Monoclonal Antibody Production and Immunolocalization of a Salinity Stress-Related Protein in Rice (Oryza sativa)

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Abstract: Among various physiological responses to salt stress, the synthesis of a lectin-related protein of 14.5 kDa was observed in rice plants (Oryza sativa L.) under treatment with 170 mmol/L NaCl. In order to better understand the role of the SALT protein in the physiological processes involving salinity, it was immunolocalized in mesopholic cells of leaf sheath and blade of a rice variety IAC-4440 following monoclonal antibodies production by hybridome culture technique. This variety turned out to be an excellent model for that purpose, since it accumulates SALT protein even in absence of salt treatment and it has been classified as moderately sensitive to salinity and a superior grain producer. This feature was relevant for this work since it allowed the use of plants without the deleterious effects caused by salinity. Immunocytochemistry assays revealed that the SALT protein is located in the stroma of chloroplasts under non-stressing condition. Since the chloroplast is the main target affected by salinity and considering that the SALT protein does not present any apparent signal peptide for organelle localization, its lectin like activity seems to play an important role in the establishment of stable complexes, either to other proteins or to oligosaccharides that are translocated to the chloroplast.

Key words: salt stress; SALT protein; immunocytochemistry; chloroplast; rice

Salinity can occur naturally or as a result of inadequate irrigation resources or drainage. Thus, injuries to seed germination and plant growth can be observed (Zeng and Shannon, 2000). The response of rice to salinity varies according to variety or cultivar showing morphological, physiological, and biochemical changes. There are two possible toxic effects of salinity: the osmotic effect and nonspecific toxicity of specific ions on defined cellular systems (Borges et al, 2004; Marcondes et al, 2009). Owing to the need to increase the rice crop area viewing food improvements, the response of rice plants to salinity become increasingly important for their cultivation under salt-stressed conditions.

SALT protein was first described in rice plants in response to salt stress by bi-dimensional electrophoresis profiles (Claes et al, 1990). By the time the salT gene was isolated and characterized, an ABRE domain (ABA-responsive-element) was found in the promoter sequence (Garcia et al, 1998). DNA sequence homology was detected within the genome of other plants as wheat, barley, oil palm, and tomato, suggesting that the salT gene or part of its sequence is conserved both in mono- and dicotyledonous plants (Roy et al, 1993). The expression of SALT protein is wider than previously expected. It is not restricted to environmental stresses but seems to act as a global response/sensor mechanism (Souza-Filho et al, 2003). Furthermore, purified recombinant SALT protein was reported as presenting lectin activities such as mannose- and glucose-ligand (Branco et al, 2004).

Lectins are carbohydrate binding proteins that bind glycans of glycoproteins, glycolipids, or polysaccharides with high affinity. Due of their specific binding, they serve as recognition molecules within cells, between cells, or between organisms. Plant lectins are found in many different species, organs and tissues (Peumans and van Damme, 1995). Molecular, biochemical, cellular, physiological and evolutionary evidences have been indicated that lectins have important biological roles in plant defense (Chrispeels and Raikhel, 1991; Hirano et al, 2000; Zhang et al, 2000). Nothing is known concerning the protective role of SALT protein during the processes of salt and osmotic stress. However, the determination of this protein as a lectin has opened new perspectives on its possible function as response to salinity stress and also in plant abiotic defense mechanisms.

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In this work, we report the production, characterization and selection of monoclonal antibodies against SALT protein followed by its immunolocalization in rice plants.

MATERIALS AND METHODS

Production of monoclonal antibodies

Immunization and hybridization

Recombinant SALT protein expressed by *Escherichia coli* BL21 (DE3) was purified from polyacrilamide gel and intradermally injected to female BALB/c mice in two occasions with an interval of seven days: In a first step in a single dose and in a second step in three consecutive doses. After the last inoculation, serum from immunized mice was tested by Western blotting (Towbin et al, 1979). After a month, splenocytes suspension were obtained by gentle puncture of the spleen cells and mixed to NS-0 mouse myeloma cells in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12, Invitrogen) based on a 4:1 ratio (5.4×10^7 splenocytes to 1.6×10^7 NS-0). Cell fusion was carried out according to the method of Köhler and Milstein (1975) with a slight modification (Goding, 1980; Coligan et al, 1991) using 50% (w/v) polyethylene glycol (MW 4000, Merck) dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen). Hybridome cells were selected by feeding with HAT Media Supplement (Sigma-Aldrich) containing 5 mmol/L hypoxantine, 20 µmol/L aminopterin, and 0.8 mmol/L thymidine followed by distribution on macrophage feeder layer and cultivation at 37°C under 7% CO2. Fifty days after cell fusion, the medium was replaced by HT (Sigma-Aldrich) and by the time for DMEM/F12 supplemented with 10% fetal calf serum and 50 µmol/L β-mercaptoethanol.

Screening for reactivity of hybridome culture supernatants and cloning

At the 10th day after cell fusion, the culture supernatants from 96-well plates were tested for their antibody activity by ELISA (Engvall and Perlmann, 1971). Positive clones were isolated by limiting dilution method using regular DMEM/F12. This procedure was extensively repeated until obtaining single positive hybridome clones in a sole well. The experiment was monitored by visual analysis using an inverted optical microscope (Axiovert 135M, Zeiss).

Production of anti-SALT monoclonal antibodies

Isolated positive hybridomes were expanded using 1 mL of regular DMEM/F12 in 24-well plates and each supernatant was recovered. Culture supernatants obtained were used in these studies. Furthermore, in order to obtain large quantities of antibodies, ascites were acquired from BALB/c mice primed by intraperitoneal injection of 500 µL Pristane (2,6,10,14-tetramethylpentadecane, Sigma-Aldrich). After a week, mice received intraperitoneal injection of at least 1×10^6 hybridome cells. Two weeks after this inoculation, ascitic fluid was recovered from abdominal cavity and specific monoclonal antibodies were purified after centrifugation.

Characterization of selected anti-SALT

ELISA was performed applying the Iso-Gold Rapid Mouse-Monoclonal Isotyping Kit (anti-IgG1, -IgG2a, -IgG3, -IgM, -IgA, -IgLκ and -IgLλ, BioAssay Works) to determine serological class and subclass of monoclonal antibodies and their titration.

Plant material

Seeds of *Oryza sativa* L. cv. IAC-4440 were grown in moist vermiculite containing Yoshida nutritive solution (Yoshida et al, 1976). After 50 days, plants were transferred to fresh solution under hydroponic condition. Four weeks later, a stress condition was established by adding 170 mmol/L NaCl to fresh nutritive solution. Salinity was maintained for 48 h until the plant harvest.

Specificity of anti-SALT

SALT-Mab specificity was detected against vegetable SALT by ELISA, dot blotting using squeezed raw plant material, and Western blotting after SDS-PAGE (Laemmli, 1970) or native-PAGE (Davis, 1964). Total plant proteins were extracted with phosphate buffer-saline (PBS) (pH 7.2) containing 0.1 mmol/L phenylmethanesulfonyl fluoride (PMSF, Sigma). After maceration, crude extract was centrifuged at 12 000×g for 10 min at 4°C. Proteins were quantified by Bicinchoninic acid assay (Smith et
Transfer of proteins to nitrocellulose membranes was carried out as described by Towbin et al. (1979).

Immunolocalization

The superior portion of older leaf and basal portion of leaf sheath were used in the experiments. Sections of each material were individually fixed in 75 mmol/L cacodylate buffer, pH 7.4, containing 0.1% glutaraldehyde and 4% paraformaldehyde for 90 min at room temperature. Samples were washed 3×60 min with cacodylate buffer and were dehydrated with a methanol dilution series (from 50% to 90%) and embedded in LR GOLD resin (Sigma). Ultrafine sections (60 nm) were obtained using diamond knives and were mounted on 400 mesh nickel grid (Sigma) previously treated with 0.5% Formvar (Sigma) in acetone. Samples were immunolabelled by immersion in 50 µL as follows: 1) 50 mmol/L NH₄Cl in PBS+BSA [10 mmol/L phosphate and 150 mmol/L NaCl (pH 7.5) plus 1% bovine serum albumin] for 2 h to block nonspecific protein-binding sites; 2) SALTMAb 1:1000 in PBS+BSA for 2 h; 3) 5×10 min with PBS+BSA; 4) goat anti-mouse IgG-gold (10 nm, Sigma) 1:100 in PBS+BSA for 2 h; 5) 5×10 min with PBS+BSA; and 6) 5×10 min deionized water. After washing, immunolabeling was visualized by staining with lead and uranyl acetate for 20 and 4 min, respectively. The sections were directly examined through their observation under a transmission electron microscope EM900 (Zeiss).

RESULTS

Monoclonal antibodies

In spite of many positive hybridomes had been produced, three of them were selected and completely isolated to be used: SALTMAb14, SALTMAb56 and SALTMAb79 (Table 1). The three antibodies presented the same class, IgG, and the best titers were achieved until 1:10000 for SALTMAb14 and SALTMAb56, or 1:1000 for SALTMAb79.

Throughout the process of testing and isolation of positive hybridomes, the specificity of monoclonal antibodies against denaturated recombinant SALT protein was tested by ELISA. However, it is always necessary to use different assessment methods to test and confirm the specificity of monoclonal antibodies obtained. In this case, confirmation of specificity was obtained using the methods of dot blotting and Western blotting. In addition, the main interest was the application of antibodies for immunolocalization of SALT protein in rice plants. Thus, it was important to determine if those antibodies recognize epitopes of the protein in its native conformation and also if they recognize the plant SALT protein.

Through Western blotting, SALTMAb14, SALTMAb56 and SALTMAb79 had their specificity tested against denatured recombinant SALT protein isolated from the polyacrylamide gel (Fig. 1) and against native recombinant protein extracted from the host E. coli (Fig. 1). The three monoclonal antibodies obtained reacted with the SALT protein under both conditions in spite of the reaction with SALTMAb14 have been weaker than the others. An interesting factor is that under native conditions, the labeled band was detected at the top of the gel which concentration was 4% (Fig. 2). This fact is possibly

<p>| Table 1. Isotyping and titles for monoclonal antibodies produced against SALT protein. |</p>
<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Isotype</th>
<th>Heavy chain</th>
<th>Light chain</th>
<th>Title a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALTMAb14</td>
<td>IgG1</td>
<td>κ</td>
<td>10×</td>
<td>6×</td>
</tr>
<tr>
<td>SALTMAb56</td>
<td>IgG2b</td>
<td>κ</td>
<td>12×</td>
<td>8×</td>
</tr>
<tr>
<td>SALTMAb79</td>
<td>IgG2b</td>
<td>κ</td>
<td>8×</td>
<td>4×</td>
</tr>
</tbody>
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a Intensities (x=0.063) based on optical density at 490 nm. Positive values were considered when OD ≥ 2× the blank value.

Fig. 1. Electrophoretic and Western blotting analysis by native-PAGE using monoclonal anti-SALT.

Denatured SALT form SDS-PAGE (1) and protein extract (20 µg) from E. coli containing pSaltPet induced by IPTG (2) in the presence of SALTMAb14 (A), SALTMAb56 (B), and SALTMAb79 (C). The superior lines indicate the edge between loading (4%) and resolution (12%) gels.
due to formation of protein complexes or clusters that prevented their penetration through the mesh formed by the polyacrylamide gel.

From the previous tests, SALTMAb56 was chosen for subsequent tests due to its good titration (Table 1), its good labeling by Western blotting, and the highest amount of ascitic fluid containing this antibody. The specificity for vegetable SALT protein was tested under native conditions through dot blotting and Western blotting. In these trials, it was compared protein extracts from recombinant *E. coli* containing only the pET3d vector induced with IPTG, *E. coli* transformed with the construct pSaltPet (Claes et al, 1990; Roy et al, 1993; Garcia et al, 1998; Souza-Filho et al, 2003; Branco et al, 2004) induced with IPTG, and from rice plants under stress condition with 170 mmol/L NaCl. Through these tests we observed that the plant SALT protein could be detected in its native conformations (Fig. 2). Again, plant native SALT protein was detected at the top of the gel (Fig. 2). These results assure us that SALTMAb56 antibody was a good choice for use in immunocytochemistry assays.

**Immunocytochemistry.**

In sections of the leaf sheath (Fig. 3-A, B, C and F) and leaf blade (Fig. 3-D), one can observe the distribution of gold particles in the stroma of

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**Fig. 2. Electrophoretic and Western blotting analysis by native-PAGE using SALTMAb56.**

Denatured SALT form SDS-PAGE (1) and protein extracts (20 µg) from *E. coli* containing pET3d (2) or pSaltPet (3), induced by IPTG, and from rice plants in the absence (4) or presence (5) of 170 mmol/L NaCl. The arrow indicates the band in the well of loading gel (4%).

**Fig. 3. Subcellular localization of SALT protein in mesophilic cells of leaf sheath and blade in rice cv. IAC-4440, showing their distribution into chloroplasts (arrows).**

Electron micrographs of transversal section of leaf sheath (A, B, C and F) and leaf blade (D) labeled with SALTMAb56 (1:1000). Bars equivalent to 0.17 µm (A, E and F) or to 0.33 µm (B, C and D).
Comparison of the electron micrographs allow the observation that the arrangement of particles was more intense in leaf sheath chloroplasts than in leaf blade chloroplasts of mesopholic cells. Changes in the localization of the SALT protein were not observed. No staining was found in mitochondria, endoplasmic reticulum (Fig. 3-E), cell wall, and apoplast (Fig. 3-F).

**DISCUSSION**

The production of monoclonal antibodies aimed at restricting the specificity to SALT protein to avoid nonspecific reactions in the experiments of immunolocalization in rice plants. The complete isolation of three clones of hybridomas, SALTMAb14, SALTMAb56 and SALTMAb79 was successfully obtained by the limiting dilution technique. Based on the results of isotyping, one can say that at least two distinct species of monoclonal antibodies were isolated. We cannot guarantee that SALTMAb56 and SALTMAb79 are different species, since both have the same class and subclass for the heavy chain isotype, and the same light chain isotype.

The titers obtained for SALTMAb14 and SALTMAb56 were similar and higher than that obtained for the SALTMAb79. However, the immunodetection of the SALT protein through Western blotting obtained by the three antibodies showed that SALTMAb14 made a mark less intense than the others. Considering that the same concentrations of protein were applied and that the same titers of antibodies were used, possibly SALTMAb14 has a lower specificity than other antibodies or their efficiency may be lower when used in that technique, in contrast to the results obtained by ELISA.

Although monoclonal antibodies have been produced against the denatured recombinant SALT protein, they were able to recognize the SALT protein present in extracts of rice plant. The epitope necessary for recognition and binding of each antibody was preserved in the SALT protein under native and denaturing conditions. This information was vital and ensured the efficiency of monoclonal antibodies in immunocytochemistry assays, although only SALTMAb56 has been used.

The recognition of a band through Western blotting in the region corresponding to the top of the loading gel (4%) indicated that the SALT protein did not penetrate the polyacrylamide mesh under native conditions. At first, one might imagine that the SALT protein was precipitated and became insoluble into the well during the gel loading, becoming immobile. It is known that the solubility of a protein is a function of pH in which it is based on its isoelectric point (pI). The pI of a protein corresponds to the characteristic pH in which the positive charge balances the negative charge. The neutral molecule remains motionless when an electric field is present. However, the SALT protein has pI 5.5 and thus its electrostatic property would not be neutral in pH 6.8 according to the sample buffer that had been applied. The only explanation for the fact that this protein does not penetrate the polyacrylamide mesh is the possible formation of stable complexes or clusters. Bernabé (1999) showed that the SALT protein is a lectin binding to mannose and glucose, thus able to form dimers both in E. coli and in rice plants. The formation of SALT-dimers with higher lectin activity supports the hypothesis of cluster formation by the SALT protein in native protein extracts, which can be destroyed when the sample is denatured by boiling and under the action of chemical agents.

A low level of SALT expression in control plants (non-stressed) could be detected in Taichung Native I cultivar by Northern blotting (Claes et al, 1991; Garcia et al, 1997) and by in situ hybridization (Garcia et al, 1998). In our analysis, SALT could be detected also in control plants of IAC-4440 Brazilian cultivar by Western blotting (Fig. 2). At first, this result was disturbing since the conditions were carefully controlled to avoid stress on the plants, except the salt stress when desired. However, Castro (2000) had detected the expression of SALT RNA under the same conditions of control by Northern blotting and, together, our results strongly indicate that the salT shows high constitutive expression in the IAC-4440 cultivar, regardless the stress-induced activation. This feature of the IAC-4440 cultivar has been rather relevant for this work since it allowed the use of this cultivar under control conditions during the immunocytochemistry assays, without deleterious
effects on cellular organization due to salt stress (Marcondes et al, 2009).

The immunocytochemistry experiments resulted in surprising information once that SALT protein was finally found in the stroma of chloroplasts. This protein is encoded by nuclear genome, synthesized in the cytosol and showed no sign to target it to organelles (Hiratsuka et al, 1989).

The SALT protein cannot be imported into the chloroplast itself as it shows no known signal of targeting required. However, besides its size (14.5 kDa), SALT provides a valuable property that is its affinity for mannose and glucose (Danpure, 1995; Chabregas et al, 2001). As already discussed, this feature would allow the formation of stable complexes with sugars and/or with other proteins. The stable association with sugars or lipids could facilitate their entry into the stroma through permeases present in the inner membrane, after their passage through the non-selective pores present in the outer membrane (Silva-Filho et al, 1996; Bruce, 1998). However, this does not seem very plausible and on the other hand an alternative hypothesis consists in the association of SALT with proteins that actually follow the path to target the stroma, based on their leader sequence. A third possibility would be the uptake into stroma facilitated by chaperonins specifically targeted to chloroplasts (Neupert, 1997).

The hypothesis discussed above are only speculative, but whatever the form of interaction, these associations must be quite specific, since the SALT protein seems to be unique to chloroplasts. The determination of this specificity may be inherent to SALT tertiary structure, perhaps due to the internal aminoacid repeats dispersed through its sequence (Claes et al, 1990) and finally improved by the specific lectin interaction that it presents (Lei and Wu, 1991).

Furthermore, the homology observed between salt and gos9 (Pater and Schilperoort, 1992) in rice and among genes present in different species of mono- and dicotyledonous (Roy et al, 1993) may be indicative of the existence of a homologous multigene family which salt belongs. By accepting this fact, it may be that in IAC-4440 there is a copy of the salt gene encoding a SALT protein quite different, containing the signal peptide that was not found in SALT belonging to Taichung Native I cultivar. Although different, this new protein would present a high degree of homology with SALT or simply save the sequence that constitutes the epitope required for recognition by SALTMAb56. Moreover, the gene that encodes a protein containing the signal peptide could be expressed constitutively, while the other copy would have its expression induced by stress. Thus, the physiological point is that the plant would be prepared to confer constitutively protection to chloroplasts when they are subjected to salt stress, while an additional protection would be triggered after an induced stress.

By analyzing the cellular ultrastructure of stressed rice plants, it was observed that the chloroplast is the principal organelle affected by salinity (Marcondes and Garcia, 2009). The most important change observed in the chloroplasts is the disorganization of thylakoid membranes. Thus, at least in terms of salinity, it is possible to speculate that the primary protective role of SALT would be fulfilled on the chloroplast which in turn is the primary target affected by salinity. Finally, we conclude that there is a direct relationship among the previously described accumulation of sodium (Claes et al, 1991), the cytomorphological changes in chloroplasts of mature tissues of rice plants induced by salinity (Marcondes and Garcia, 2009), and the current disclosure about the accumulation of SALT protein also in chloroplasts.

REFERENCES


Castro V R M. 2000. Study of accumulation of SALT protein in roots of rice plants (Oryza sativa L.) in response to different
Jackson MARCONDES, et al. Monoclonal Antibody Production and Immunolocalization of a Salinity Stress-Related Protein


