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Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress

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ABSTRACT

The inherent immobility of rice (*Oryza sativa* L.) limited their abilities to avoid heat stress and required them to contend with heat stress through innate defense abilities in which heat shock proteins played important roles. In this study, Hsp26.7, Hsp23.2, Hsp17.9A, Hsp17.4 and Hsp16.9A were up-regulated in Nipponbare during seedling and anthesis stages in response to heat stress. Subsequently, the expressing levels of these five sHsps in the heat-tolerant rice cultivar, Co39, were all significantly higher than that in the heat-susceptible rice cultivar, Azucena. This indicated that the expressive level of these five sHsps was positively related to the ability of rice plants to avoid heat stress. Thus, the expression level of these five sHsps can be regarded as bio-markers for screening rice cultivars with different abilities to avoid heat stress. Hsp18.1, Hsp17.9A, Hsp17.7 and Hsp16.9A, in the three rice cultivars under heat stress were found to be involved in one protein complex by Native-PAGE, and the interactions of Hsp18.1 and Hsp 17.7, Hsp18.1 and Hsp 17.9A, and Hsp17.7 and Hsp16.9A were further validated by yeast 2-hybridization. Pull down assay also confirmed the interaction between Hsp17.7 and Hsp16.9A in rice under heat stress. In conclusion, the up-regulation of the 5 sHsps is a key step for rice to tolerate heat stress, after that some sHsps assembled into a large hetero-oligomeric complex. In addition, through protein–protein interaction, Hsp101 regulated thiamine biosynthesis, and Hsp82 homology affected nitrogen metabolism, while Hsp81-1 were involved in the maintenance of sugar or starch synthesis in rice plants under heat stress. These results provide new insight into the regulatory mechanism of sHsps in rice.

1. Introduction

Unlike animals, the inherent immobility of rice plants limited their abilities to avoid stress and required them to contend with heat stress through their innate defense abilities [1] in which heat shock proteins (Hsps) might play important roles [2]. Hsps, which possessed molecular chaperone activities, were key factors contributing to cellular homeostasis in cells under both normal and adverse growth conditions [3].

Hsps were highly conserved proteins, suggesting the parallel functions in different organisms [4]. Hsps were divided into high-molecular-mass proteins, comprising Hsp100, Hsp90, Hsp70, Hsp60, and small-molecular-mass proteins consisting of Hsp20 or small heat shock proteins (sHsps) [5]. SHsps were designated as a group of proteins with a molecular mass of 15 to 42 kDa in both prokaryotic and eukaryotic cells [6]. In mammalian cells, sHsps were known to be involved not only in enhancing cell survival in response to stress but also in the regulation of other cellular function, including apoptosis and differentiation, via their participation in the modulation of cellular redox states [7]. In rice, the expression of some sHsp genes was regulated differentially by abiotic stresses and abscisic acid (ABA), implying that these sHsp genes may play important roles in rice plant development and abiotic stress responses [8]. Based on the comprehensive sequence and expression profile analysis, Ouyang et al. [9] suggested that the expression patterns of OsHsp20 genes differed not only in different developmental stages but also in different variety levels. Sarkar et al. [5] also revealed that 23 sHsp genes were differentially expressed under several stresses and at different stages in the life cycle of rice plant by using microarray analysis and RT-PCR. High temperature increased the expression of sHsps in rice during seedling [10] and caryopsis development [11] and anthesis [12].

However, the investigation of functional characterization of sHsps in plant was limited. Recently, some interesting results on rice sHsp had been reported, which allowed us to partly predict the functions of

Abbreviations: Hsps, heat shock proteins; sHsps, small heat shock proteins; GS, glutamine synthetase; IEF, iso-electrophoresis focus; β-Me, β-mercaptoethanol; CI, confidence interval; PVP, polyvinylpyrrolidone

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sHsp. Transgenic rice lines with overexpression of the sHsp17.7 gene clearly showed higher drought tolerance compared to untransformed control rice plants during the seeding stage [13]. In addition, overexpression of a rice chloroplast sHsp in *Escherichia coli* conferred better tolerance not only to heat stress but also to oxidative stress [14]. In maize, mitochondrial sHsps protected Complex I electron transport during NaCl stress [15]. Moreover, the chloroplast sHsp had been found to protect photosynthesis of rice during heat [16], oxidative [17] and heavy metal stress [18]. Taken together, these results indicated that rice sHsps could be induced by abiotic stresses and sHsp improved tolerance to abiotic stresses. However, the relationship between sHsp expression levels and ability of plant adaptation to environmental stresses was poorly understood.

After inducing by stresses, sHsp stably binds heat-denatured proteins and then cooperate with Hsp70, an ATP-dependent chaperone, to reactivate a metaprotein [19,20]. Stable binding of several non-native proteins to Hsp25 creates a reservoir of folding intermediates for reactivation [21]. Thus, it can be inferred that cell protection by Hsps in plants under abiotic stresses was achieved by the binding of sHsps to other proteins. However, there was no direct evidence to prove this inference in rice because limited focus had been given to intensive study of the complex of sHsps in rice in response to heat stress.

In addition, two-dimensional Blue Native/SDS-PAGE (BN-PAGE) showed greatly higher resolution and had been generally used for identification of protein complexes [22]. However, compared with BN-PAGE, a potential advantage seemed to be the milder conditions of Native-PAGE, which might allow us to determine protein complexes under a condition that was close to the physiological situation of cells since proteins' electrophoretic migration depended perfectly on protein interior charges [23]. Therefore, it was assumed that more tethered rice soluble protein complexes could be identified using Native-PAGE than BN-PAGE.

In this study, rice varieties (*Oryza sativa* L.) with different abilities to tolerate heat stress were employed as materials to analyze the expression levels of sHsps, and their interactions with other proteins in response to heat stress by using a combination of techniques, including two-dimensional gel electrophoresis (2-DE), Native-PAGE, RT-PCR, yeast two-hybridization, pull-down and mass spectrometry. The objectives of this study were to: (i) determine the abundance of the main sHsp in different rice cultivars; and (ii) investigate the Hsp complexes, especially sHsp complexes, in rice in response to heat stress.

2. Materials and methods

2.1. Plant growth and heat-stress treatment

In this study, three rice varieties (*O. sativa* L), Nipponbare, Azucena (Susceptible to heat) and Co39 (Tolerant to heat), with different abilities to tolerate heat stress [24], were used as materials. Rice seedlings were grown in a greenhouse (28 °C ± 2° day/22°C ± 2° night) under natural light conditions (14 h light/10 h darkness period) in nutrient solution after germination at 30 °C for 48 h [25]. For high-temperature treatments, 3-week-old seedlings were exposed to 42 °C (Treatment) or 28 °C (Control) for 12 h and 24 h, respectively. Nipponbare plants during anthesis stage were exposed to 42 °C (Treatment) or 28 °C (Control) for 6 h and 12 h. Leaves were sampled and stored at −80 °C immediately after treatment.

2.2. IEF and SDS-PAGE

Total protein for 2-DE experiment was extracted from leaves using the methods of He and Li [26]. Leaf proteins (300 μg) were loaded on prepared iso-electrophoresis focus (IEF) tube gels (17 cm length and 2 mm diameter), which contained 12 M urea, 5.5% (w/v) acrylamide, 3% NP-40 and 7.5% Biolyte (pH 3–10 and 5–8; Bio-Rad), 0.02%w/v ammonium persulfate, and 0.15%v/v N, N, N, N-N-tetraethylethylenediamine. The IEF was performed at 200 V, 300 V, 400 V, 500 V and 600 V for 30 min, followed by 800 V for 16 h and 1000 V for 4 h. After IEF, the gels were immediately equilibrated for 25 min in equilibration buffer (60 mM Tris–HCl pH 6.8, 2.5% SDS, 9% (v/v) glycerol and 5% (v/v) β-mercaptoethanol (β-Me)). The second-dimension electrophoresis was carried out by using 12.5% resolving gel and 5% stacking gel. The gels were then stained with Coomassie.

2.3. Evaluation of protein expression abundance

The images of 2-D gels were analyzed by ProteinMaster 6.0 software (FortuneSun corporation, China). 2-D gels were aligned and matched after spot detection and background subtraction (lowest on boundary mode), and spot volumes were quantitatively determined (total spot volume normalization mode). The 2-DE experiments were performed in triplicate using three separate samples.

2.4. Image analysis and MALDI-TOF/TOF MS analysis

In MALDI-TOF/TOF MS analysis, protein spots were digested with sequencing-grade trypsin (Promega, USA) as described previously [27]. The resulting peptides were desalted with C18 ZipTips (Millipore), mixed with 5 mg/ml alpha-cyanocinnamic acid in 70% acetonitrile and 0.1% trifluoraoacetic acid, and spotted onto a MALDI sample plate. Mass spectra were acquired on a MALDI TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems) in both the MS and MS/MS modes. Data were analyzed using MASCOT software (Matrix Science, UK). NCBI or Swiss-Prot was selected as the database. Typical search parameters were set as: mass tolerance, 0.5 Da; missed cleavages, 1; enzyme, trypsin; fixed modifications, carbamido-methylation; variable modification, oxidation (M); taxonomy, *O. sativa*. For a match to be considered a valid identification, a confidence interval (*CI*) greater than 95% was required [27].

2.5. Native-PAGE and SDS-PAGE

Total protein extracted from leaves for Native-PAGE were carried out following the protocol described by Kügler et al. [28] and Sun et al. [29] with some modifications. Briefly, rice leaves were ground in liquid nitrogen for at least 30 min. The powder was rapidly transferred to centrifuge tubes and the solubilization buffer (500 mM 6-aminohexanoic acid, 50 mM imidazole/HCl PH7.0, 1 mM EDTA, 0.6% polyvinylpyrrolidone (PVP), 1 mM PMSF, 1 mM phenantroline) was added subsequently [30]. The mixture was incubated on ice for 15 min. After mild lysis, the protein solution was separated by centrifugation for 25 min at 15,000 rpm. The supernatant could then be quickly frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were measured according to Bradford [31].

The protocol for Native-PAGE and SDS-PAGE analysis was performed as described by Pan et al. [32]. Briefly, the first-dimension Native-PAGE gels consisted of an 8% separating gel and a 4% stacking gel. Prior to electrophoresis, triton X-100 was added to a final concentration of 2.5%. The mixtures were allowed to stand for 5 min on ice before the addition of sample buffer (100 mM Tris–HCl pH 6.8, 50% glycerol, 0.2% bromophenol blue). Native-PAGE was carried out at 4 °C at a constant voltage of 80 V in Tris–glycine buffer. The NativeMark™ Unstained Protein Standard (Invitrogen) was used for Native marker. After 1-D Native-PAGE, the gels were stained with imidazole–sodium dodecyl sulfate–zinc reverse staining [33]. The protein lanes were cut out according to the results of staining and then were equilibrated in equilibration buffer (60 mM Tris–HCl pH 6.8, 3% SDS, 10% (v/v) glycerol and 8% (v/v) β-mercaptoethanol) for not less than 35 min. Second-dimension SDS-PAGE was performed at room temperature with 12% resolving gel and 5% stacking gel, and then the gels were stained with Coomassie. The experiments were also performed in triplicate using three separate samples.