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A proteomics approach for identifying osmotic-stress-related proteins in rice

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Abstract

Osmotic stress can endanger the survival of plants. To investigate the mechanisms of how plants respond to osmotic stress, rice protein profiles from mannitol-treated plants, were monitored using a proteomics approach. Two-week-old rice seedlings were treated with 400 mM mannitol for 48 h. After separation of proteins from the basal part of leaf sheaths by two-dimensional polyacrylamide gel electrophoresis, 327 proteins were detected. The levels of 12 proteins increased and the levels of three proteins decreased with increasing concentration or duration, of mannitol treatment. Levels of a heat shock protein and a dnaK-type molecular chaperone were reduced under osmotic, cold, salt and drought stresses, and ABA treatment, whereas a 26S proteasome regulatory subunit was found to be responsive only to osmotic stress. Furthermore, proteins whose accumulation was sensitive to osmotic stress are present in an osmotic-tolerant cultivar. These results indicate that specific proteins expressed in the basal part of rice leaf sheaths show a coordinated response to cope with osmotic stress.

Keywords: Rice; *Oryza sativa*; Graminacea; Proteomics; Osmotic stress; Mannitol; Rice; Basal part of leaf sheath

1. Introduction

Plants are continuously exposed to biotic and abiotic stresses that endanger their survival. Among abiotic stresses, osmotic stress is one of the most severe, caused by drought, high salinity and cold stresses in nature. Plants respond to osmotic stress at the morphological, anatomical, cellular, and molecular levels. To cope with osmotic-related stresses, plants have developed various responses such as production of osmolites for osmotic adjustment, synthesis of Na^+/H^+ antiporters for ion sequestration, and many other mechanisms (Bohnert et al., 1995). AtHKT1, which is a Na^+ transporter, mediates osmolality balance between xylem vessels and xylem parenchyma cells (Snarpi et al., 2005). The operation of these responses usually requires three steps: osmotic stress recognition, signal transduction, and production of components for the physiological response (Tamura et al., 2003). There have been many reports on osmotic stress recognition and signal transduction in bacteria and yeast. Both in *Escherichia coli* and yeast, osmotic stress is detected by an osmosensor (Maeda et al., 1994; Mizuno, 1998). In yeast, osmotic signals perceived by two osmosensors are transduced to a mitogen activated protein kinase through Pbs2 (Reiser et al., 2000). In plants, a hybrid-type histidine kinase (AtTHK1) functions as an osmosensor and transmits the stress signal to a downstream mitogen-activated protein kinase (MAPK) cascade (Urao et al., 1999).

Lu and Neumann (1999) reported that when rice seedlings were exposed to osmotic stress modulated by polyethylene glycol 6000, growth in emerging first leaves of

Abbreviations: ABA, abscisic acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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the intact plant was inhibited. Early inhibition of leaf growth was not related to changes in root size, osmotic potential gradients, or cell wall-yielding characteristics in the leaf-expansion zone of stressed seedlings. In *Arabidopsis*, Deak and Malamy (2005) demonstrated that osmotic stress represses the formation of autonomous lateral roots from lateral root primordia, while lateral root initiation was not greatly affected. Abscisic acid (ABA) and a newly identified gene, LRD2, are involved in osmotic repression of lateral root formation. Further examination revealed that both ABA and LRD2 control root system architecture even in the absence of osmotic stress. This finding indicated that the same molecular mechanisms that mediated responses to environmental cues could also be regulators of intrinsic developmental programs in the root.

Zonia and Mnnik (2004) investigated whether tobacco pollen tube cell volume changes in response to osmotic perturbation by activation of the phospholipid signaling pathway. Several intermediates in the phospholipid signaling pathway were detected during pollen tube growth. Hypo-osmotic stress induced a rapid increase in phosphatidic acid and a decrease in phosphatidylinositol phosphate. The fact that these signaling molecules are present during normal growth suggested that the mechanism for osmotic response involved components of the biomechanical networks driving pollen tube cell elongation. In osmotically stressed wheat coleoptiles, reduced rates of phenylalanine ammonia-lysase and tyrosine ammonia-lysase activities suppress phenylalanine biosynthesis, resulting in a reduced level of wall-bound ferulic acid. This decrease in wall-bound ferulic acid may lead to reduced levels of diferulic acid, an important contributor to maintaining cell wall extensibility (Wakabayashi et al., 1997). Clearly, there is much to learn at the biochemical and molecular levels about how plants respond to osmotic stress.

Proteomics is a powerful tool for separating complex protein mixtures, and has been employed to analyze protein changes in response to environmental changes. Abbasi and Komatsu (2004) have investigated rice proteins induced by salt stress using proteomics. The expression of superoxide dismutase was a common response to cold, drought, salt, and ABA stresses. Fructose bisphosphate aldolases, photosystem II oxygen evolving complex protein, and oxygen evolving enhancer protein 2 were expressed in leaf sheaths and leaf blades but not in roots. This result indicated that specific proteins, expressed in specific regions of rice, showed a coordinated response to salt stress. Riccardi et al. (1998) examined drought-responsive proteins of two maize lines and their first filial generation hybrid. There was significant quantitative variation in 78 out of 413 leaf proteins with 38 of them exhibiting differential expression in the two genotypes. In rice seedlings, phosphorylation of proteins induced by cold stress has been analyzed by 2D-PAGE (Komatsu et al., 1999). Although several proteins were found to be phosphorylated upon cold stress, a fragment originating from the RuBisCO large subunit accumulated to high levels after cold stress and was phosphorylated. These reports show that a proteomics approach is useful for analyzing the physiological function of stress-induced proteins.

Rice is not only a very important agricultural resource but also a model plant for biological research because its genome is smaller than those of other cereals, making it suitable for efficient genetic analysis and transformation. Because protein analysis is the most direct approach for defining the function of genes, analysis of the proteome linked to genome sequence information is a very useful strategy for functional genomics (Komatsu and Tanaka, 2004). To date, there are several reports about systematic proteomic analysis of rice protein abundance under abiotic stresses. In this study, proteins from the basal part of rice leaf sheaths were screened by a proteomics approach to investigate the response of rice to osmotic stress.

2. Results and discussion

2.1. Rice shoot elongation is inhibited by osmotic stress

The osmotic potential of soil alters the depth of root systems, the rate of root elongation, and the number of lateral roots (van der Weel et al., 2000); however, there are only a few reports about phenotypic changes in rice under osmotic stress (Lu and Neumann, 1999). To understand about phenotypic changes in rice under osmotic stress, treatment was done using two-week-old rice seedlings based on the sensitivity of rice plants. At the beginning of this study, rice seedlings were homogeneous in terms of shoot height. When two-week-old rice seedlings, cv. Nipponbare, were treated with mannitol at different concentrations for 48 h or at 400 mM for different intervals, shoot elongation was obviously inhibited and the leaf blade gradually withered from the tip to the bottom. This damage to rice seedlings was more severe as the mannitol concentration increased (Fig. 1a, left). Rice seedling heights were reduced with increasing concentrations of mannitol treatment for 48 h (Fig. 1b, left). The pattern of this damage was continuous when rice seedlings were exposed to mannitol for longer periods of time (Fig. 1a, right and Fig. 1b, right). Particular morphological adaptations may be vital in specific plant species under osmotic stress, but these adaptations are not common to all plants. In this study, inhibition of growth in emerging first leaves and inhibition of shoot elongation were shown to be dependent on mannitol dose and length of treatment. This result may be due to osmotic stress inducing hydraulic limitations to water uptake that limit water availability for the volume increase required for expanding cells. The damage to seedlings and inhibition of shoot elongation were started at 400 mM from 48 h after mannitol treatment, so in the following study, this condition was used.

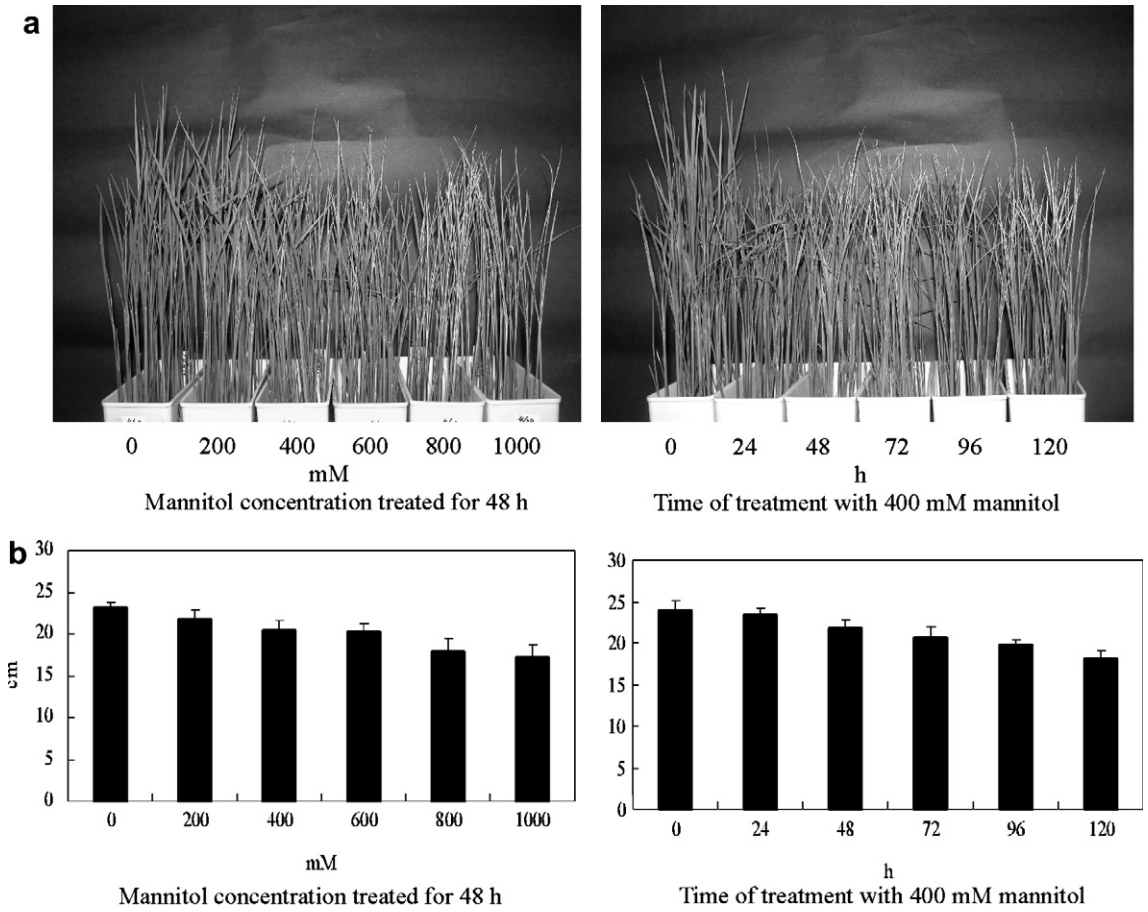

Fig. 1. Effect of osmotic stress induced by mannitol on the growth of rice. Two-week-old rice seedlings, cv. Nipponbare, were treated with 200, 400, 600, 800 and 1000 mM mannitol at different concentrations for 48 h (a, left and b, left) or at 400 mM for 24, 48, 72, 96 and 120 h (a, right and b, right). Graphs show the changes of shoot height including leaf blades and leaf sheaths under mannitol treatment at different concentrations and intervals. The bars in the graphs represent an average shoot height of 10 seedlings ± SD.

2.2. Fifteen proteins are changed in the basal part of rice leaf sheaths by mannitol treatment

The basal section of leaf sheath may undergo more subtle changes in protein expression than the distal end, where significant morphological changes are evident. In this study, basal parts of leaf sheath were used for proteomics experiment. After two-week-old seedlings were subjected to 400 mM mannitol for 48 h, proteins were extracted from the basal part of leaf sheaths and separated by 2D-PAGE. After CBB staining, 327 proteins were detected by digital image analysis (Fig. 2a). Among these, 15 proteins significantly responded to osmotic stress by up- or down-regulation: 12 proteins (spots 1, 2, 3, 4, 6, 9, 10, 11, 12, 13, 14 and 15) increased in amount and 3 proteins (spots 5, 7 and 8) decreased (Fig. 2b). The amino acid sequences of the differentially regulated proteins were analyzed by protein sequencing and mass spectrometry (Table 1). Homology searches were carried out using the FASTA or Mascot search tools. N-terminal sequences were successfully obtained for 8 of the 15 proteins (spots 1, 2, 5, 7, 8, 9, 12 and 14). These proteins were identified as the 26S proteasome regulatory subunit, a calreticulin precursor, a heat shock protein, a dnaK-type molecular chaperone, an uroporphyrinogen decarboxylase, two functionally unknown proteins encoded by *Oryza sativa* genomic DNA and an unidentified protein. The remaining seven proteins were N-terminally blocked. Internal sequencing identified spot 11 as a glutathione *S*-transferase (GST). Among the other seven proteins, two proteins (spots 4 and 15) were not identified, while four proteins (spots 3, 6, 10 and 13) were identified as an endosperm luminal binding protein (BiP), a lipid transfer protein, glyoxalase I and a 20S proteasome α subunit.

The identity of 12 of the 15 proteins differentially regulated by osmotic stress could be determined from amino acid sequence similarity or from the deduced amino acid sequence of gene sequences deposited in databases. Ten of the identified proteins are recognized as important components for stress response such as two proteasome-degradation system related proteins, five endoplasmic reticulum (ER) related proteins, two proteins related to detoxification, and uroporphyrinogen decarboxylase, which is a cell death related protein (Mock and Grimm, 1997) (Table 2). Two proteins matched annotated sequences for putative proteins of unknown function and the three remaining

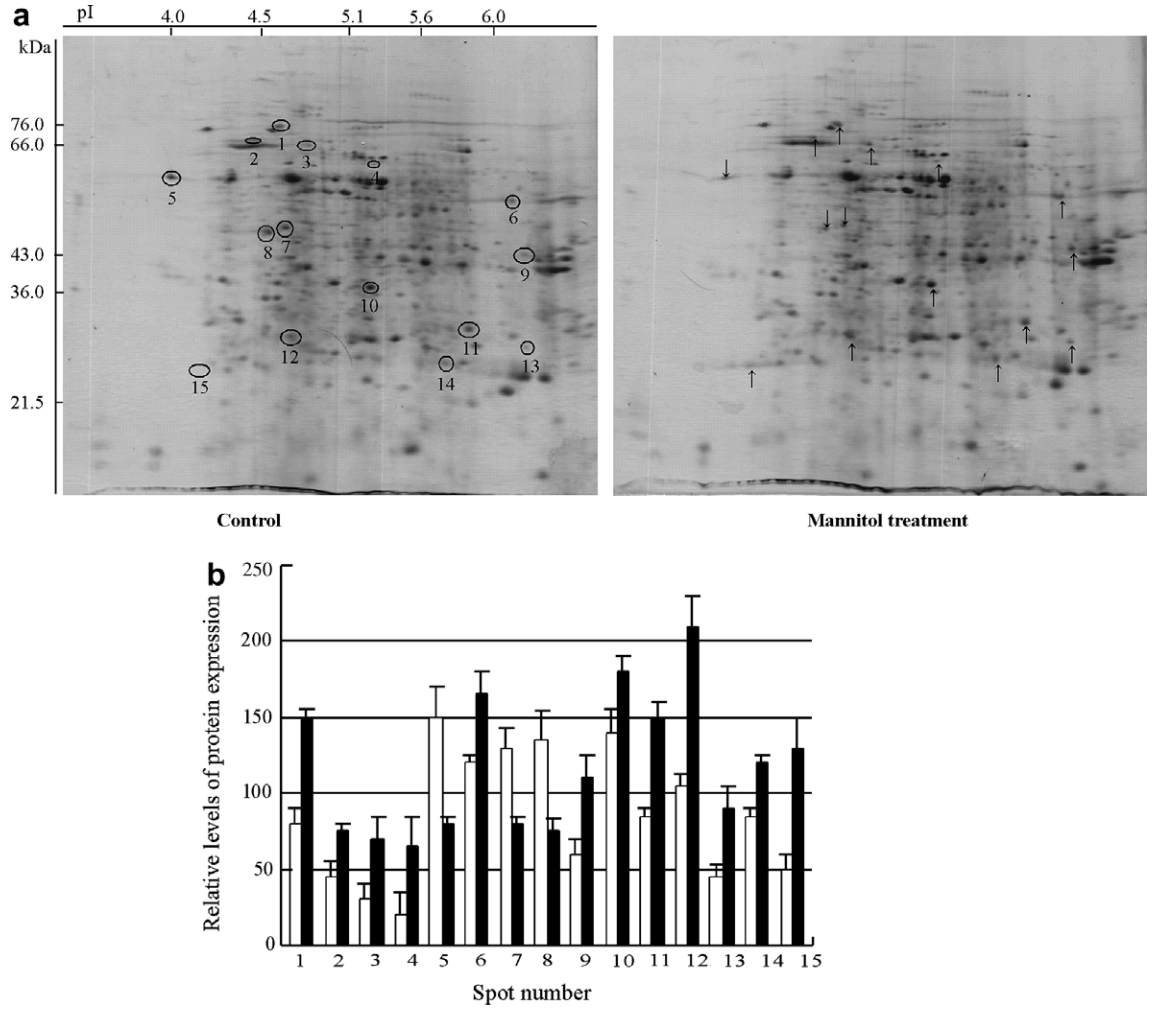

Fig. 2. Protein patterns changed by osmotic stress in the basal part of rice leaf sheaths. Two-week-old rice seedlings were treated without (a, left) or with 400 mM mannitol for 48 h (a, right). Proteins (300 μg) were extracted from the basal part of leaf sheaths, separated by 2D-PAGE and stained by CBB. Arrows indicate the proteins changed by mannitol treatment and circles mark the positions of the same proteins from control. Following scanning, the gel patterns were analyzed using the 2D-Elite software, and the relative abundance ratio of proteins was analyzed (b). *X*-axis denotes the spot number, and *Y*-axis denotes the relative levels of protein expression. The bars in the graphs represent an average volume ± SD of three times experiment. White bar shows control and black bar mannitol treatment.

proteins responding to osmotic stress were not similar to other proteins in existing databases. These results suggest that the main target of osmotic stress is the ER and that the proteasome-degradation system and detoxification system are involved in the osmotic stress response of plants.

2.3. Protein expression under conditions of osmotic stress applied by mannitol treatment

The effect of osmotic stress on proteins in the basal part of leaf sheaths was examined by treating rice seedlings with mannitol. Two-week-old seedlings were subjected to 200, 400, 600, 800 and 1000 mM mannitol for 48 h. The proteins in the basal part of leaf sheaths were extracted and separated by 2D-PAGE. Great variation in the level of protein accumulation was detected during the osmotic stress treatment (Fig. 3a). Ten proteins including two proteasome-degradation system related proteins (spots 2 and 13), a lipid transfer protein (spot 6), a glyoxalase I (spot 10), an uroporphyrinogen decarboxylase (spot 12), a functionally unknown protein (spot 14) and an unidentified protein (spot 15) showed maximal accumulation in response to 400 mM mannitol treatment (Fig. 3b). Two proteins including BiP (spot 3) and GST (spot 11) reached maximal levels with the 200 mM mannitol treatment, and were still expressed at levels higher than the control at 400 mM (Fig. 3b). Taken together, the expression profile of proteins whose accumulation was dependent on mannitol concentration further substantiated our conclusion that proteins changed by mannitol were not an artifact.

To study changes in protein accumulation with increasing exposure to osmotic stress, two-week-old seedlings of rice were treated with 400 mM mannitol for different intervals. Proteins in the basal part of leaf sheaths were extracted after 24, 48, 72, 96 and 120 h of treatment and separated by 2D-PAGE. Several differences in protein

Table 1
Identification of proteins induced by osmotic stress in the basal part of rice leaf sheath

Spot No.[a]	p*I*[b]	kDa[b]	Sequences[c]	Homologous protein (%)	AC No.[e]
1	4.6	76	N-XDPLFYEPF	*Oryza sativa* genomic DNA (100)	AL606610
2	4.4	68	N-VLEATMI	26S proteasome regulatory subunit (100)	O94444
3	4.8	66	N-blocked/MS[d]	Endosperm luminal binding protein (BiP) (32)	AF006825
4	5.3	60	N-blocked/MS	Not hit	–
5	4.0	57	N-EVFFQEKFE	Calreticulin precursor (100)	Q9SP22
6	6.3	51	N-blocked/MS	Lipid transfer protein (24)	AF051369
7	4.7	48	N-TAXERAKRTL	Heat shock protein (90)	J01089
8	4.6	46	N-STAQTKXEID	dnaK-type molecular chaperone (80)	S48024
9	6.4	43	N-AKIKIXVN	Not hit	–
10	5.2	37	N-blocked/MS	Glyoxalase I (101)	AB017042
11	5.8	32	I-IVPLDFSKGE	Glutathione *S*-transferase (GST) (100)	O82451
12	4.7	30	N-DLPADFY	Uroporphyrinogen decarboxylase (100)	Q9HLB9
13	6.4	29	N-blocked/MS	20S proteasome α subunit (74)	AB026558
14	5.7	27	N-GVEVXVKAAV	*O. sativa* genomic DNA (90)	AB037970
15	4.1	26	N-blocked/MS	Not hit	–

[a] Spot numbers are given in Fig. 2.
[b] Molecular mass (kDa) and p*I* are from the gel in Fig. 2.
[c] N-terminal (N-) and internal (I-) amino acid sequences as determined by Edman degradation.
[d] MALDI-TOF MS.
[e] Accession number.

Table 2
Characterization of proteins induced by osmotic stress in the basal part of rice leaf sheath

Spot no	Homologous protein	In this study					Previous study
		Mannitol	NaCl	ABA	cold	drought	
1	Functional unknown protein	↑	↑	–	↑	–	(–)
2	26S proteasome regulatory subunit	↑	↑	–	–	–	Smalle et al. (2003)
3	BiP	↑	↑	–	–	–	Rutkowski and Kaufman (2004)
4	Undetermined protein	↑	–	–	–	–	(–)
5	Calreticulin precursor	↓	↓	–	–	↓	Zhu et al. (1997)
6	Lipid transfer protein	↑	↑	↑	↑	–	Imin et al. (2006)
7	Heat shock protein	↓	↓	↓	↓	↓	Fink (1999); Zhu et al. (1997)
8	dnaK-type molecular chaperone	↓	↓	↓	↓	↓	Zhu et al. (1997)
9	Undetermined protein	↑	↑	↑	↑	↑	(–)
10	Glyoxalase I	↑	↑	↑	↑	–	Singla-Pareek et al. (2003)
11	GST	↑	↑	↑	↑	–	Skipsey et al. (2000)
12	Uroporphyrinogen decarboxylase	↑	↑	–	↑	↑	Mock and Grimm (1997)
13	20S proteasome α subunit	↑	↑	–	–	–	Smalle et al. (2003)
14	Functional unknown protein	↑	↑	↑	↑	–	(–)
15	Undetermined protein	↑	↑	–	↑	–	(–)

accumulation patterns as a result of osmotic stress were detected (Fig. 4a). Seven proteins including BiP (spot 3), lipid transfer protein (spot 6), glyoxalase I (spot 10), GST (spot 11), uroporphyrinogen decarboxylase (spot 12), a functionally unknown protein (spot 14), and an unidentified protein (spot 4) reached their maximum levels of accumulation at 48 h with a subsequent decline to the level of the control (Fig. 4b). Although the levels of two proteasome-degradation system related proteins (spots 2 and 13), a functionally unknown protein (spot 1) and two novel proteins (spots 9 and 15) were not at their maxima with the 400 mM mannitol treatment for 48 h, the levels of these five proteins were higher than those of the control (Fig. 4b). Generally, a time course analysis of protein changes with mannitol at 400 mM also revealed the significant effects of osmotic stress on the targeted proteins.

On the other hand, accumulation of chaperone proteins, namely, a calreticulin precursor (spot 5), a heat shock protein (spot 7) and a dnaK-type molecular chaperone (spot 8), gradually decreased with increasing concentrations of mannitol (Fig. 3). These chaperone proteins were at their highest levels in the absence of osmotic stress, and declined in abundance with increasing time of mannitol treatment (Fig. 4). These three proteins are members of a family of molecular chaperones that assist in protein folding and prevention of protein denaturation (Zhu et al., 1997). Water deficit, as a result of osmotic stress, is expected to lead to increased protein aggregation and denaturation, making production of molecular chaperones more necessary. In contrast, our result showed that calreticulin, heat shock protein and dnaK-type molecular chaperone decreased in abundance during this time course, suggesting that

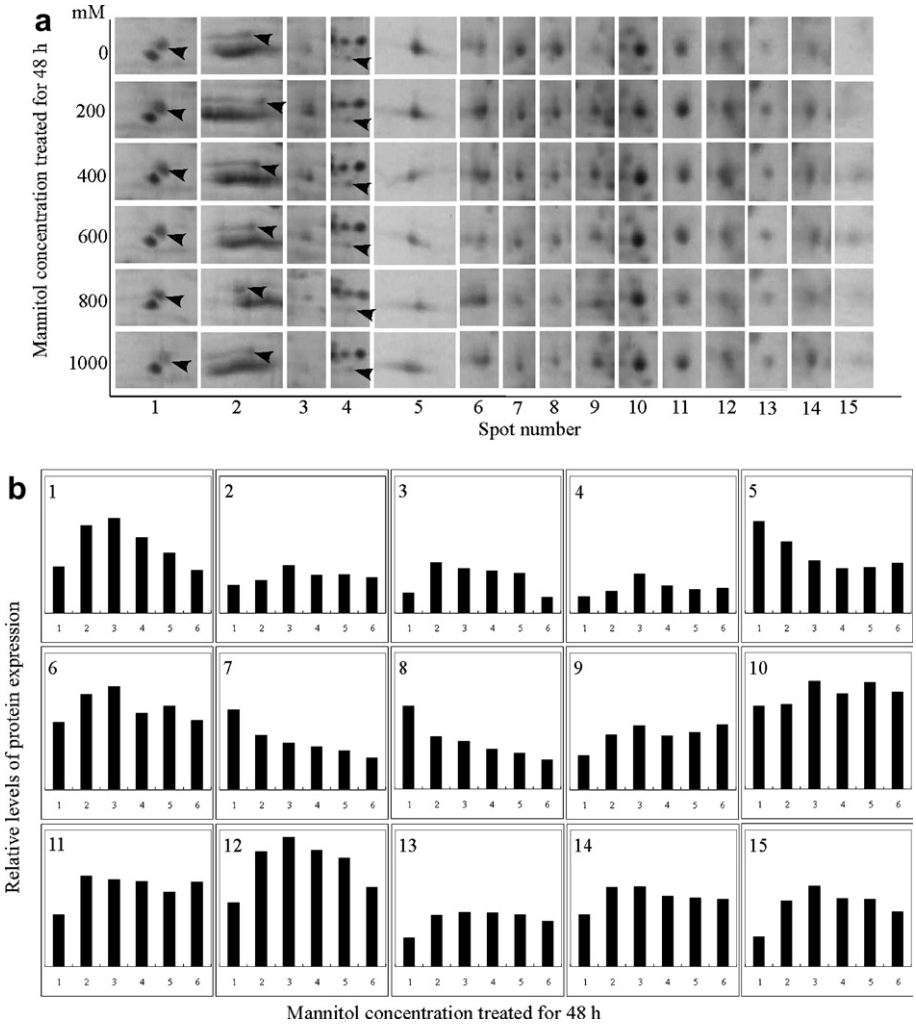

Fig. 3. Protein accumulation in the basal part of leaf sheaths in response to mannitol at different concentrations. Rice seedlings were treated without or with mannitol at 200, 400, 600, 800 or 1000 mM for 48 h. Proteins were extracted from the basal part of leaf sheath, separated by 2D-PAGE, and stained by CBB. The changed protein spots in Fig. 2 were displayed (a). Following scanning, the gel patterns were analyzed using the 2D-Elite software, and the relative abundance ratio of proteins was analyzed (b). *X*-axis denotes the concentration at which rice seedlings were treated, and *Y*-axis denotes the relative levels of protein expression. Columns 1, 2, 3, 4, 5 and 6 represent the relative abundance ratio of changed proteins with mannitol treatment at 0, 200, 400, 600, 800 or 1000 mM for 48 h, respectively. In the case of 2 protein spots in 1 panel, arrowhead was added. The bars in the graphs represent an average volume of three times experiment. Spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 show unknown protein encoded by O.sativa genome DNA, 26S proteasome regulatory subunit, BiP, undetermined protein, calreticulin precursor, lipid transfer protein, heat shock protein, dnaK-type molecular chaperone, undetermined protein, glyoxalase I, GST, uroporphyrinogen decarboxylase, 20S proteasome α subunit, unknown protein encoded by O.sativa genome DNA and undetermined protein.

molecular chaperones may act within the first 24 h of mannitol treatment.

2.4. Protein response of rice to different stresses

Salt, cold and drought stresses imposed on plants also can cause osmotic stress. ABA is a signaling molecule involved in many plant stress responses (Xiong and Zhu, 2002; Zhu et al., 1997). To compare the similarity of response to other abiotic stresses, two-week-old rice seedlings were treated with 150 mM NaCl, 50 μM ABA, 5 °C cold or drought stresses for 48 h. Proteins from the basal parts of leaf sheaths were extracted and separated by 2D-PAGE. CBB-stained gels were analyzed to quantify the accumulation level of proteins in response to these different stresses. Great variability in protein accumulation levels was detected (Fig. 5).

Fifteen proteins that responded to mannitol treatment were also detected with the other stress treatments (Table 2). With salt treatment, 12 proteins including spots 1, 3, 5, 6, 7, 8, 9, 10, 11, 12 13 and 14 showed significant changes compared with the control. With ABA treatment, seven proteins including spots 6, 7, 8, 9, 10, 11 and 14 accumulated to significantly higher levels than the control. Cold stress changed the levels of 10 proteins compared with the control conditions including spots 1, 6, 7, 8, 9, 10, 11,

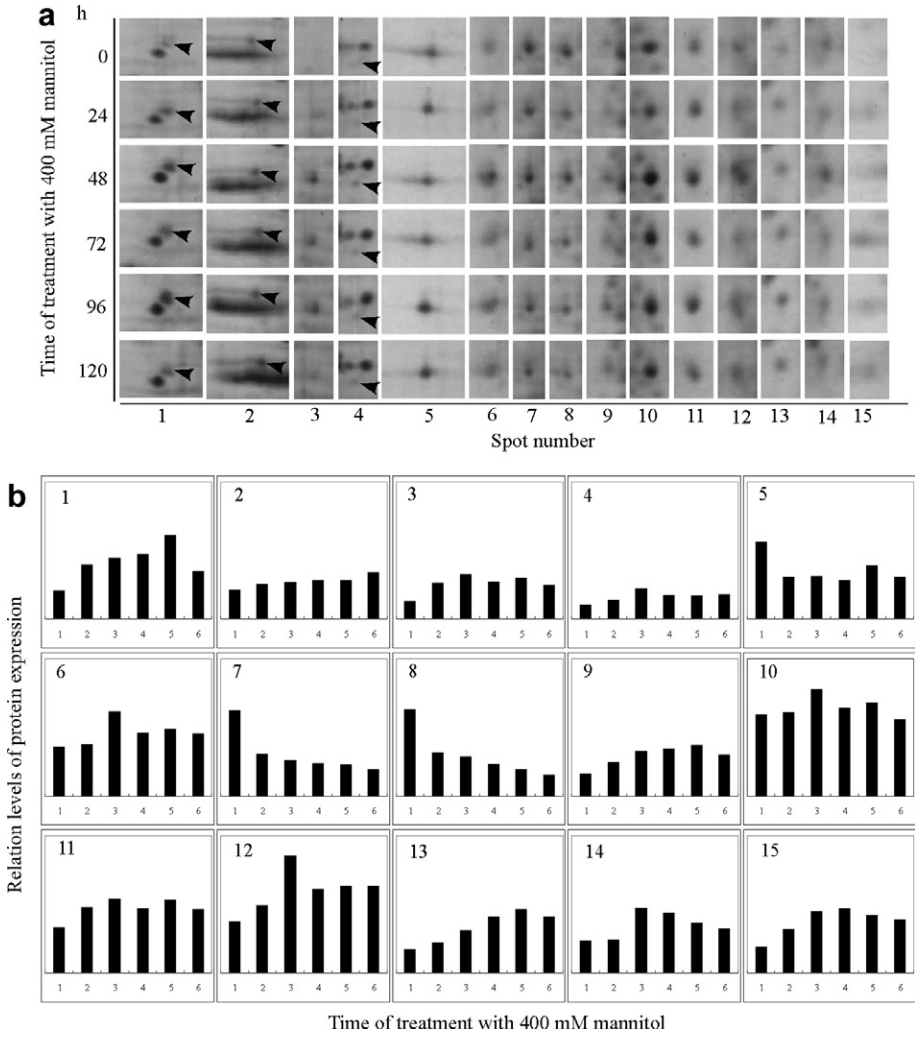

Fig. 4. Protein accumulation in the basal part of leaf sheaths in response to mannitol treatment for different intervals. Rice seedlings were treated without or with 400 mM mannitol for 24, 48, 72, 96 or 120 h. Proteins were extracted from the basal part of leaf sheaths, separated by 2D-PAGE, and stained by CBB. The changed protein spots in Fig. 2 were displayed (a). Following scanning, the gel patterns were analyzed using the 2D-Elite software, and the relative abundance ratio of proteins were analyzed (b). *X*-axis denotes the time intervals for which rice seedlings were treated, and *Y*-axis denotes the relative levels of protein expression. Columns 1, 2, 3, 4, 5 and 6 represent the relative abundance ratio of changed protein with 400 mM mannitol for 0, 24, 48, 72, 96 or 120 h, respectively. In the case of 2 protein spots in 1 panel, arrowhead was added. The bars in the graphs represent an average volume of three times experiment. Spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 show unknown protein encoded by *Oryza sativa* genome DNA, 26S proteasome regulatory subunit, BiP, undetermined protein, calreticulin precursor, lipid transfer protein, heat shock protein, dnaK-type molecular chaperone, undetermined protein, glyoxalase I, GST, uroporphyrinogen decarboxylase, 20S proteasome α subunit, unknown protein encoded by *O. sativa* genome DNA and undetermined protein.

12, 14 and 15. Under drought conditions five proteins changed in abundance compared with the control conditions including spots 5, 7, 8, 9 and 12.

The glyoxalase system is ubiquitous in nature and consists of two enzymes: glyoxalase I and glyoxalase II, which act co-ordinately to convert 2-oxoaldehydes into 2-hydroxyacids using reduced glutathione as a cofactor. The glyoxalase pathway involving glyoxalase I and glyoxalase II enzymes is required for glutathione-based detoxification of methylglyoxal. Methylglyoxal is a primary physiological substrate for glyoxalase I. Besides detoxification of methylglyoxal, the glyoxalase system could also play a role in providing tolerance under stress by recycling glutathione that would be ''trapped'' spontaneously by methylglyoxal to form hemithioacetal, thereby maintaining glutathione homeostasis (Creighyton et al., 1988). Transgenic plants over-expressing glyoxalase I showed significant tolerance to methylglyoxal and high salt (Veena and Sopory, 1999; Singla-Pareek et al., 2003). Our results suggest an important role of glyoxalase I in conferring tolerance to plants under mannitol and salt stresses, because the osmotic potential of NaCl and mannitol solution was same.

With all of the stress treatments, levels of a heat shock protein (spot 7) and a dnaK-type molecular chaperone (spot 8) were reduced, while an unidentified protein (spot 9) increased under all stresses. The heat shock protein and the dnaK-type molecular chaperone are members of the

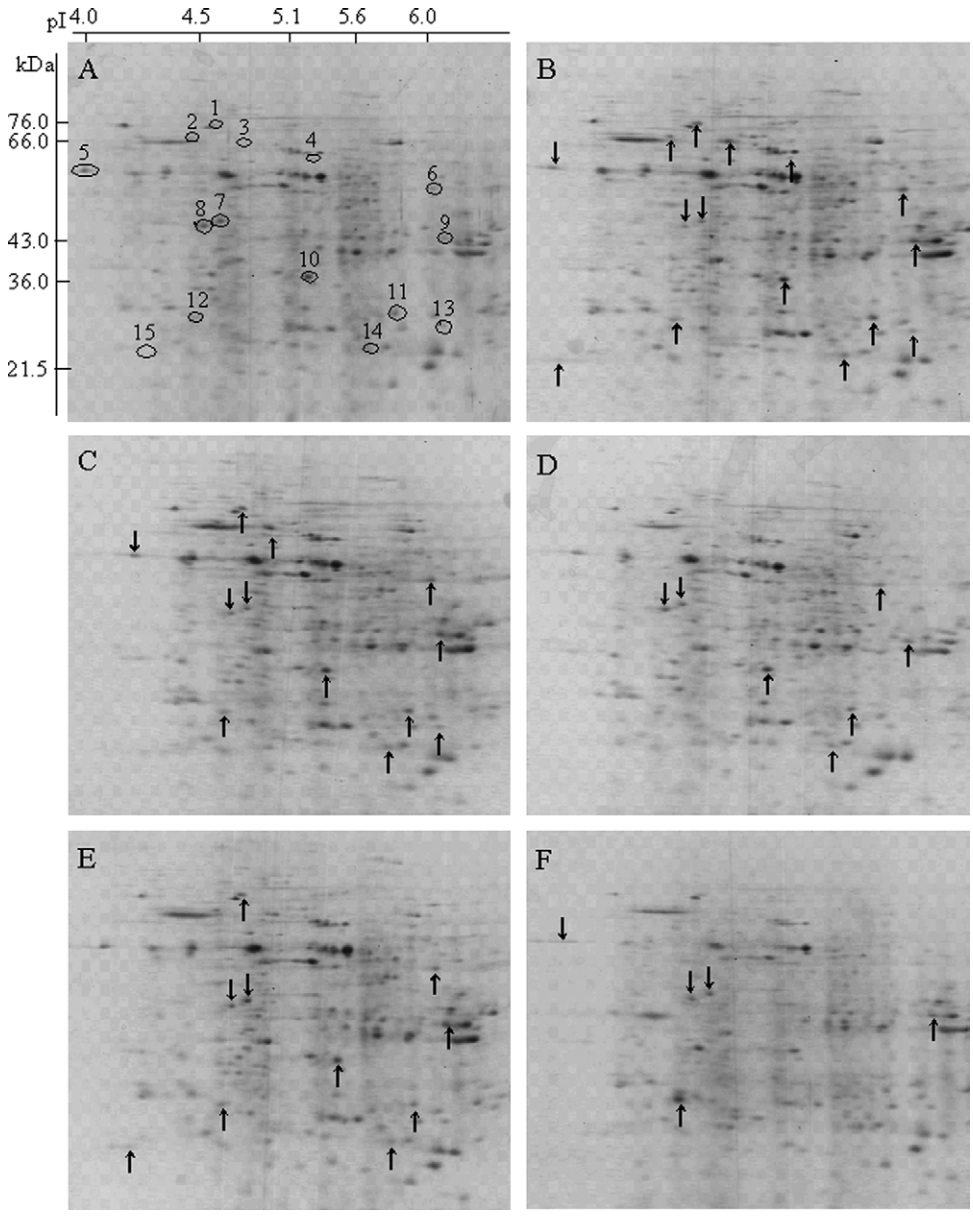

Fig. 5. Changes in protein abundance due to different stresses. Rice seedlings were treated without (A) or with 400 mM mannitol (B), 150 mM NaCl (C), 50 μM ABA (D), 5 °C cold (E), or drought (F) for 48 h. Proteins (300 μg) were extracted from the basal part of leaf sheaths, separated by 2D-PAGE, and stained by CBB. Circles indicate the proteins with changed accumulation in Fig. 2 and arrows mark the proteins regulated by the different stresses.

HSP/dnaK family. These proteins are likely to play a role in stabilizing and facilitating the refolding of proteins that have been denatured during exposure to various stresses (Bukau and Horwich, 1998; Fink, 1999). It is uncertain why the levels of two molecular chaperones were unexpectedly reduced as a result of stress treatment. This is an interesting observation which indicates stress conditions in the ER lumen and cytoplasm raise different responses. We look forward to further studies that will provide more information about the signaling pathway for the ER stress response in rice.

On the other hand, there were three proteins that showed a response only with osmotic stress: a 26S proteasome regulatory subunit (spot 2), BiP (spot 3) and an unidentified protein (spot 4). The 26S proteasome regulatory subunit is an essential part of the proteasome complex responsible for removing most short-lived intracellular proteins, especially those modified with polyubiquitin chains. One of the subunits of this proteasome, rpn10, is considered to be the main ubiquitinated protein receptor in the entire complex and functions to insure substrate-specificity and to stabilize the regulatory particle (Smalle et al., 2003). The endoplasmic reticulum (ER) consists of a three-dimensional structure in eukaryotic cells where proteins for the secretary pathway are synthesized. Proper folding and assembly of proteins synthesized in the ER are necessary for transport to their final destinations. When folding or assembly of proteins in the ER is disordered, unfolded proteins accumulate in the ER and expression of ER-resident proteins such as BiP and folding enzymes is induced. This phenomenon is conserved among eukaryotic cells and

is referred to as the ER stress response or the unfolded protein response (Rutkowski and Kaufman, 2004). Our results suggest that the protein degradation system may be activated during osmotic stress. All proteins that respond to mannitol did not respond to NaCl, cold and drought. Other osmotic stresses elicit different quantitative changes from mannitol. Although mannitol is a useful model treatment, it does not mimic exactly osmotic stresses.

2.5. Comparison of the effect of osmotic stress on proteins in two different rice cultivars

A comparative study on morphological and proteomic differences in response to osmotic stress was carried out between Nipponbare and an osmotic stress-tolerant cultivar, Zhonghua 8 (Alli and Komatsu, 2006). Two-week-old seedlings were treated with mannitol at different

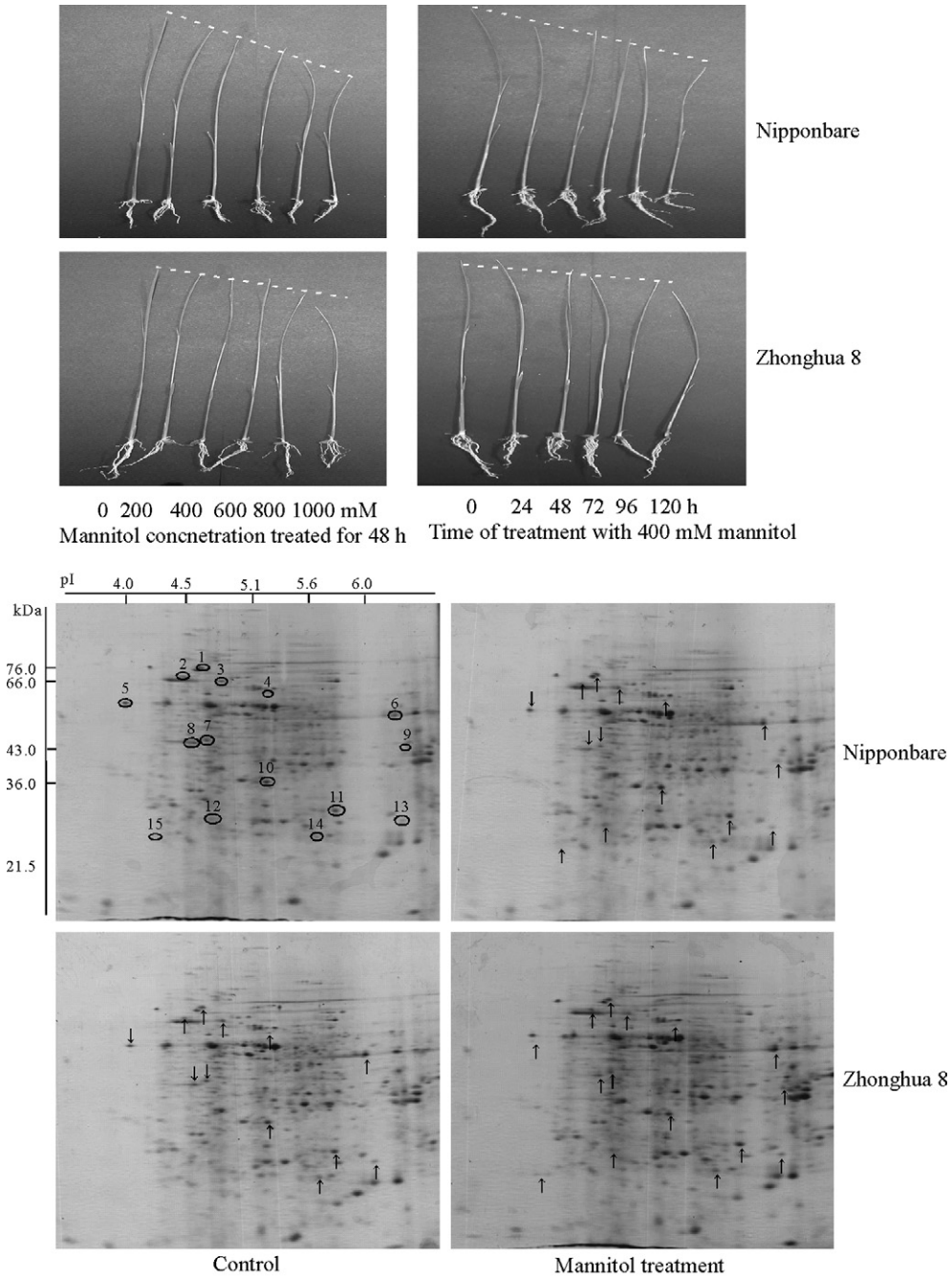

Fig. 6. A comparative response of rice varieties to osmotic stress. Rice seedlings cvs. Nipponbare (a and b upper) and Zhonghua 8 (a and b lower) were treated with mannitol at different concentrations for 48 h or at 400 mM for different intervals. Photographs show the changes of shoot height under mannitol treatment at different concentrations for 48 h or at 400 mM for different intervals (a). The proteins were extracted from the basal part of leaf sheaths treated without (control) or with 400 mM mannitol for 48 h, separated by 2D-PAGE, and stained by CBB (b). Proteins induced by mannitol are indicated by arrows. The numbers refer to the spot numbers as given in Fig. 2.

concentrations for 48 h or at 400 mM for different time intervals. Under osmotic stress, the growth of both cultivars was repressed (Fig. 6a) as shown previously in potted plants (Fig. 1). As expected, the osmotic stress-tolerant genotype, Zhonghua 8, suffered less from osmotic stress than Nipponbare, especially with lower concentrations of mannitol or with shorter treatment intervals (Fig. 6a). Proteins from the basal part of leaf sheaths were extracted, separated by 2D-PAGE and stained by CBB. Protein patterns from Nipponbare and Zhonghua 8 were qualitatively similar (Fig. 6b). Fifteen proteins that responded to mannitol treatment were also detected in Zhonghua 8. Out of these 15 proteins, 3 proteins accumulated to lower amounts than the Zhonghua 8 controls: a calreticulin precursor (spot 5), a heat shock protein (spot 7) and a dnaK-type molecular chaperone (spot 8). Nine proteins, out of the 15 target proteins, accumulated to higher levels compared to Nipponbare controls: a 26S proteasome regulatory subunit (spot 2), a 20S proteasome α subunit (spot 13), BiP (spot 3), a lipid a transfer protein (spot 6), 2 functionally unknown proteins (spots 1 and 14) and an unidentified protein (spot 4).

Glyoxalase I and GST also accumulated in the osmotic-tolerant cultivar, Zhonghua 8. Glyoxalase I and GST are two glutathione-dependent enzymes that are enhanced in plants during cell division and in response to diverse stress treatments (Skipsey et al., 2000) including osmotic stress as discussed previously. Both BiP and the lipid transfer protein that were induced by mannitol in Nipponbare also accumulated in the osmotic-tolerant cultivar, Zhonghua 8. In mammalian cells, treatment with high levels of glucose results in lipid accumulation, impaired glucose-stimulated insulin secretion, apotosis, and pronounced induction of the ER stress marker genes, BiP and Chop10 (Wang et al., 2005). Imin et al. (2006) reported that lipid transfer protein was changed in rice anthers by low temperature. Our results indicate that treatment with high levels of mannitol may result in lipid accumulation through ER stress. Levels of the 26S proteasome regulatory subunit and the 20S proteasome α subunit increased in the osmotic-tolerant cultivar. The disposal of misfolded proteins from the ER lumen is one of the quality control mechanisms present in the protein secretory pathway. Through the ER-associated degradation pathway, misfolded substrates are targeted to the cytosol where they are degraded by the proteasome (McCracken and Brodsky, 2003). An important role of the proteasome regulatory system may be in regulating the responses to signals promulgated by osmotic stress.

3. Concluding remarks

In this study, mannitol was used to determine the effect of osmotic stress on protein accumulation. Proteins were analyzed by a proteomic approach and the potential relationships with osmotic stress were discussed. Accumulation of 15 proteins was significantly changed under osmotic stress, and at least 6 proteins were identified as important components in the glyoxalase, lipid accumulation and the proteasome regulatory pathways through ER stress induced by osmotic stress. The similarity in response for dose- and time-dependent experiments and comparisons among different stress treatments, different tissues and different cultivars underscores the likelihood that the proposed pathways are involved in plant responses to osmotic stress. These findings have important implications for understanding the biochemical and molecular mechanisms of plant adaptation and response to osmotic stress.

4. Experimental

4.1. Plant materials and treatment

Rice (*Oryza sativa* L.) cvs. Nipponbare and Zhonghua 8 seedlings were used in this study. Seedlings were grown in the granulated nutritional soil (Kutrha Chemical, Tokyo, Japan) under white fluorescent light (600 μmol m^{-2} s^{-1}; 12 h light period/day) at 25 °C and 75% relative humidity in a growth chamber. Experiments were conducted in a plastic seedling pots in growth chamber. At 2 weeks after sowing, seedling pots were transferred to plastic containers containing the solutions of mannitol (Wako, Osaka, Japan) at 200 mM to 1000 mM for 24 h to 120 h. Similarly, seedling pots were transferred to plastic containers containing the solutions of 150 mM NaCl or 50 μM ABA. For the drought experiment, drought was initiated by withholding water at 2 weeks after sowing and the samples were collected when plants had lost 50% fresh weight (Abbasi and Komatsu, 2004). For cold treatment, plants were transferred to a growth chamber at 5 °C and grown for 48 h. Twenty plants were collected randomly and the proteins from basal part of leaf sheath were separated by 2D-PAGE.

4.2. Preparation of protein extract

A portion (200 mg) of basal part of leaf sheath was homogenized in 300 μl of lysis buffer containing 8 M urea, 2% NP-40, 0.8% ampholine (pH 3.5–10), 5% 2-mercaptoethanol and 5% polyvinylpyrrolidone-40 (O'Farrell, 1975), using a glass mortar and pestle on ice. The homogenates were centrifuged at 20,600*g* for 5 min. The supernatant was centrifuged at 20,600*g* for 5 min again and subjected to 2D-PAGE.

4.3. Two-dimensional polyacrylamide gel electrophoresis

Proteins (300 μg) were separated by 2D-PAGE in the first dimension by isoelectric focusing (IEF) tube gels and in the second dimension by SDS-PAGE. An IEF tube gel of 11 cm length and 3 mm diameter was prepared. IEF gel solution consisted of 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate and TEMED. Electrophoresis was

carried out at 200 V for 30 min, followed by 400 V for 17 h and 600 V for 1 h. After IEF, SDS–PAGE in the second dimension was performed using 15% polyacrylamide gels with 5% stacking gels. The gels were stained with Coomassie brilliant blue (CBB), and image analysis was performed. The position of individual proteins on gel was evaluated automatically with Image Master 2D Elite software (Amersham Biosciences, Piscataway, NJ, USA). The p*I* and Mr of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA).

4.4. Image acquisition and data analysis

CBB-stained gels were analyzed with Image Master 2D-Elite software. Specially, spot detection, spot measurement, background subtraction and spot matching were performed. Following automatic spot detection, gel images were carefully edited. Prior to performing spot matching between gel images, one gel image was selected as the reference gel. After automatic matching, the unmatched spots of the member gels were added to the reference gel. The amount of a protein spot was expressed as the volume of the spot which was defined the sum of the intensities of all the pixels that make up the spot. In order to correct the variability due to CBB staining and reflect the quantitative variations in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all of the spots present in gel. The resulting data from image analysis were transferred to 2D Elite software for querying protein showing quantitative or qualitative variations.

4.5. Cleveland peptide mapping

To determine internal amino acid sequence, Cleveland peptide mapping was used. Following separation by 2D-PAGE, gel pieces containing protein spots were removed and the protein was electroeluted from the gel pieces using an electrophoretic concentrator (Nippon Eido, Tokyo, Japan) at 2 W constant powers for 2 h. After electroelution, the protein solution was dialyzed against deionized water for 2 d and lyophilized. The protein was dissolved in 20 μl of SDS sample buffer (pH 6.8) and applied to sample wells in a SDS–PAGE gel. The sample solution was overlaid with 20 μl of a solution containing 10 μl of *Staphylococcus aureus* V8 protease (0.1 μg/μl; Pierce, Rockford, IL, USA) in deionized water, and 10 μl of SDS sample buffer (pH 6.8). Electrophoresis was performed, until the sample and protease were stacked in the stacking gel. It was interrupted for 30 min to digest the protein, and then continued (Cleveland et al., 1977).

4.6. N-terminal and internal amino acid sequence analysis and homology searches

Following separation by 2D-PAGE or by the Cleveland method, proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Pall Bio Support division, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido) and detected by CBB staining. The stained protein spots were excised from the PVDF membrane and applied to the upper glass block of the reaction chamber in a gas-phase protein sequencer (Procise cLC; Applied Biosystems, Foster City, CA, USA). Edman degradation was performed according to the standard program supplied by Applied Biosystems. The amino acid sequences obtained were compared with those of known protein in the Swiss-Prot, PIR, Genpept and PDB databases with the Web-accessible search program FASTA (http://www.dna.affrc.go.jp).

4.7. Analysis using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

CBB-stained proteins were excised from gels, washed with 25% MeOH, 7% AcOH for 12 h at room temperature, and destained with 50 mM NH_4HCO_3 in 50% methanol for 1 h at 40 °C. After drying under vacuum, gel spots were incubated in 50 μl of a reduction solution containing 10 mM EDTA, 10 mM dethiothreitol and 100 mM NH_4HCO_3 at 60 °C for 1 h. The gel spots were dried under vacuum and incubated in 50 μl of alkylation solution containing 10 mM EDTA, 10 mM iodoacetamide and 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. After washing with water, the gel spots were minced, dried in vacuo, and digested in 10 mM Tris–HCl (50 μl, pH 8.0) containing 1 pM trypsin (Sigma, St.Louis, MO, USA) at 37 °C for 10 h. CH_3CN (100 μl containing 0.1% CF_3CO_2H) was added to each gel piece and sonicated. Purification of the generated peptides was achieved using Nutip C-18 (Glygen, Columbia, MD, USA). The purified peptides were added to α-cyano-4-hydroxycinamic acid matrix and dried onto a plate for analysis using MALDI-TOF MS (Voyager-DE RP, Applied Biosystems). Calibration was external and data were collected in the linear mode. Matching of empirical peptide mass values with theoretical digest and sequence information obtained from the database was performed and using Mascot Version 2.0 software (Matrix Science Ltd., London, UK).

For MALDI-TOF MS analysis, 4 criteria were used to assign a positive match with a known protein: (1) the deviation between the experimental and theoretical peptide masses needed to be less than 50 ppm; (II) at least six different predicted peptide masses needed to match the observed masses for an identification to be considered valid; (III) the matching peptides needed to cover at least 30% of the known protein sequence; and (IV) individual ions scores >51 identity or extensive homology ($P < 0.05$).

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References

Abbasi, F.M., Komatsu, S., 2004. A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics 4, 2072–2081.

Alli, G.M., Komatsu, S., 2006. Proteomic analysis of rice leaf sheath during drought stress. J. Proteome Res. 5, 396–403.

Bohnert, H.J., Nelson, D.E., Jensen, R.G., 1995. Adaptations to environmental stresses. Plant Cell 7, 1099–1111.

Bukau, B., Horwich, A.L., 1998. The Hsp 70 and Hsp60 chaperone machines. Cell 92, 351–366.

Cleveland, D.W., Fischer, S.G., Kirchner, M.W., Laemmli, U.K., 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102–1106.

Creighyton, D.J., Magliorini, M., Pourmotabbed, T., Guha, M.K., 1988. Optimization of efficiency in the glyoxalase pathway. Biochemistry 27, 7376–7384.

Deak, K.I., Malamy, J., 2005. Osmotic regulation of root system architecture. Plant J. 43, 17–28.

Fink, A.L., 1999. Chaperone-mediated protein folding. Physiol. Rev. 79, 425–429.

Imin, N., Kerim, T., Weinman, J.J., Rolfe, B.G., 2006. Low temperature treatment at the young microspore stage induces protein changes in rice anthers. Mol. Cell. Proteomics 5, 274–292.

Komatsu, S., Karibe, H., Hamada, T., Rakwal, R., 1999. Phosphorylation upon cold stress in rice (*Oryza sativa* L.) seedlings. Theol. Appl. Gen. 98, 1304–1310.

Komatsu, S., Tanaka, N., 2004. Rice proteome analysis: a step toward function analysis of the rice genome. Proteomics 4, 938–949.

Lu, Z., Neumann, P.M., 1999. Water stress inhibits hydraulic conductance and leaf growth in rice seedling but the transport of water via mercury-sensitive water channels in the root. Plant Physiol. 120, 143–152.

McCracken, A.A., Brodsky, J.L., 2003. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). Bioessays 25, 868–877.

Maeda, T., Wurgler-Murphy, S.M., Saito, H., 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369, 242–245.

Mizuno, T., 1998. His-Asp phosphotransfer signal transduction. J. Biochem. 123, 555–563.

Mock, H.P., Grimm, B., 1997. Reduction of uroporphyrinogen decarboxylase by antisense RNA expression affects activities of other enzymes involved in tetraphyrrole biosynthesis and leads to light-dependent necrosis. Plant Physiol. 113, 1101–1112.

O'Farrell, H.P., 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007–4021.

Reiser, V., Salah, S.M., Ammerer, G., 2000. Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. Nat. Cell Biol. 2, 620–627.

Riccardi, F., Gazeau, P., deVienne, D., Zivy, M., 1998. Protein changes in response to progressive water deficit in maize. Plant Physiol. 117, 1253–1263.

Rutkowski, D.T., Kaufman, R.J., 2004. A trip to the ER: coping with stress. Trends Cell Biol. 14, 20–28.

Singla-Pareek, S.L., Reddy, M.K., Sopory, S.K., 2003. Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proc. Natl. Acad. Sci. USA 100, 14672–14677.

Skipsey, M., Andrews, C.J., Townson, J.K., Jepsom, I., Edwards, R., 2000. Cloning and characterization of glyoxalase I from soybean. Arch. Biochem. Biol. 374, 261–268.

Smalle, J., Kurepa, J., Yamg, P., Emborg, T.J., Babiychuk, E., Kushnir, S., Vierstra, R.D., 2003. The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signaling. Plant Cell 15, 1–16.

Snarpi, Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Hrie, R., Chan, W.Y., Leung, H.Y., Hattori, K., Konomi, M., Osumi, M., Yamagami, M., Schroeder, J.I., Uozumi, N., 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na unloading from xylem vessels to xylem parenchyma cells. Plant J. 44, 928–938.

Tamura, T., Hara, K., Yamaguchi, Y., Koizumi, N., Sano, H., 2003. Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants. Plant Physiol. 131, 454–462.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., Shinozaki, K., 1999. A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. Plant Cell 11, 1743–1754.

Veena, R.V.S., Sopory, S.K., 1999. Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. Plant J. 17, 385–395.

Wakabayashi, K., Hoson, T., Kamisaka, S., 1997. Osmotic stress suppresses cell wall stiffening and the increase in cell wall-bound ferulic and diferulic acids in wheat coleoptiles. Plant Physiol. 113, 967–973.

Wang, H., Kouri, G., Wollheim, C.B., 2005. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. J. Cell Sci. 118, 3905–3915.

van der Weel, C.M., Spollen, W.F., Sharo, R.E., Baskin, T.I., 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J. Exp. Bot. 51, 1555–1562.

Xiong, L., Zhu, J.K., 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ. 25, 131–139.

Zhu, J.K., Hasegawa, P.M., Bressan, R.A., 1997. Molecular aspects of osmotic stress. Crit. Rev. Plant Sci. 16, 253–277.

Zonia, L., Mnnik, T., 2004. Osmotically induced cells swelling versus cell shrinking elicits specific changes in phospholipid signals in tobacco pollen tubes. Plant Physiol. 134, 813–823.