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## High salinity reduces the content of a highly abundant 23-kDa protein of the mangrove *Bruguiera parviflora*

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**Abstract** A significant decrease in the amount of a protein, whose migration in two-dimensional gel electrophoresis corresponds to an apparent molecular mass of 23 kDa and  $pI=6.5$ , was observed in leaves of NaCl-treated *Bruguiera parviflora* (Roxb.) Wt. & Arn. ex Griff. seedlings. This particular salt-sensitive protein, designated as SSP-23, almost disappeared after 45 days of treatment in 400 mM NaCl as compared to untreated seedlings (0 mM NaCl) where the presence of the protein was significant. A polyclonal antibody raised against the 23-kDa protein was used to determine the subcellular localization of this protein in leaves by cross-reaction with proteins from isolated chloroplasts, mitochondria, peroxisomes and cytosol fractions on Western blots. SSP-23 was confirmed to be localized in the cytosol by immunoblotting. The disappearance of SSP-23 as a result of high NaCl treatment suggests that this protein is salt-sensitive and has a possible role in salt adaptation.

**Keywords** *Bruguiera* · Mangrove · Osmotic adjustment · Protein (salt-sensitive) · Salt stress

**Abbreviations** 2-D: Two dimensional · PAGE: Polyacrylamide gel electrophoresis · SDS: Sodium dodecyl sulfate · SSP: Salt-sensitive protein

### Introduction

Salt stress negatively impacts agricultural yield throughout the world, affecting production whether it is for subsistence or economic gain (Flowers 2004). To cope with salt stress, plants respond with physiological and biochemical changes designed to retain water in spite of the high external osmoticum and to maintain photosynthetic activity even when stomatal opening is reduced to counter water loss.

Mangroves are an assortment of woody trees and shrubs, which flourish in an inhospitable zone between land and sea along the tropical coastline of the globe. True mangroves occur only in the mangrove habitat and their existence is rare elsewhere. Mangroves are taxonomically diverse; true mangroves include about 54 species in 20 genera belonging to 16 families (Das et al. 2002). The most striking feature of mangroves is their ability to tolerate NaCl to sea water level (approx. 500 mM). Mangroves are divided into two distinct groups on the basis of their salt-management strategies, namely, “secretors”, having salt glands or salt hairs for excretion of excess salt, and “non-secretors”, lacking such morphological features. *Bruguiera parviflora* is included in the latter group, which grows in a range of salinities from brackish water up to two times concentrated sea water. Due to high salt concentrations in the growth medium, mangroves are confronted with the problem of maintaining turgor pressure and protecting their metabolism from high inorganic ion concentrations (Flowers et al. 1977). This is accomplished by the accumulation of inorganic ions within the vacuole and/or by the synthesis of inorganic compounds (Hasegawa et al. 2000). In *B. parviflora*, it is suggested that sugar,

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proline and polyphenol act as compatible solutes to regulate water potential (Parida et al. 2002). In addition to the synthesis of these osmolytic compounds, specific proteins and translatable mRNA induced and increased by salt stress have been reported (Hurkman et al. 1989; Bray 1993; Xu et al. 1996; Swire-Clark and Marcotte 1999; Xu et al. 2001). It has also been reported that the total soluble protein content of leaves decreases in *B. parviflora* as a result of NaCl treatment, but without any noticeable changes in untreated controls (Parida et al. 2002). This decrease might have resulted from an adverse effect of NaCl on the synthesis of certain low-molecular-weight proteins. However, the exact nature of these low-molecular-weight proteins, especially in response to NaCl stress, has not been fully elucidated. In this communication, we use Western blotting of protein fractions from isolated chloroplasts, mitochondria and vacuoles to show the existence of a cytosolic 23-kDa protein that declines in response to NaCl treatment in the mangrove *B. parviflora*.

## Materials and methods

### Plant materials and culture conditions

Propagules of *Bruguiera parviflora* (Roxb.) Wt. & Arn. ex Griff. were collected from the Bhitarkanika mangrove forest of Orissa, India (latitude 20°4' to 20°8' N and longitude 86°45' to 87°50' E). Seedlings were raised, in a greenhouse, in non-saline and non-brackish water under photosynthetically active radiation (PAR) of 1,220–1,236  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Two-month-old healthy seedlings were selected for hydroponic culture in Hoagland's nutrient medium, pH 6.0 (Hoagland and Arnon 1940), salinized with four levels of NaCl (0, 100, 200, 400 mM). The cultures were aerated continuously with an air-bubbler. The hydroponic cultures were maintained at  $22 \pm 2^\circ\text{C}$  and 80% relative humidity in a culture room under a 14-h photoperiod (300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The culture medium was replaced with fresh medium weekly. The fourth pair of leaves from the top of the shoot was harvested after 7, 14, 30 and 45 days from control (without NaCl) and treated seedlings for extraction and estimation of protein. Two independent experiments were carried out for each extraction, estimation and analysis of NaCl-induced alterations in protein profile.

### Extraction and estimation of protein

Leaf samples were harvested in parallel from control and NaCl-treated plants after 7, 14, 30 and 45 days for the analysis of soluble proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Fourth pairs of leaves were harvested from three plants per treatment to prepare three replicate samples (0.5 g)

from each treatment and time point. Samples (0.5 g) were homogenized with 2 ml of a buffer containing 50 mM Tris–glycine (pH 8.3), 0.5 M sucrose, 50 mM EDTA, 0.1 M KCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) 2-mercaptoethanol in a chilled pestle and mortar at 4°C. The homogenate was centrifuged in a refrigerated centrifuge (Sigma 2K15, Germany) at 14,000 g for 10 min. Protein concentration in the supernatant samples was estimated according to the method of Bradford (1976). The supernatants were stored in small aliquots at  $-85^\circ\text{C}$  for SDS–PAGE.

### Analysis of leaf protein profiles by SDS–PAGE

Protein samples were resolved in 12.5% SDS–PAGE gels following the procedure of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250 (Sigma). The gels were photographed and scanned using a densitometer (GS-710; Bio-Rad) and analyzed with Quantity One software from Bio-Rad. The apparent molecular weights of the protein bands were estimated using molecular weight markers from Genei, Bangalore, India.

### Two-dimensional (2-D) PAGE, isolation and purification of the salt-sensitive protein, SSP-23

Total proteins from leaves of control plants were separated by 2-D PAGE following the procedure of O'Farrell (1975) with slight modifications. For 2-D analysis, total proteins were extracted by the phenol-partitioning method (Hurkman and Tanaka 1986) and precipitated at  $-20^\circ\text{C}$  with methanol containing 0.1 M ammonium acetate. Proteins were solubilized in 9.5 M urea, 2% Nonidet P-40 (w/v), 2% (w/v) ampholytes (1:1 mixture of pH 3.5–10 and pH 5–8 solutions) and 5% (v/v)  $\beta$ -mercaptoethanol. The first-dimension isoelectric focusing (IEF) was done on cylindrical gels (3 mm in diameter, 11 cm long) containing 4% acrylamide, 2% Nonidet P-40 (w/v), 2% ampholytes (mixture as above) and 9 M urea. For the first dimension, 1 mg protein was loaded per gel. Electrode solutions were 0.4% (w/v) NaOH at the cathode and 0.06% (w/v) phosphoric acid at the anode. Isoelectric focusing was done for 16 h at 1,000 V. Gels were then equilibrated in 50 mM Tris–HCl buffer (pH 6.8) containing 2% (w/v) SDS, 10% glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, and 0.01% (w/v) bromophenol blue. The second dimension was carried out in 12.5% SDS–polyacrylamide gels. 2-D PAGE gels were stained with Coomassie Brilliant Blue R-250, and the spots containing the protein to be purified were excised from the gels with a minimum of polyacrylamide. Spots from 15 gels were collected and de-stained by incubation in 50% methanol (v/v). Then, gel fragments were homogenized, equilibrated for 30 min in Laemmli buffer (Laemmli 1970), incubated for 5 min at  $100^\circ\text{C}$ , and loaded onto 12.5% SDS–PAGE gels. After

electrophoresis, the purified protein was electroblotted onto nitrocellulose membranes using a Mini Transblotter (Bio-Rad) following the procedure of Towbin et al. (1979). Blots were stained with 0.1% Ponceau S in 1% acetic acid followed by de-staining with 1% acetic acid.

#### Preparation of anti-SSP-23 antiserum

Pieces of nitrocellulose carrying the protein of interest, obtained after 2-D PAGE and blotting were dissolved in dimethyl sulfoxide (DMSO), suspended with complete adjuvant (1:1 ratio) and sonicated. The solution was used to raise anti-SSP-23 antibodies in rabbit according to the procedure described by Walzel et al. (1991). The first boost was given 4 weeks after the first injection; the second was given after an additional 3 weeks. A high titer against the protein, which could be used for immunodetection, was obtained by enzyme-linked immunosorbent assay (ELISA) following the method of Haines and Patel (1997). ELISA gave the titer value of SSP-23 antiserum against SSP-23 protein to be 1:200.

#### Western blotting of SSP-23

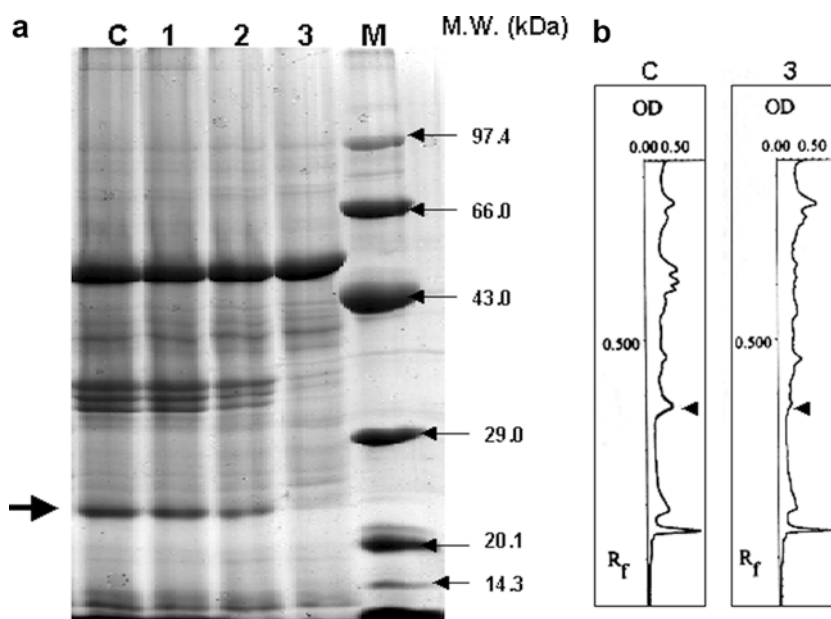
Purified protein samples (control and NaCl-stressed plants) were electroblotted onto nitrocellulose membranes, using 20 mM Tris-HCl (pH 8.3) containing 150 mM glycine and 20% methanol as the electrode buffer in a Mini Transblotter (Bio-Rad) following the procedure of Towbin et al. (1979). Blots were incubated with anti-SSP-23 antiserum (1:200 dilution) and immunodetection was done according to Zemel and Gepstein (1985), using peroxidase-linked goat anti-rabbit antibodies and 3-amino-9-ethylcarbazole as substrate.

#### Isolation of subcellular fractions and localization of SSP-23 by immunoblotting

About 30 g of leaves was homogenized in buffer consisting of 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 50 mM HEPES-KOH (pH 7.8), and 0.2% BSA (buffer 1) using a tissue homogenizer (Ultra Turax, Ika, Germany). The homogenate was filtered through four layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 1,200 g for 3 min. The supernatant fraction was used for isolation of cytosol, mitochondria and peroxisomes following the procedure of Moore and Proudlove (1983; expected purity of the different fractions 90–95%), whereas the pellet fraction was used for isolation of intact chloroplasts following the modified method of Robinson (1982; reported integrity of chloroplasts 96–99%). The pellet was resuspended in 18 ml of buffer 1, loaded over 14 ml of the same medium (buffer 1) containing 40% Percoll, and then the tubes were centrifuged at 200 g for 3 min. The supernatant was discarded and the pellet of intact chloroplasts was resuspended in buffer 1.

The supernatant fraction obtained in the first step was centrifuged for 20 min at 12,500 g. The obtained supernatant was the cytosol fraction. The pellet was gently resuspended in a medium containing 0.3 M mannitol, 0.2% BSA, 1 mM EDTA, and 20 mM Mops (pH 7.2), and then purified in a continuous gradient formed by 24% (v/v) Percoll in 0.25 M sucrose, 0.2% BSA and 20 mM Mops (pH 7.2). The gradient was centrifuged at 40,000 g for 30 min in fixed-angle rotor. Afterwards, the mitochondrial and peroxisomal fractions were carefully collected and washed from Percoll in a 20-fold volume of buffer (0.3 M sucrose, 20 mM Mops, pH 7.2). After 30 min centrifugation at 8,000 g, purified mitochondria and peroxisomes were

**Fig. 1a,b** Effects of varying levels of NaCl on the protein profile of leaves of *Bruguiera parviflora* after 45 days of treatment. **a** Lanes: C, 1, 2, 3, control, 100, 200 and 400 mM NaCl-treated leaves, respectively; M molecular weight markers. The proteins were analyzed by 12.5% SDS-PAGE; 40 µg of protein was loaded per lane and stained with Coomassie Brilliant Blue R-250 after electrophoresis. The arrow on the left indicates SSP-23. **b** Densitometric scanning of lanes C and 3 in **a** shows changes in optical density of protein bands in control and 400 mM NaCl-treated samples after 45 days of treatment. The arrowheads indicate the SSP-23 peak



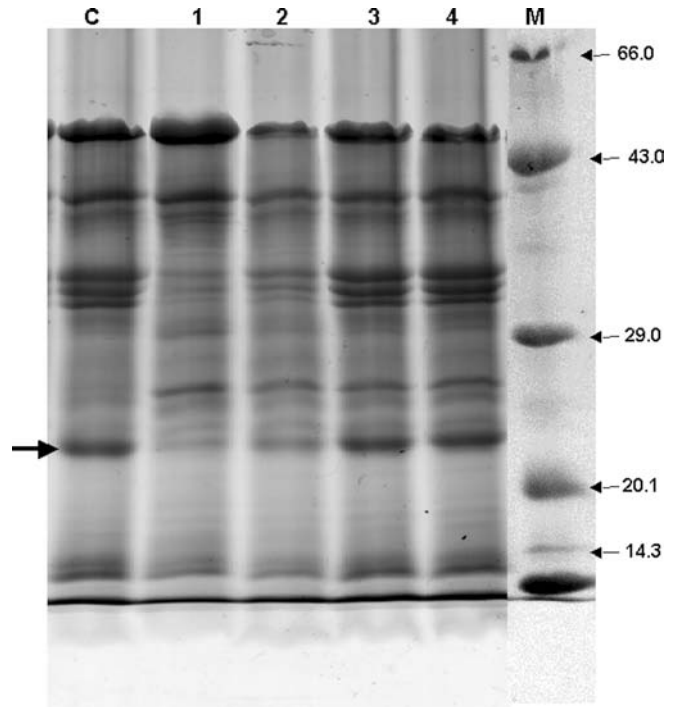
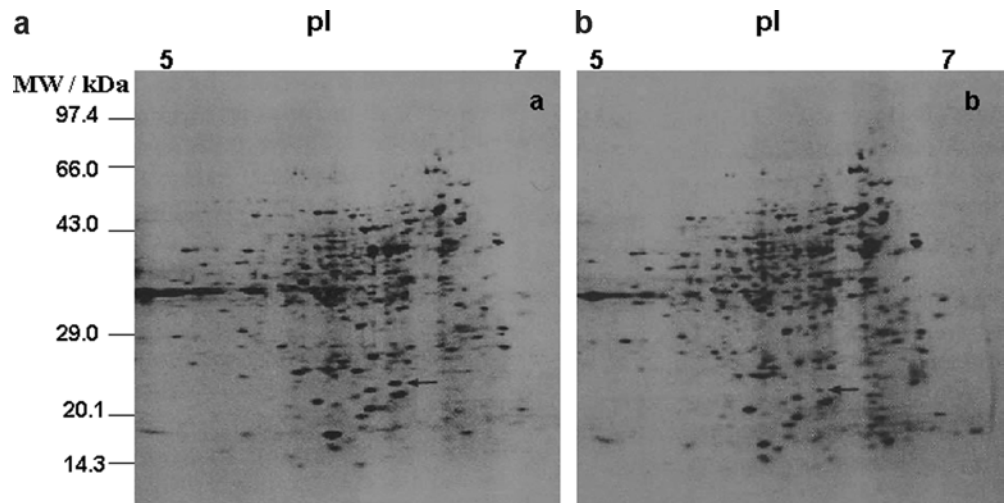
resuspended in the same buffer. Protein was determined according to the method of Bradford (1976).

The chloroplastic, mitochondrial, peroxisomal and cytosolic fractions were solubilized with 2× sample buffer (Laemmli 1970), and SDS-PAGE and immunoblotting were performed as described above.

## Results

Total proteins were extracted from leaves of control and NaCl-treated plants after 7, 14, 30 and 45 days of treatment and analyzed by SDS-PAGE. The control protein profiles remained unchanged in 7-, 14-, 30- and 45-day samples, whereas the intensities of several protein bands of molecular weights 17, 23, 32, 33 and 34 kDa decreased as a result of NaCl treatment (Fig. 1a). The degree of decrease of these protein bands seemed to be roughly proportional to the external NaCl concentration (Fig. 1a). The most prominent decrease was observed in the case of a 23-kDa polypeptide, which almost disappeared after 45 days in the 400 mM NaCl treatment (Fig. 1). Densitometric scanning of the gel showed that the SSP-23 band had a sharp peak in control samples whereas the same band became flattened in 400 mM NaCl-treated samples (Fig. 1b). Essentially the same profile was observed by Western blotting, i.e., a gradual decrease in the expression of SSP-23 (Fig. 3), emphasizing the fact that SSP-23 is a salt-sensitive protein. Two-dimensional gel electrophoresis revealed that the 23-kDa polypeptide had a pI of 6.5 (Fig. 2). The appearance and disappearance of these polypeptides could be reversed during salinization and desalination in *B. parviflora*. When plants treated with 400 mM NaCl for 45 days were transferred to 0 mM NaCl and kept at this concentration for 45 days, the 23-kDa polypeptide, which had disappeared due to NaCl treatment, reappeared (Fig. 3). The degree of reappearance of this polypeptide was time-dependent (Fig. 3).

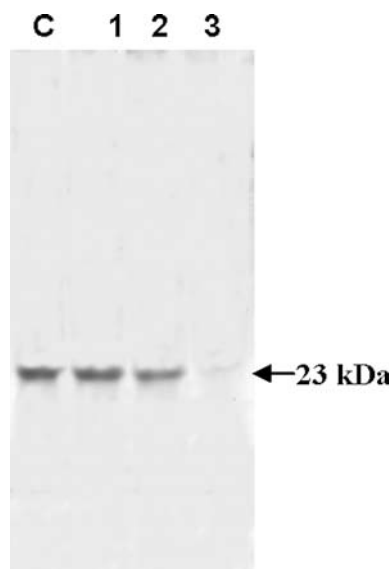
**Fig. 2a,b** Two-dimensional IEF/SDS-PAGE patterns of proteins extracted from leaves of control (a) and 400 mM NaCl-treated (b) *B. parviflora* plants. For the first dimension, 80 µg of protein of both control and NaCl-treated samples was loaded at the upper basic end of the focusing gel. Arrows indicate SSP-23. The locations of the molecular standards are indicated on the left. The isoelectric points are indicated at the top of the figure



**Fig. 3** Changes in the protein profile in leaves of *B. parviflora* after 7, 14 and 30 days recovery from salt stress. Lanes: C and 1, proteins extracted from control and 400 mM NaCl-treated (45 days) leaves; 2, 3 and 4, proteins extracted from leaves after 7, 14 and 30 days of desalination, respectively; M, molecular weight markers. The proteins were analyzed by 12.5% SDS-PAGE; 40 µg of protein was loaded per lane and stained with Coomassie Brilliant Blue R-250 after electrophoresis. The arrow on the left indicates the 23-kDa polypeptide, which increases after desalination

### Specificity of the anti-SSP-23 antibody

SSP-23 was purified by 2-D PAGE and cutting out the corresponding single spots. Polyclonal antibodies were raised against the purified SSP-23 protein. As seen in Fig. 4, Western blot analysis showed that SSP-23 cross-reacted with the anti-SSP-23 antibodies. However, the possible existence of closely related homologues or iso-

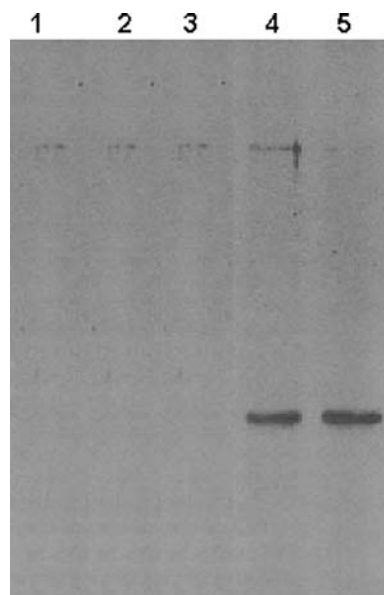


**Fig. 4** Western blots of proteins extracted from leaves of *B. parviflora* plants that were treated with 0 mM (lane C), 100 mM (lane 1), 200 mM (lane 2) and 400 mM NaCl (lane 3) for 45 days. Anti-SSP-23 antibodies were used for immunodetection

forms to which the antibody may bind cannot be ruled out.

#### Subcellular localization of SSP-23 in leaves

Protein fractions from isolated chloroplasts, mitochondria, peroxisomes and cytosol were analyzed by Western



**Fig. 5** Western blots of protein fractions from isolated chloroplasts (lane 1), mitochondria (lane 2), peroxisomes (lane 3), and cytosol (lane 4) of control leaves of *B. parviflora*. Lane 5, Western blot of purified SSP-23. Anti-SSP-23 antibodies were used for immunodetection

blotting using anti-SSP-23 antibody. Western blot analysis revealed that only cytosolic fractions cross-reacted with the anti-SSP-23 antibody (Fig. 5).

#### Discussion

The total leaf protein content of *B. parviflora* gradually decreased with increasing concentration of NaCl, which might be due to the increased activity of acid and alkaline proteases, as evidenced earlier (Parida et al. 2004). These results suggest that the alteration in total leaf protein level must be associated with salt adaptation. We noticed a decrease in the intensities of 17-, 23-, 32-, 33- and 34-kDa proteins when *B. parviflora* was subjected to high salt treatment. The decrease in the intensity of the 23-kDa polypeptide (SSP-23) was most prominent: it almost disappeared after 45 days of treatment in 400 mM NaCl. Further, SSP-23 reappeared when the treated seedlings were desalinized for 30 days in non-saline Hoagland solution (0 mM NaCl). Our results tend to agree with those of Unni and Rao (2000) who reported that certain chloroplast outer-membrane proteins of molecular weights 22, 38, 40, 42, 62 and 68 kDa markedly decrease in the presence of salt in *Rhizobium*. However, in *B. gymnorhiza*, the intensity of a 33-kDa protein with pI 5.2 increased as a result of NaCl treatment (Sugihara et al. 2000). SDS-PAGE analysis of peanut (*Arachis hypogaea* L.) proteins revealed that plants grown under NaCl show induction (127 and 52 kDa) or repression (260 and 38 kDa) of the synthesis of few polypeptides (Hassanein 1999). Our results contrast with earlier reports of increased accumulation of a 22-kDa protein (pI 7.5) in salt-stressed radish leaves (Lopez et al. 1994). Further, Tamas et al. (2001) showed that NaCl induced the accumulation of four polypeptides of 61, 51, 39 and 29 kDa in maize roots. By immunodetection of chloroplastic, mitochondrial, peroxisomal and cytosolic protein fractions we confirmed that SSP-23 is localized in the cytosol (Fig. 5). We reported earlier that proteins are hydrolyzed by proteases to release amino acids for osmotic adjustment during NaCl stress in *B. parviflora* (Parida et al. 2002, 2004). Moreover, the proposed functions of free amino acid accumulation include osmotic adjustment, protection of cellular macromolecules, nitrogen storage, maintaining cellular pH, detoxification of cells, and scavenging of free radicals. Our observations suggest the possible involvement of SSP-23 in osmotic adjustment in the cytosol of *B. parviflora* under saline conditions. N-terminal amino acid sequencing of the SSP-23 and sequence alignment with proteins of known functions will provide valuable information on the nature of the protein and its role in salt-adapted plant species.

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