-containing protein detected in this study may be active in rice drought-stress responses.

A cytochrome P450, CYP707A family member was identified as ABA 8′-hydroxylase, which degraded ABA under dehydration stress condition. The knockout mutant of *CYP707A3* gene led to drought-tolerant phenotype (Umezawa et al. 2006). However, the ectopic expression of *PtCYP714A3* from *Populus trichocarpa* improved salt tolerance in transgenic rice (Wang et al. 2016). Moreover, the expression of *Os08g01480*, encoding CYP-like protein, in Arabidopsis, caused the tolerance to heavy metal, salt, and dehydration stress (Rai et al. 2015). The up-regulated cytochrome P450 (LOC_Os10g05020) suggests the involvement of this protein in osmotic-stress response.

Stripe rust-resistance protein is encoded from *Yr10*, which is one of the disease-resistant (*R*) genes in plants. It has evolutionary-conserved and unique CC–NBS–LRR sequence (Liu et al. 2014). This gene is conserved among plant species, including wheat, maize, sorghum, and rice. Another NBS–LRR disease-resistance protein, LOC_Os07g29820, was also found to be up-regulated in SR line, but down-regulated in SS line, which was similar to the expression of another defense protein,

Table 1 Proteins commonly found in both stress-susceptible and stress-resistant rice lines

Locus[a]	Description[a]	Functional group	ID score[b]	Peptide[b]	MH+ (Da)[b]
LOC_Os01g56200	BTBA2—Bric-a-Brac, Tramtrack, Broad Complex BTB domain with Ankyrin repeat region	Defense	7.289999962	EQGQESNK	919.8600159
LOC_Os02g50370	Helicase domain-containing protein	Transcription	10.27000046	AFPGPSKDDK	1061.202812
LOC_Os04g35864	DDT domain-containing protein	Transcription	5.159999847	QSVQSNSLGK	1046.382186
LOC_Os07g29820	NBS–LRR disease-resistance protein	Defense	5.519999981	SLRGLGAMK	948.2815457
LOC_Os10g05020	Cytochrome P450	Metabolic process	9.850000381	MGSRLEVIVADR	1345.188315
LOC_Os11g34920	Stripe rust-resistance protein Yr10	Defense	13.14999962	KTDDLVSR	934.3509976

[a]Locus numbers and descriptions were retrieved from the Rice Genome Annotation Project database

[b]ID scores and predicted peptide and protein masses were obtained using the Mascot program

LOC_Os01g56200. These proteins may have the function not only in biotic stress responses, but also in abiotic responses.

DDT domain has been characterized as a domain in bromodomain PHD finger transcription factors (BPTFs) (Doerks et al. 2001). It was shown to have the DNA-binding function. However, the function of DDT domain-containing protein encoded from *LOC_Os04g35864* has not been reported.

Specific proteins detected only in the SR line include transposable elements, and proteins involved in metabolic processes and signaling

Transposable elements

The genes encoding the proteins that accumulated only in the drought-tolerant line in response to osmotic stress may be useful as drought-tolerance genes. Protection from environmental stresses may be mediated by epigenetic events, such as the induction of the expression of adjacent genes by transposable elements. More than one-fifth of 49 proteins detected only in the SR line (Supplementary Table 4) consisted of a combination of retrotransposons and transposons. Transposable elements (TEs) are classified as Class I (copy-and-paste mechanism via an RNA intermediate or retroelement) or Class II (cut-and-paste mechanism via a DNA intermediate) transposons, and are major components of eukaryotic genomes (Anca et al. 2014; Chadha and Sharma 2014). In addition, the LTR retrotransposons, which may mediate somaclonal variation, are the major plant TEs (Grandbastien 2015; Wessler 1996). For example, copper and heat shock stresses induce TE activities, leading to instability in the *Magnaporthe oryzae* genome (Chadha and Sharma 2014). The *Hordeum vulgare DEMETER* gene (*HvDME*) contains an LTR retrotransposon element. Its expression is induced in drought-tolerant barley exposed to drought conditions, resulting in differential DNA methylation in drought-sensitive (e.g., 'Caresse') and drought-tolerant (e.g., 'Demetra') cultivars (Kapazoglou et al. 2013). The activation of TEs is one of the mechanisms that enables self-protection and self-repair. It also stimulates the expression of other genes responsible for stress responses (Grandbastien 2015).

Plant metabolism

Several proteins involved in metabolic processes were up- or down-regulated following treatment with osmotic stress (Supplementary Table 4). When plant cells experience abiotic stress, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most prominent proteins targeted for oxidative modification (Hildebrandt et al. 2015). This enzyme has an important role in converting glycerate-3-phosphate to glyceraldehyde-3-phosphate. Glycerate-3-phosphate is an electron acceptor that receives electrons from NADPH and protects photosystem II from reactive oxygen species (Takahashi and Murata 2006). In our study, GAPDH (LOC_Os04g38600) abundance increased in only SR lines treated with osmotic stress for 2 h, implying that the enzyme has a role in protecting photosystem II. This is similar to the findings for two wheat cultivars with contrasting drought tolerances. The GAPDH enzyme is up-regulated after 48 h of PEG6000 treatment in both wheat genotypes (Cheng et al. 2015). However, in our study, the maintenance of stable GAPDH levels for up to 24 h of osmotic stress was observed only in the SR line (Fig. 3B), suggesting that photosystem II is protected from the effects of osmotic stress more in the SR line than in the SS line. A proteome-level study of *Thellungiella halophila* chloroplasts exposed to different saline conditions uncovered several salt-responsive proteins, including the GAPDH β subunit (GAPB) (Chang et al. 2015). Overexpression of

GAPB in transgenic *A. thaliana* increases the chlorophyll concentration, dry weight, water content, and survival rate.

Enolase, the enzyme in glycolytic pathway, was the protein involving in metabolic process with significant reduction found only in SR line. In a previous study, enolase protein abundance was significantly higher in drought-tolerant Chinese spring wheat than in a drought-sensitive cultivar after a 48-h PEG6000 treatment (Cheng et al. 2015). This pointed out that drought tolerance in different species might use different metabolic pathways for adaptation.

Both GAPDH and enolase changes suggested the adaptation in carbohydrate metabolism to drought stress in SR line. The regulation of photosynthetic efficiency under drought stress leads to the maintenance of grain yield in rice (Ambavaram et al. 2014). Sugar accumulation is also the mechanisms for tolerance to abiotic stresses, including drought (Pandey and Shukla 2015), salt (Udomchalothorn et al. 2009), and chilling stresses (Morsy et al. 2007).

Plant signaling and defense

Osmotic stress caused the changes in proteins with signaling and defense functions differently between SS and SR lines. These suggested that these two lines had different signaling pathways and use different defense responses to cope with osmotic stress.

Other mechanisms

Transcription and post-transcriptional regulation for osmotic-stress response have been investigated in various plant species. In this study, at least two transcription factors, Trihelix transcription factor GTL1 and WKRY106 were found to have a change in protein levels in SR line, but not in SS line, suggesting the role in the regulation of osmotic-stress tolerance. Further validation is required for the future study. The reduction of GTL1 in SR line is consistent with the previous study in Arabidopsis, showing that *A. thaliana* GT-2 LIKE 1 (*AtGTL1*) loss-of-function mutant (gtl1-4) exhibited a higher integrated water use efficiency and a higher survival rate after exposure to water deficit conditions (Yoo et al. 2010).

Post-transcriptional regulation of gene expression is controlled by gene activities in mitochondria. In this experiment, pentatricopeptide repeat (PPR) protein was up-regulated after drought stress. Mitochondrial pentatricopeptide repeat (PPR) proteins are associated with many plant biological processes, including RNA sequence changes, translation, and seed and embryo development.

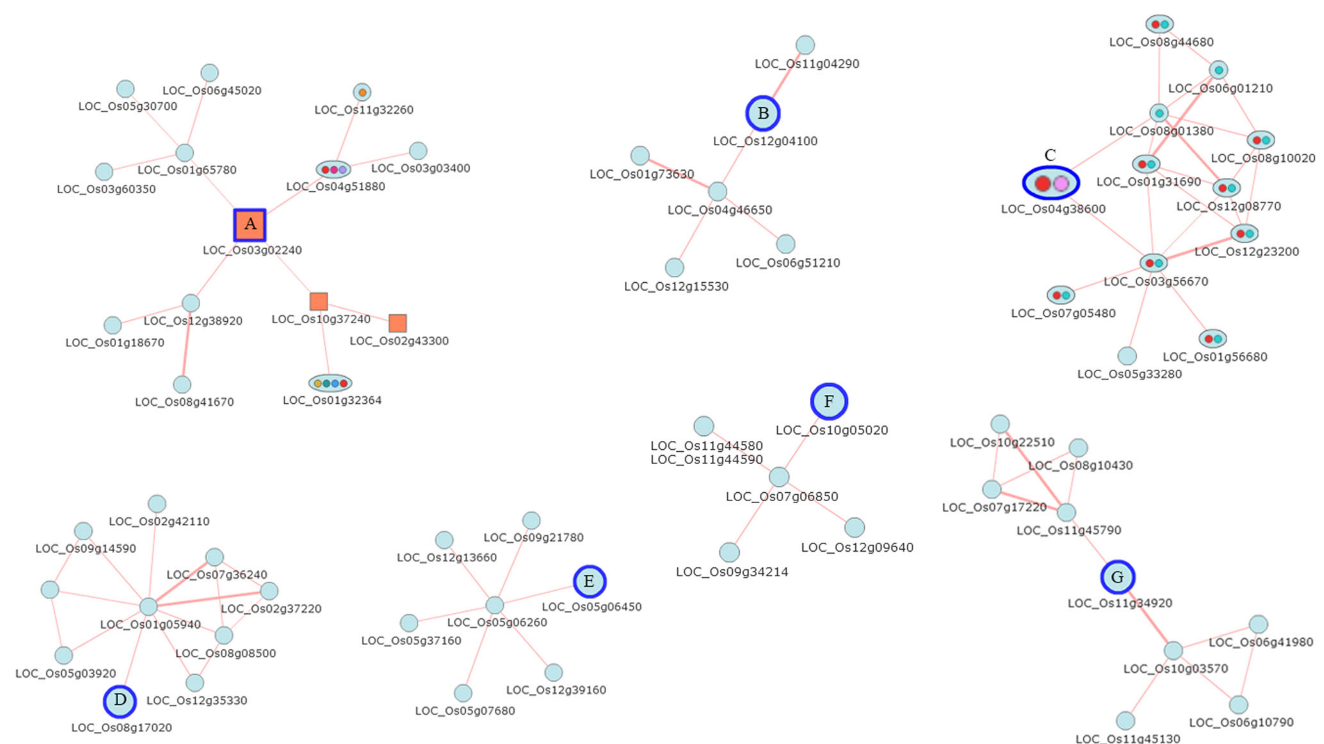

Fig. 3 Co-expression networks of the significant changed proteins from SR line. A–G Genes significantly expressed in SR lines. Squares represent the transcription factors. Blue circles indicate nodes in the network, while the green, red, and pink circles in the ellipses represent the metabolic pathways in which the node genes (ellipses) are involved

Salt, ABA, and oxidative stresses inhibit plant growth in an *A. thaliana* mutant (*ppr40*), and results in the accumulation of reactive oxygen species. Because PPR proteins are very important to plant organelles, defects in these proteins lead to retarded growth, diverse defects in embryo morphology, and irregular photosynthesis (Cushing et al. 2005; Manna 2015; Meierhoff et al. 2003; Pusnik et al. 2007).

We identified one protein with transport functions (i.e., SEC 14 cytosolic factor family protein). A comparison of transcriptomes among several sorghum genotypes revealed that SEC14 cytosolic factor protein is more abundant in the nitrogen stress-tolerant sorghum genotypes than in the susceptible sorghum lines. In addition, the production of this protein can lead to greater membrane stability and stress tolerance (Gelli et al. 2014).

Co-expression network reveals GAPDH involving photosynthetic adaptation during drought stress

A group of 57 genes identified only in the SR line was subjected to co-expression network analysis using the RiceFREND (Sato et al. 2013). Seven proteins showed the co-expression network, as shown in Fig. 3. The seven proteins with the co-expressed gene network were transcription factor GTL1 (Fig. 3A), cytochrome P450 (LOC_Os12g04100) (B), GAPDH (C), LOC_Os08g17020 (expressed protein, D), Tubulin/ftsz domain-containing protein (E), cytochrome P450 (LOC_Os10g05020) (F), and stripe rust-resistance protein Yr10 (G). Nodes F and G were also expressed in SS line, while nodes A–E were the proteins significantly changed only in SR line.

Among significant proteins expressing only in SR line, with co-expressed gene network, GAPDH was the only protein showing the connection to the genes in metabolic pathways, especially the genes in photosynthesis. This suggested the importance of the GAPDH in osmotic-stress response. We conducted semi-quantitative RT-PCR to validate the expression of *GAPDH* genes. We used *EF1-α* as an internal control, while *DREB2A* served as a stress-responsive gene control. Although *GAPDH* expression was up-regulated in both SS and SR leaves treated with drought conditions for 2 h, the increase was greater in the SR leaves (Fig. 4). This was consistent with the increase in *GAPDH* protein abundance during our proteome-level analysis (Fig. 5). These observations indicate that these genes are regulated at the transcriptional level.

In many species, the up-regulation of GAPDH is associated with drought tolerance. For example, the drought-tolerant 'Ningchun 47' wheat cultivar increases GAPDH accumulation under drought conditions (Cheng et al. 2015), while the *GAPB* expression level increases in *T. halophila* under salt-stress conditions. A previous study revealed that GAPDH plays an important role in maintaining photosynthetic activities and plant development under salt-stress conditions (Chang et al. 2015). The overexpression of a potato *GAPDH* gene in transgenic tobacco enhances drought tolerance, while reduced expression of this gene leads to a severe stress phenotype under drought-stress conditions, suggesting the importance of GAPDH in drought tolerance (Kappachery et al. 2014).

Ferredoxin–NADP reductase (FNR) is important for balancing electron transport and redox homeostasis in chloroplasts. Therefore, the gene expression of FNR was investigated in both lines, as shown in Fig. 4. The decrease of *FNR* expression was detected only in SR line after stress. The abundance of this enzyme decreases after drought-stress treatments in transgenic tobacco (Gharechahi et al. 2015), *P. cathayana* (Xiao et al. 2009), wheat (Budak et al. 2013), and rice (Nouri et al. 2015), which is consistent with our results (Fig. 4). In contrast, the expression of ferredoxin genes significantly increases in potato after an incubation at a moderately high temperature (30 °C) (Hancock et al. 2014). In addition, maize FNR levels increase after being treated with 25 mM NaCl (Zörb et al. 2009) or 16% PEG (Tai et al. 2011). However, in some wheat cultivars, FNR exhibits no significant changes when grown under drought-stress conditions (Nikolaeva et al. 2010). These findings imply that different species use different mechanisms to balance electron flow in photosynthetic processes under osmotic-stress conditions.

Both GAPDH and FNR are involved in regulating plant NADP(H) levels (Hald et al. 2008). Drought stress induces a decrease in stomatal conductance, leading to lower Calvin cycle activities, and ultimately an increase in stromal NADPH levels. In addition, the repair of photosystem II from photodamage is suspended by the decreasing of Calvin cycle activities and the disruption of Calvin cycle diminish their utilization of NADPH which induces ROS accumulation (Takahashi and Murata 2005; Zavafer et al. 2015). Therefore, maintaining an NADP(H) homeostasis is needed for preventing PSII damage. In addition, reduced FNR levels under drought conditions also contribute to NADPH homeostasis, delaying a NADPH production,

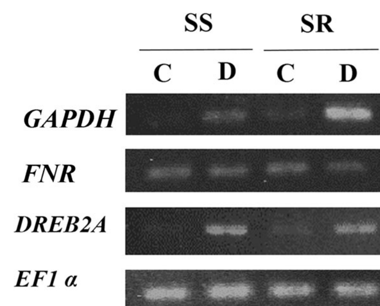

Fig. 4 Semi-quantitative expression of *GAPDH* and *FNR* in SS and SR leaves after air-dry treatment

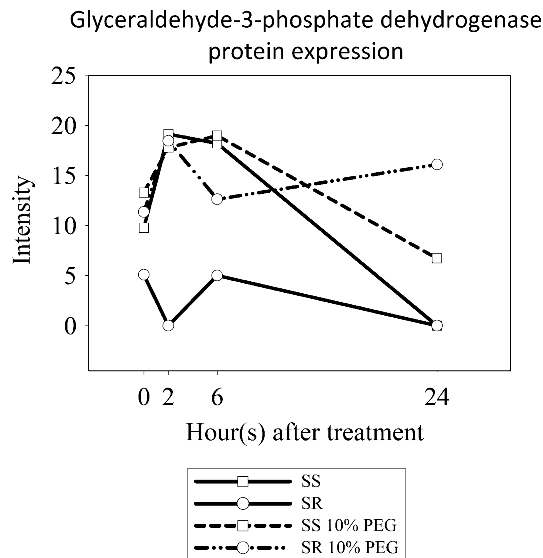

Fig. 5 Protein expression patterns for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after an osmotic-stress (10% PEG6000) treatment for 0, 2, 6, and 24 h

whereas increased GAPDH level will decrease NADPH/NADP ratios which lead to protection of PSII. Consistent with our result, SR line shows a greater reduction after the stress, whereas *FNR* slightly down-regulated after dehydration (Fig. 4). In drought-tolerant transgenic tobacco plants producing cyanobacterial flavodoxin, the abundance of FNR decreases after exposure to drought stress (Gharechahi et al. 2015). In conclusion, SR rice showed that *GAPDH* were up-regulated, while *FNR* reduced under the stress (Fig. 4) which imply that during the stress, plants try to use NADP(H) homeostasis mechanism to prevent photosystem damage by stress.

Conclusion

Our proteome-level analysis revealed several candidate proteins with important roles in drought responses. We determined that the genes encoding GAPDH and FNR, which are key enzymes influencing NADP(H) homeostasis, are affected by osmotic-stress treatments. These findings suggest that drought tolerance in rice may be mediated by photosynthesis-related adaptations involving NADP(H) homeostasis. Our research confirms the value of conducting proteome-level investigations to further characterize the potential mechanism regulating drought tolerance.

Author contribution statement Six authors contribute to this manuscript as follows: study conception and design: SC and NC. Acquisition of data: NC, MM, NP, and SR. Technical support: NP and SR. Analysis and interpretation of data: NC and SC. Drafting of manuscript: NC and SC. Critical revision: MM and SC. Final approval of the article: SC.

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