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Protein profiles in response to salt stress in leaf sheaths of rice seedlings

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ABSTRACT: Salt-induced changes in protein synthesis in leaf sheaths of the Thai rice cv. Leaung Anan were investigated using one-dimensional SDS-PAGE and two-dimensional PAGE (2DE). In SDS-PAGE, two protein bands (22 and 31 kDa) whose expression was specifically increased under salt stress were identified. Enhanced expression of these protein bands was consistently observed in plants grown under greenhouse conditions saline during different time of the year. In 2DE, at least thirteen different proteins were induced and ten different proteins were decreased by salt. Western blot analysis of the 31 kDa of rice seedlings of three cultivars: salt-tolerant Pokkali, moderately tolerant Leuang Anan, and salt-sensitive KDML 105, showed different expression patterns. The protein was strongly induced by salinity in leaf sheaths of treated tolerant Pokkali and Leuang Anan, compared to control plants. In contrast, its level in the sensitive KDML 105 remained unchanged with salt treatment. Moreover, rice cv. Leaung Anan showed higher expression of the 31 kDa protein than Pokkali. These results suggest that the 31 kDa polypeptide is a salt-induced protein in rice leaf sheaths.

KEYWORDS: Polyclonal antibody, Rice, Salt-induced proteins.

INTRODUCTION

Salinity, which is generally detrimental to plant growth, adversely affects the metabolism of plants and causes important modifications in gene expression in plants. Such modifications may lead to accumulation or depletion of certain metabolites resulting in an imbalance in the levels of a relatively small set of cellular proteins, which could increase, decrease, appear or disappear after salt treatment.

Over the past few years, much attention has been concentrated on resolving the identity of salt stress proteins, in order to identify and understand the role of proteins in rice salt tolerance. However, at present their functions are still unclear. Several workers have detected a number of proteins induced by salt, reflecting the complexity of biochemical and physiological responses. There are many reports showing that these protein changes are accompanied with the biological changes of the adaptation process, which make the organism more fit in the altered environment[1,2]. Several proteins have been characterized to play prominent roles in the regulation of K^+ and/or Na^+ fluxes[3]. However, most of them do not always confer tolerance to salinity[4]. Changes in protein profiles also depend on the plant parts studied and the nature of the plant species[5].

Salt stress proteins have been reported in many plant species, such as in barley roots[6,7,8]: 20-24 kDa, pI 6.3-7.2 and 26 kDa, pI 6.3 and 6.5; in *Brassica*[9]: 56.1-70.8 kDa, 93.8 kDa; in rice: roots[4,10,11], 14.5 kDa, 15 kDa, pI 5, shoots[12]: 15 and 26 kDa, cultured cells[12]: 26 and 27 kDa, and germinating seeds[13]: 23 kDa; in *Raphanus sativus*[14]: 22 kDa; in cultured tobacco cells[1]: 18, 19.5, 21, 26, 34, 35.5, 37, 58 kDa, and in tomato roots[15]: 21 kDa, pI 5.7; 21.5 kDa, pI 5.5; 22 kDa, pI 5.4; and 32 kDa, pI 6.4.

However, the effect of salt stress on protein changes in rice leaf sheath has rarely been studied. This study focused on moderately salt tolerant Thai rice cv. Leuang Anan seedlings. The protein profiles induced by salt stress in this line were studied by SDS-PAGE and 2DE. Additionally, a polyclonal antibody raised against a 31 kDa salt-induced protein of salt-treated leaf sheaths of this line was produced and used to study the pattern of protein expression in leaf sheaths of three rice cultivars, namely, Pokkali, Leuang Anan, and KDML 105 using western blot technique.

Materials and Methods

Plant Materials and Salinity Treatments

Rice (*Oryza sativa* L. cv. Leuang Anan; moderately salt tolerant Thai rice) seeds were hydroponically grown in a glasshouse together with two standard cultivars: Pokkali (a salt tolerant Indian cultivar) and Khao Dawk Mali 105 (KDML 105; a sensitive Thai cultivar). Methods of growing plants and salinization of the nutrient solution were modified from Gregorio et al.[16]. Seeds were surface-sterilized with 1.5% (w/v) calcium hypochlorite for 30 min, rinsed well with distilled water, imbibed for 48 h and placed on plastic grids above a 4-liter black plastic pot containing distilled water. After 3 days, when seedlings were well-established, the distilled water was replaced by the non-salinized nutrient solution.[17] The nutrient solution was adjusted daily to pH 5.0 with 1 N KOH or 1 N HCl and renewed every 7 days. When the plants were 14 days old, NaCl was introduced, initially at the level of 6 dS m^{-1} for 3 days, then 12 dS m^{-1} for 10 days (the electrical conductivities of 6 and 12 dS m^{-1} are equivalent to approximately 3 and 6 g NaCl L^{-1}, respectively[16]). The roots, leaf laminae, and leaf sheaths were then sampled, frozen in liquid nitrogen and stored at –70 °C.

Protein Extraction

For SDS-PAGE, root, leaf lamina, and leaf sheath tissues of each cultivar were ground to powder under liquid nitrogen and melted in ice-cold extraction buffer [50 mM Tris-HCl, pH 8, 10 mM NaCl, 1% SDS, 5% 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM DTT], followed by centrifugation at 10,000 x g at 4 °C for 15 min. Protein content of the clear supernatants obtained after centrifugation were determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard. Extracts were stored at -20 °C.

For 2DE, leaf sheaths of rice cv. Leuang Anan were ground to powder under liquid nitrogen and melted in ice-cold double distilled water. The homogenate was centrifuged at 10,000 x g for 15 min at 4 °C. Desalting was performed by precipitating all proteins from the supernatant by using the 2-D clean-up kit (Amersham Biosciences, Uppsala, Sweden). Dried protein pellets were solubilized in rehydration buffer [8M urea, 0.5% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) IPG buffers]. The amount of protein was determined according to the Bradford method.[18]

One-Dimensional SDS-PAGE

Proteins, 5 μg of each sample, were separated by SDS-PAGE according to the method of Laemmli[19]. The separation was performed with a 10% separating gel and a 4% stacking gel using PROTEAN II Multi Cell (Bio-Rad). Electrophoresis was started at 10 mA constant current until the tracking dye entered the separating gel and continued at 25 mA until the tracking dye reached the end of the gel. After electrophoresis, the gel was stained with silver nitrate, according to the method of Damerval et al.[20]. Relative molecular weight (MW) of each protein was determined by using a standard curve generated from the standard proteins.

Two-Dimensional PAGE

The first-dimensional isoelectric focusing gel electrophoresis (IEF) was conducted at 20 °C with an IPG phor™ IEF System and a DryStrip kit (Amersham Biosciences, Uppsala, Sweden). Each 7 cm IPG strip (pH 3-10, non-linear) was rehydrated with 125 μL of rehydration buffer for 13 h and sample (5 μg) was subsequently loaded. Isoelectric focusing was performed at 500 V for 1 h, followed by 1000 V for 0.5 h, and 8000 V for 1 h. The focused strips were equilibrated twice for 30 min in 10 mL equilibration buffer [50 mM Tis HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS] containing 100 mg DTT] with gentle shaking. During the second equilibration, 250 mg iodoacetamide was used instead of DTT.

The second dimensional SDS-PAGE was performed by SDS-PAGE (10% total monomer, with 2.6% crosslinker) using a PROTEAN II Multi Cell (Bio-Rad, Hercules, USA). The focused strips were transferred to the tops of the gels and two slabs were simultaneously run with initial current of 10 mA for 10 min and continued at 25 mA until the tracking dye reached the end of the gel. The protein spots were visualized by staining with silver nitrate. Isoelectric points (pI) were evaluated automatically with Image Master 2D Platinum version 5 software (Amersham Biosciences, Uppsala, Sweden) and the relative molecular weight of each protein spot was determined as described above. All 2-D protein gel analyses were performed at least three times.

Production of Polyclonal Antibodies to 31 kDa Protein

Antibody raised against 31 kDa protein was prepared by subcutaneous injection into each mouse. Before immunization, a small portion of blood was taken for preparation of pre-immune serum. For the first injection, the 31 kDa protein band of salt-stressed leaf sheaths of Leuang Anan, which was stained by silver nitrate, was excised and washed three times with PBS. The gel slices were ground and then lyophilized. The sample powders (0.1 g) were then dissolved in 100 μL PBS buffer, pH 7.4, and emulsified with an equal volume of Freund's complete adjuvant (Bio-Rad) by the ultra-sonicator. After 2 weeks, the mice were boosted again with a homogenized mixture of an equal volume of Freund's incomplete adjuvant and the homogenized

suspension. These procedures were repeated at biweekly intervals until the polyclonal antibody was detected by western blot analysis. The mouse was bled and the immune serum was stored in 50 µl aliquots at -70 °C.

Western Blot Analysis

Twenty µg of protein per sample from leaf sheaths of untreated and salt-treated plants, was separated by SDS-PAGE mini-gel and electrotransferred onto a nitrocellulose membrane using the mini trans-blot (Bio-Rad, Richmond, USA) in transfer buffer [48 mM Tris base, 30 mM glycine, 20% methanol (v/v)] at 100 mA for 1 h. The blot was shaken-incubated for 1 h in a Tris-buffered saline solution [10 mM Tris base and 150 mM NaCl, pH 8.0] containing 0.05% Tween 20 (TTBS) with 5% (w/v) skim milk. Then, the blot was incubated with the mouse antiserum raised against the 31 kDa protein of salt-stressed leaf sheaths of Leuang Anan at a dilution of 1:9 in TTBS for 1 h and washed three times for 5 min each in TTBS. The blot was then probed with the secondary antibody [alkaline phosphatase-goat-anti-mouse IgG (H+L)], and the reactive band was visualized by transferring the membrane to the substrate solution (NBT-BCIP; Bio-Rad). A membrane strip containing marker proteins was stained with 0.1% Amido black and used for MW estimation.

Results and Discussion

Effects of NaCl on the Protein Patterns using SDS-PAGE and 2DE

In an attempt to understand the molecular basis of salt tolerance, proteomics using a combination of SDS-PAGE and 2DE was used to identify proteins involved in salt stress response in the Thai rice cv. Leuang Anan. Detection of proteins whose levels are altered by salt stress was also done by comparing patterns from control and salt-treated plants. Proteins were extracted from 14-day-old rice seedlings, which were treated with 6 dS m^{-1} for 3 days then 12 dS m^{-1} for 10 days of NaCl, and were separated by SDS-PAGE and 2DE. A set of control plants was grown without added NaCl under the same conditions as the salinized plants.

Comparing the protein profiles in control and salt-treated leaf sheaths and roots using SDS-PAGE revealed that the 12 dS m^{-1} NaCl treatment did induce significant changes in the pattern of proteins. It was found that the intensity of the 90 kDa protein band in roots (Fig. 1A) and the 22 and 31 kDa protein bands in leaf sheaths (Fig. 1B) were more abundant in salt-treated plants than in control plants. Furthermore, the enhanced expression of these proteins, which also existed in the control plants, were specifically increased and clearly observed in plants grown under saline condition during different times of the year. These results revealed that these proteins were expressed in specific regions of rice plants adapted to salt stress.

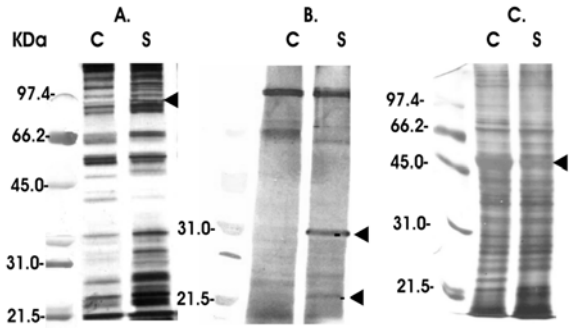

Fig 1. The SDS-PAGE profiles of polypeptides extracted from controlled (C) and salt-treated (S) roots (A), leaf sheaths (B), leaf laminae (C) of rice cv. Leuang Anan.

In contrast, patterns of proteins extracted from leaf laminae of salt-treated plants were not different from those of control plants, except that the intensity of some protein bands markedly decreased in the salt-treated leaves such as the 55 kDa protein band (Fig. 1C). This result was similar to a previous work[21]. Various investigators[22-24] suggested that the protein content decrease is attributed to the decreased rate of protein synthesis, the increased activities of hydrolyzing enzymes, the decreased availability of amino acids, or the denaturation of the enzymes involved in amino acid and protein synthesis. However, the decreased level of proteins content in this study remains to be investigated.

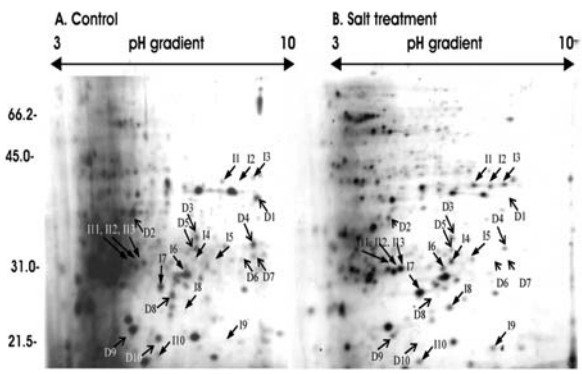

Fig 2. Silver-stained two-dimensional protein patterns of leaf sheaths of Leuang Anan rice seedlings cv. under salt stress (EC 12 dS m^{-1}) (B) and control conditions (A). The first-dimensional separation was performed using Immobiline Dry Strips 3-10 NL, 7 cm run on Ettan IPG phor IEF System. The second-dimensional separation was performed using a 10% polyacrylamide gel on a Bio-Rad mini vertical system. Thick arrows indicate proteins induced by salt treatment, thin ones show proteins decreased by salt treatment.

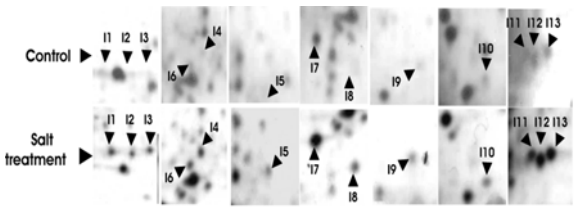

Fig 3. Details of silver-stained 2D gels of salt-induced leaf sheath proteins of rice cv. Leuang Anan under salt stress (EC 12 dS m^{-1}) and control conditions. The labels I1-I13 in the top and bottom panels indicate thirteen proteins whose intensities were increased by salt treatment.

To investigate the components of these salt-stressed proteins in leaf sheaths of rice cv. Leuang Anan and to determine the approximate pI, proteins were separated on 2D-PAGE, stained with silver nitrate and analyzed using Image Master 2D Platinum version 5 software. By comparing 2-D electrophoretic gels of proteins extracted from control and salt-treated plants, it was clear that at least twenty different polypeptides showed a significant change in abundance in response to salt stress. Fig. 2 illustrates that half of these proteins were more expressed and others were depressed, as indicated by the arrows. The thirteen increased proteins are I1 to I13: 42 kDa, pI 7.1, 7.4 and 7.7; 32 kDa, pI 6.6 and 7.0; 31 kDa, pI 6.4; 28 kDa, pI 5.9; 26 kDa, pI 6.5; 22 kDa, pI 7.4; 20 kDa, pI 5.9 and 31 kDa, pI 5.4, 5.5 and 5.6 respectively (Fig. 3). The ten decreased proteins are D1 to D10: 40 kDa, pI 7.8; 38 kDa, pI 5.4; 36 kDa, pI 6.6, 34 kDa, pI 7.7 and 6.6; 33 kDa, pI 7.7 and 7.9; 27 kDa, pI 6.1; 24 kDa, pI 5.5; and 23 kDa, pI 5.8, respectively (Fig. 4).

Interestingly, the 31 kDa increased protein might consist of at least four components: with pI of 5.4, 5.5, 5.6, and 6.4. Spot numbers I11-I13 were more expressed and showed about 3-fold increased expression, while at least one protein of 24 kDa (D9) showed about 11-fold decreased expression (based on the % volume of the spots in treated and control). Additionally, five proteins (D1, D6-D8 and D10) appeared only on the control gel. In a previous report, proteomic analysis of rice suspension culture cells treated with rice blast fungus[25] also detected a 31.1 kDa (pI 4.98) protein, which was identified as putative receptor-like kinase. The same author also found an increase in the amount of 33.8 kDa proteins with pI of 4.73 and 4.79, which was identified as salt induced protein, SalT. However, the proteins of similar molecular weight in this study (31 kDa protein with an pI of 5.4, 5.5, 5.6, and 6.4) were found to be increased with salt stress. Salinity induced 26 kDa proteins were reported in barley roots[7,8], rice shoots and cultured cells[12], and 21 kDa protein was found in cultured tobacco cells[1]. That salinity decreased a 23 kDa protein was also reported in germinating rice seeds,[13] whereas the 22 kDa proteins were found in *Raphanus sativus*[14] and in tomato roots[15], but at different pI.

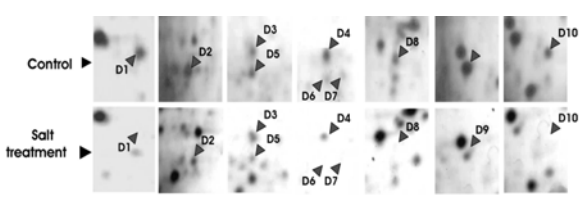

Fig 4. Details of silver-stained 2D gels of salt-decreased leaf sheath proteins of rice cv. Leuang Anan under salt stress (EC 12 dS m^{-1}) and control conditions. The labels D1-D10 in the top and bottom panels indicate ten proteins whose intensities were decreased by salt treatment.

Study of the Pattern of 31 kDa Protein Expression in Leaf Sheath of Different Rice Cultivars

The polyclonal antibody against the 31 kDa protein band of salt-stressed leaf sheaths of Leuang Anan was raised in mice. The antibody was used to probe blots of polyacrylamide gel protein extracts from leaf sheaths of the control and salt-treated plants (12 dS m^{-1}) of three rice cultivars, namely, Pokkali, Leuang Anan, and KDML 105. Western blot analysis showed the specificity of the antibody with the 31 kDa region in rice leaf sheaths of both control and salt-treated leaf sheaths. Furthermore, the results showed that the 31 kDa protein showed different expression patterns in the three rice cultivars. The 31 kDa salt-induced protein was highly enhanced in leaf sheath of salt-treated plants in the tolerant Pokkali and Leuang Anan cultivars, but its level in the sensitive KDML 105 remained unchanged. In addition, the expression levels observed

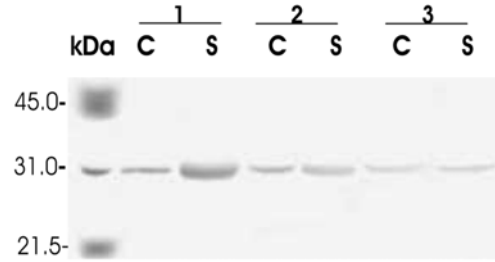

Fig 5. Immunoblot analysis of crude protein extracts from control (C) and salt-treated (S) leaf sheaths of three rice cvs.: Leuang Anan (1), Pokkali (2), and KDML 105 (3). Twenty micrograms protein per sample was separated by SDS-PAGE, transferred to nitrocellulose membranes and probed using antiserum raised against the 31 kDa protein of salt-treated leaf sheaths (dil 1:9).

in salt-treated leaf sheaths of Leuang Anan were higher than in Pokkali (Fig. 5).

Salt-induced proteins in rice leaf sheaths were also observed by several authors[4,10,26]. de Souza Filho et al.[4] demonstrated that SALT protein (14.5 kDa) expression is induced in roots, sheaths and laminae of rice plants exposed to 170 mM NaCl. Claes et al.[10] reported that salinity induced changes in 15 kDa protein synthesis are often more pronounced in both leaf sheaths and in roots of salt-sensitive rice cv. Taichung native 1. They suggested that water was lost more readily from the sheath to replace that lost from leaves or roots, or that salt was transferred from leaves and roots into the sheath to maintain a critical osmotic balance in the two more important tissues. Whereas, Abbasi and Komatsu[26] found that five specific salt-responsive proteins, which were expressed in specific regions of rice, were up-regulated in rice leaf sheath and expressed in other tissues. One (LSY262, an unidentified spot) was expressed in leaf sheath and root, three (44.8 kDa, pI 7.2, fructose biphosphate aldolases; 35.4 kDa, pI 4.8, Photosystem II oxygen evolving complex protein and 27.1 kDa, pI 5.4, oxygen evolving enhancer protein 2) in leaf blade and the other (LSY363, an unidentified spot) in leaf blade and root, but at a low level of detection. They also found that these salt-responsive proteins, which were found in rice leaf sheath, were also enhanced by other stresses such as low temperature and drought.

There are many reports concerning the correlation between salt protein levels and the level of salinity tolerance using the physiological parameters such as photochemical efficiency of photosynthesis and net photosynthesis rate[4,27]. Moreover, Na^+/K^+ ratio and chlorophyll content can be used as an indication of the stress tolerance capacity in rice and used for screening salt-tolerant lines of rice[28,29]. Based on Na^+/K^+ ratio, in our previous studies[28], the sensitive line had less capacity to exclude Na^+ ions than the tolerant lines. KDML 105 was classified as a sensitive line, and Pokkali and Leuang Anan as tolerant lines. Thus, it was possible to establish a positive correlation between level of salinity tolerance and the 31 kDa protein expression level.

In conclusion, the data presented here revealed that salinity induced changes in protein patterns in leaf sheaths and in roots, but not in leaf laminae. Additionally, the 31 kDa protein whose level increased after salt stress could represent a salt-stress induced protein only in leaf sheaths. Moreover, this investigation reported the molecular weights of some salt responsive proteins. It is necessary to further investigate the structural and functional roles of these salt stress-responsive polypeptides to enhance our understanding of the salt responses in rice.

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