

Characterization of a Salt-Responsive 24-Kilodalton Glycoprotein in *Mesembryanthemum crystallinum*¹

H. Emilie Yen, Gerald E. Edwards, and Howard D. Grimes*

Department of Botany, Washington State University, Pullman, Washington 99164-4238

A concanavalin A (Con A)-binding polypeptide with a molecular mass of 24 kD (termed "SRgp24") was associated with the intercellular space of *Mesembryanthemum crystallinum* L. callus. When callus was grown in medium containing between 0 and 100 mM NaCl, SRgp24 was detected by Con A binding. Increasing the NaCl concentration to 200 mM caused a reduction in the amount of SRgp24 within 3 d, and returning the callus to medium without salt resulted in an accumulation of SRgp24. Immunoblot analysis showed that appreciable amounts of SRgp24 accumulated in the leaves when plants were grown under sodium-limiting conditions. Unlike most of the cell-wall Con A-binding proteins in *M. crystallinum* callus, the carbohydrate moiety of SRgp24 was resistant to endoglycosidase H digestion. After purification of SRgp24, the N terminus was sequenced and found to share 55 to 60% identity with the N terminus of osmotin, a group 5 pathogenesis-related protein (PR-5) that accumulates in salt-adapted tobacco cell suspension. Immunocytochemical assays, with affinity-purified antibodies to SRgp24, indicated that SRgp24 preferentially accumulated in the cell-wall region. We conclude that SRgp24 is a salt-responsive glycoprotein related to the PR-5 family in *M. crystallinum*.

CAM plants are well-adapted to arid habitats because of their ability to conserve water and to sustain CO₂ fixation through periods of drought stress. *Mesembryanthemum crystallinum* L., the common ice plant, is a halophyte with the capacity to induce CAM when water stressed (Winter, 1985; Bohnert et al., 1988). It is able to survive both drought and salt stress through unique stress-tolerance mechanisms. ABA also appears to have a role in regulating CAM induction, although its mechanism is not yet understood (Chu et al., 1990; McElwain et al., 1992).

When *M. crystallinum* is exposed to high external salt, Pro (Heun et al., 1981) and pinitol (Paul and Cockburn, 1989) accumulate in the cytoplasm where they function as compatible solutes. Apparently this allows a physiological range of Na ions to be maintained in the cytosol while compartmentalizing excess NaCl in the vacuole or intercellular space. Preferential exclusion of Na⁺ from the cytosol requires transport across the plasma membrane or tonoplast. Because *M. crystallinum* adapts to drought stress and is a halophyte, it serves as a model system to study the mechanisms of plant

adaptation to salt and water stress (Cushman et al., 1990). A major limitation of this system, however, is that both CAM and osmotic adjustment are induced concurrently after exposure of plants to high salt concentrations. The nature of these different adaptive mechanisms could be probed more efficiently if only one set of adaptive mechanisms were induced.

A cell culture initiated from hypocotyls of *M. crystallinum* provides an amendatory approach to this problem. We found that Pro levels increased more than 13-fold when light-grown callus was transferred to 200 mM NaCl, whereas the activity of phosphoenolpyruvate carboxylase, the key enzyme for performing CAM, remained relatively constant (Yen, 1993). Because of their ability to survive in high concentrations of NaCl without inducing CAM, these cultured cells were used to study the halotolerance mechanism of this plant at the cellular level.

The synthesis of several proteins located in either the cytosol (Hurkman and Tanaka, 1987; Zhao et al., 1989; Reviron et al., 1992), membrane fractions (Singh et al., 1987a; Hurkman et al., 1988), chloroplasts (Winicov and Button, 1991), or intercellular space (Bozarth et al., 1987; Iraki et al., 1989; Esaka et al., 1992) was found to be either up- or down-regulated by osmotic stress. In most cases, however, the function of these polypeptides is presently unknown. Among these polypeptides, a 24-kD protein termed "osmotin" accumulated in vacuolar inclusion bodies of salt-adapted cell cultures of tobacco, a glycophyte (Singh et al., 1987a). Osmotin is classified as a basic form of PR-5 protein (Cornelissen et al., 1986). In the present investigation a salt-responsive, Con A-binding protein with a molecular mass of 24 kD was found in callus cultures of *M. crystallinum*. This protein is homologous to PR-5 proteins at the N terminus. The experiments presented here offer a partial characterization of this 24-kD glycoprotein that we have termed "SRgp24," since it appears to be a salt-responsive 24-kD glycoprotein.

MATERIALS AND METHODS

Cell Culture and Plant Growth Conditions

Light-grown calli of *Mesembryanthemum crystallinum* were initiated and maintained in 1% agar slants containing a culture medium described by Treichel (1986). Calli were

¹ This research was partially supported by the U.S. Department of Agriculture-National Research Initiative Competitive Grants Program grant 91-37305-6707.

* Corresponding author; fax 1-509-335-3517.

Abbreviations: Endo H, endoglycosidase H; PR-5 protein, group 5 pathogenesis-related protein; PSB, protein solubilization buffer; PVDF, polyvinylidene difluoride; SRgp24, salt-responsive glycoprotein of 24 kD.

grown with a 16-h light (110–130 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$)/8-h dark period at temperatures of 27/18°C and were subcultured every 3 weeks. ABA and cycloheximide were filter sterilized before addition to autoclaved medium.

Plants of *M. crystallinum* were grown hydroponically as described previously (Chu et al., 1990) with slight modifications. Initial observations using *M. crystallinum* plants indicated that hydroponically grown seedlings failed to grow when NaCl was omitted from the nutrient solution; therefore, seedlings were grown with successively increasing strengths of Johnson's nutrient solution (0.125 \times at 1 week, 0.25 \times at 2 weeks, 0.5 \times at 3 weeks, and 1 \times at 4 weeks) containing 20 mM NaCl. For Na-limiting conditions, plants grown for 4 weeks in 20 mM NaCl were transferred to 1 \times nutrient solution without NaCl. Plants were maintained in this Na⁺-deficient nutrient solution for another 2 weeks before being harvested. For Na-excess conditions, 200 mM NaCl was added to 5-week-old plants growing in the 1 \times nutrient solution, and plant tissues were harvested 1 week later.

Isolation of Membrane Fractions

Three-week-old callus was ground in a chilled mortar and pestle with an equal volume (g fresh wt/mL) of grind buffer, which consisted of 8% Suc, 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, and 0.1 mM MgCl₂. The homogenate was centrifuged at 5000 rpm for 20 min. The supernatant was filtered through Miracloth and centrifuged at 100,000g for 1 h. The postcentrifugation 100,000g supernatant was precipitated in 80% acetone and resuspended in PSB (25 mM Tris, pH 6.8, 0.5% SDS). The 100,000g pellet (microsomal fraction) was resuspended in grind buffer at approximately 10 mg/mL and layered onto a linear 20 to 45% Suc gradient (10 mL total volume). The gradients were centrifuged at 100,000g for 16 h in a SW40 rotor. Five fractions were collected from the gradient corresponding to 1.07 to 1.12, 1.12 to 1.15, 1.15 to 1.16, 1.16 to 1.17, and 1.17 to 1.19 g/cm³. Membranes from each fraction were recovered by centrifuging at 100,000g for 1 h. The resulting pellets were resuspended in PSB and assayed for protein concentration using the BCA protein assay (Pierce, Rockford, IL).

For hypotonic washes, 10 mg of crude microsomal fraction were resuspended in 1 mL of grind buffer and subjected to hypotonic washes by adding 20 mL of grind buffer without Suc and centrifuging at 100,000g for 45 min. The process was repeated three more times, and the final pellet was resuspended in PSB.

Electrophoresis and Affinoblotting for Glycoprotein Visualization

An equal volume of 2 \times Laemmli sample buffer was added to protein preparations diluted in PSB and heated at 90°C for 5 min. For membrane protein preparations, samples were heated at 90°C for 5 min before addition of 2 \times sample buffer containing 8 M urea. Proteins were separated by SDS-PAGE according to the method of Laemmli (1975) except that a 7.5 to 15% acrylamide gradient, accompanied by a 7.5 to 15% glycerol gradient, or a continuous (10 or 12% acrylamide) slab gel, was used. Proteins were either stained with Coomassie brilliant blue R-250 or silver (Rabilloud et al., 1988)

to verify that equal amounts of protein were loaded in each lane. Unless stated otherwise, 25 μg of protein were loaded in each lane. After separation by SDS-PAGE, proteins were transferred to nitrocellulose as described by Towbin et al. (1979). The blot was blocked in Tris-buffered saline plus 3% gelatin for 1 h. Membrane glycoproteins were detected by Con A-horseradish peroxidase complex as described by Faye and Chrispeels (1985). Cell-wall glycoproteins were visualized with biotinylated Con A and avidin-alkaline phosphatase (Bio-Rad) because of the presence of existing cell-wall peroxidases.

Purification of SRgp24

Intact callus tissue was placed into cell-wall extraction solution (1 g/3 mL), which consisted of 100 mM CaCl₂ and 3% Suc. Tissue was incubated at room temperature for 30 min with gentle mixing. The callus was collected by filtration through two layers of Miracloth, and the Ca²⁺-extracted proteins were precipitated by adding 4 volumes of 100% ice-cold acetone and centrifuging at 20,000g for 20 min. Residual acetone was evaporated, and the pellets were resuspended in PSB. Preparative SDS-PAGE gradient gels were used to separate the Ca²⁺-extractable cell-wall proteins isolated from control callus. After the samples were electrophoretically separated, two strips from both edges of the gel were excised and stained with 0.1% Coomassie brilliant blue G-250 for 10 min, followed by rapid destaining in 50% methanol/10% acetic acid until the protein bands were visible. SRgp24 was identified by examining the stained gel strips, and the position corresponding to this region was excised from the nonstained gel, electroeluted using a Hoefer GE 200 Gel Eluter, dialyzed overnight with several changes of distilled water, lyophilized, and stored at -20°C until used. Isolated SRgp24 was subjected to SDS-PAGE to verify purity.

N-Terminal Amino Acid Sequence and Glycosyl Composition Analysis

Cell-wall proteins were resolved by a modified SDS-PAGE procedure essentially as described by Tranbarger et al. (1991). The resolved polypeptides were blotted to PVDF membrane and visualized by staining the blot with 0.1% Coomassie brilliant blue G-250 for 5 min and destaining with several changes of 50% methanol and 10% acetic acid. Bands containing SRgp24 were excised and PVDF membrane pieces were rinsed with several changes of water to remove excess Gly in the transfer buffer. Automated Edman degradation was performed directly on the PVDF membrane, and the amino acid sequence was determined at the Laboratory for Biotechnology and Bioanalysis (Washington State University, Pullman). Glycosyl composition analysis was performed by preparing the trimethylsilyl methylglycosides after methanolysis; actual analysis was done by personnel at the Complex Carbohydrate Research Center (University of Georgia, Athens).

Hydrolysis of Oligosaccharide Chain by Endo H

Cell-wall proteins were resolved on 10% acrylamide SDS-PAGE gels. Proteins were transferred to nitrocellulose paper and blocked as described previously. The nitrocellulose strips were incubated in 50 mM Na citrate (pH 6) with or without 60 milliunits of Endo H (Boehringer) for 48 h at 37°C according to the method of Grimes and Breidenbach (1987). After this incubation, the strips were washed for 24 h with Tris buffered saline plus 0.1% Tween-20 and then affinity-blotted with Con A for detection of glycosyl residues.

Antibody Production and Immunocytochemistry

Gel-purified SRgp24 was used to produce antibody from rabbits using standard procedures. Briefly, the antigen, i.e. electrophoretically purified SRgp24, was dissolved in PBS, emulsified with an equal amount of RIBI adjuvant, and injected into New Zealand White rabbits. The titer and specificity of the antiserum were determined by immunoblotting.

An affinity purification step was applied to obtain SRgp24-specific antibodies by a modification of the immunoblotting technique (Smith and Fisher, 1984). Cell-wall proteins were separated by preparatory SDS-PAGE and blotted to nitrocellulose paper as described above. After the blots were blocked in 5% milk (Nonfat Carnation Instant Milk) for 2 h, the area containing SRgp24 was excised from the blot and incubated in undiluted antiserum for 3 h. After incubation, the nitrocellulose strip was briefly washed with Tris-buffered saline to remove any nonspecific binding. Gly (100 mM, pH 2.5) was added for 5 min to dissociate the SRgp24 antibody, and the solution was immediately neutralized with one-fifth volume of 1 M Tris (pH 8.0).

Callus grown for 3 weeks in either medium without salt or medium containing 200 mM NaCl was used for immunolocalization. Tissues were fixed according to the method of Kim et al. (1988), and 1- μ m sections were prepared and immunostained as described by Tranbarger et al. (1991). An affinity-purified antibody against SRgp24 was used at a dilution of 1:25, and protein A-conjugated gold (15 nm) was used at a dilution of 1:50. Gold particles were enhanced with silver (IntenseM, Amersham) twice for 5 min each according to the manufacturer's directions. Treated sections were observed and photomicrographed with a Leitz Aristoplan microscope.

RESULTS

Identification of SRgp24

In previous work, we obtained an *M. crystallinum* callus line that retained salt-tolerant properties but did not induce CAM (Yen, 1993). Total soluble proteins from callus grown in medium without salt and medium containing 200 mM NaCl were examined on SDS-PAGE. Coomassie-stained gels revealed no dramatic changes in polypeptide patterns of abundant soluble proteins after salt treatment (data not shown). The SDS-PAGE profiles of membrane-associated proteins were also examined in these cells. Total microsomal proteins from control, salt, and salt plus ABA treatments were isolated and separated on 20 to 45% continuous Suc gradients. Five fractions were collected from the Suc gradient

corresponding to densities of 1.07 to 1.12, 1.12 to 1.15, 1.15 to 1.16, 1.16 to 1.17, and 1.17 to 1.19 g/cm³. After the samples were separated by SDS-PAGE, Coomassie brilliant blue was used to stain total membrane proteins, and Con A affinity blotting was used to detect glycosylated membrane polypeptides. Several subtle changes were detected in the polypeptide pattern of the Coomassie-stained gel between treatments (data not shown). Figure 1 shows that very distinct salt-dependent changes were observed on the Con A-stained affinity blots, especially in fraction 2 (1.12–1.15 g/cm³). After exposure of the callus to 200 mM NaCl for 3 weeks, the level of two low molecular mass proteins, estimated as 26 and 24 kD, decreased significantly with the 24-kD peptide decreasing to undetectable levels. Addition of 10⁻⁷ M ABA to the salt medium further enhanced the disappearance of the 26-kD peptide. Subsequent experiments focus on the 24-kD protein because it appeared more responsive to salt than the 26-kD protein. The 24-kD protein was subsequently named SRgp24 representing salt-responsive glycoprotein with molecular mass of 24 kD.

Hypotonic washes of microsomal membranes were performed to determine whether SRgp24 was associated with the membrane fraction or whether it was a soluble protein trapped inside membrane vesicles. The results shown in Figure 2 indicate that SRgp24 was released during hypotonic washes and demonstrates that SRgp24 is not a membrane-associated protein. Because of its distinct association with membrane vesicles with a density of 1.12 to 1.15 g/cm³ (Fig. 1), however, it would appear that SRgp24 is not an intracellular protein that becomes "trapped" during homogenization. If this were the case, then one would expect SRgp24 to be uniformly distributed throughout the Suc gradient.

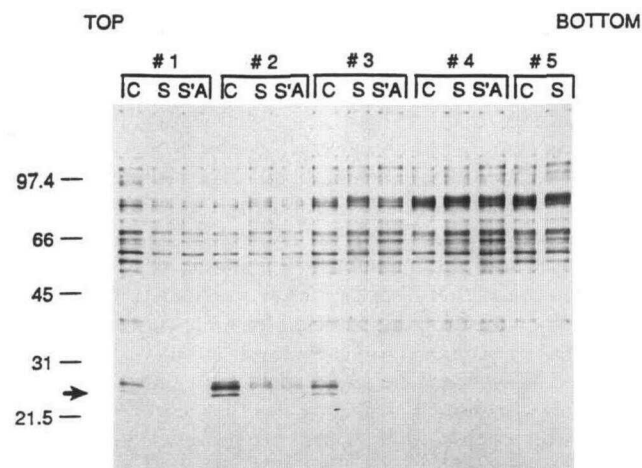


Figure 1. Con A affinity blot of membrane proteins from 3-week-old callus of control (C, minus NaCl), 200 mM NaCl (S), or 200 mM NaCl plus 10⁻⁷ M ABA (S'A). Five membrane-enriched fractions labeled 1 to 5 were collected after separation by continuous Suc gradients (20–45%), with densities of 1.07 to 1.12, 1.12 to 1.15, 1.15 to 1.16, 1.16 to 1.17, and 1.17 to 1.19 g/cm³, respectively. Proteins were separated via SDS-PAGE and blotted, and glycoproteins were detected by staining with Con A. Arrow indicates the position of SRgp24.

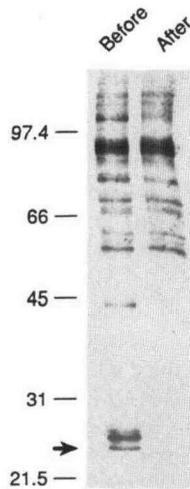


Figure 2. Con A affino blot of a microsomal preparation from control callus before and after hypotonic washes. Crude microsome (10 mg) resuspended in 8% Suc was diluted in a hypotonic solution containing 0% Suc, and the process was repeated four times. Proteins were then separated via SDS-PAGE, and the affino blot was reacted with Con A. Arrow indicates the position of SRgp24.

Cell-fractionation experiments were designed to determine the final destination of SRgp24. The possibility that SRgp24 is secreted to the cell-wall region was examined first. Callus grown on medium without salt was incubated in 100 mM CaCl_2 to release cell-wall proteins, and total cell extracts were also collected before and after this treatment. The results shown in Figure 3A (lane 2) indicate that SRgp24 is extracted from the cell-wall fraction of callus grown in medium without salt. Furthermore, SRgp24 was not detected in the total cellular extract after removal of the cell-wall proteins by CaCl_2 extraction. Figure 3B shows that SRgp24 is only present in the cell wall of callus grown without salt; SRgp24 disappears upon addition of NaCl to the medium. Figure 3B also demonstrates that several other cell-wall glycoproteins change in response to the presence or absence of salt.

Since Na^+ and Ca^{2+} are both cations, it is possible that high concentrations of salt in the culture medium may dissociate some proteins from the cell wall and cause them to be released into the medium. If this occurred, it would (by artifact) appear that SRgp24 is down-regulated by 200 mM NaCl. When the proteins extracted from the culture media of callus grown in both the presence and absence of salt were analyzed, however, there was no detectable SRgp24 in either case (data not shown). This indicates that SRgp24 is, in fact, a cell-wall protein that is down-regulated by high salt concentrations.

Physiological Characterization of SRgp24

The response of SRgp24 to various concentrations of NaCl was tested by transferring callus to medium containing between 0 and 200 mM NaCl and allowing the callus to grow for an additional 3 weeks. Figure 4 demonstrates that SRgp24 that accumulated in callus grown in 0 up to 50 mM NaCl started to decrease with callus grown in 100 mM NaCl and

decreased to undetectable levels when callus was grown in 200 mM NaCl. Other distinct changes in cell-wall glycoproteins were also observed with callus grown under increasing concentrations of salt. Polypeptides marked 3 and 4 on Figure 4, estimated as 40 and 39 kD, respectively, increased in response to 10 mM NaCl and became quite abundant when the concentration of NaCl was increased to 50 mM NaCl or higher. On the other hand, polypeptides 1 and 2 on Figure 4, estimated as 100 and 97 kD, respectively, were sensitive to addition of NaCl to culture medium (Fig. 4). Other changes in Con A-binding polypeptides are also evident.

Time-course experiments were performed to determine the kinetics of SRgp24 response to salt stress and whether the down-regulation is reversible after release from salt stress. Figure 5 shows that SRgp24 disappeared within 3 d when callus was grown for 3 weeks in medium without salt and then transferred to medium containing 200 mM NaCl. Likewise, SRgp24 appeared within 3 d when callus was grown for 3 weeks in medium containing 200 mM NaCl and then transferred into medium without salt. The rapid disappearance of SRgp24 under salt stress was not due to a dilution effect because callus grew normally in the first 3 d and the fresh weight remained virtually the same (Yen, 1993). The results from time-course experiments suggest that SRgp24 responds to changes in the salt concentration within 3 d.

Figure 6 demonstrates that factors other than NaCl can affect the accumulation of SRgp24. To test whether water stress per se affects the accumulation of SRgp24, 400 mM mannitol was added to create a nonionic osmotic stress,

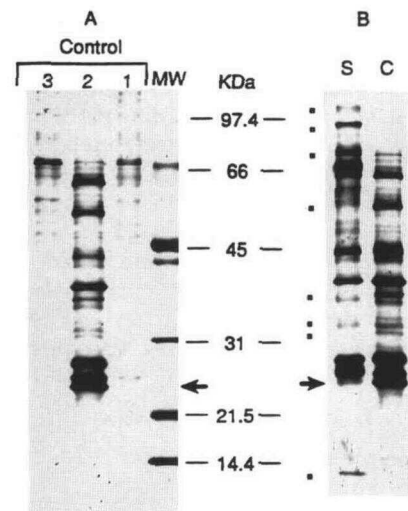


Figure 3. A, Localization of SRgp24 in various cell fractions of callus of *M. crystallinum* grown in medium without salt (control). Lane 1, Total cellular glycoproteins before 100 mM CaCl_2 extraction. Lane 2, Ca^{2+} -extractable cell-wall glycoproteins. Lane 3, Total cellular glycoproteins after Ca^{2+} extraction. MW, Biotinylated protein standards. B, Changes in Ca^{2+} -extractable cell-wall glycoproteins after salt treatment. Cell-wall proteins were isolated from 3-week-old callus grown in media containing no salt (C) or 200 mM NaCl (S), and the affino blot was reacted with biotinylated Con A. Arrow indicates the position of SRgp24. Dots indicate other polypeptides that increase or decrease with salt treatment.

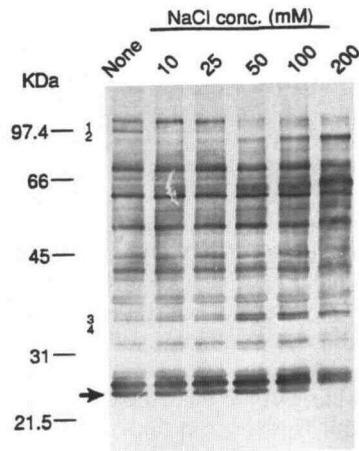


Figure 4. Dose-response curve of SRgp24 to salt concentration in the medium. Calli were grown in medium containing different concentrations of NaCl (0–200 mM) for 3 weeks. Ca^{2+} -extractable proteins from each treatment were separated by SDS-PAGE, and glycoproteins were detected by biotinylated Con A after transfer to nitrocellulose. Arrow indicates the position of SRgp24. Numbers 1 to 4 on the left refer to other cell-wall glycoproteins (with approximate molecular mass values of 72, 70, 40, and 39, respectively) apparently regulated by salt concentration.

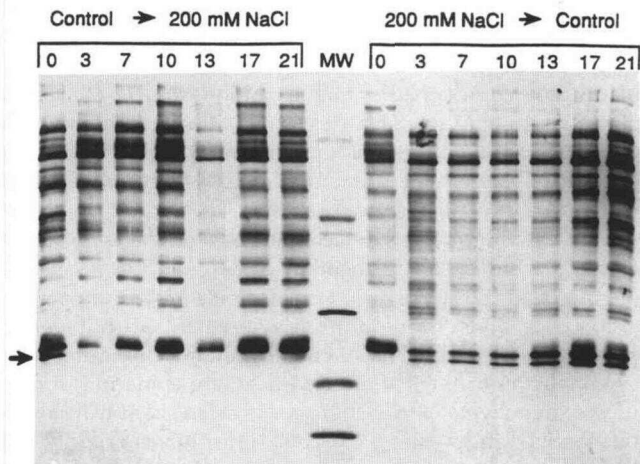


Figure 5. Con A affino blot of Ca^{2+} -extractable cell-wall glycoproteins during culture growth. Left, Callus was grown in control medium lacking salt for 3 weeks and then transferred to medium containing 200 mM NaCl, and samples were collected 3, 7, 10, 13, 17, and 21 d after transfer. The disappearance of SRgp24 begins from the first time point collected (3 d). The biotinylated protein standards (MW) are 97, 66, 45, 31, 21, and 14 kDa, respectively. Right, Callus was grown in medium with 200 mM NaCl for 3 weeks and then transferred to media without NaCl (control). Samples were harvested 3, 7, 10, 13, 17, and 21 d after transfer to new medium. The synthesis of SRgp24 begins from the first time point collected (3 d).

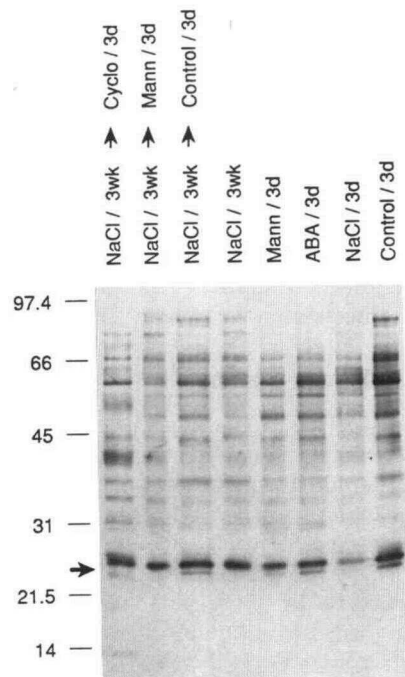


Figure 6. Effects of 10^{-7} M ABA, 400 mM mannitol (Mann), or 0.75 $\mu\text{g}/\text{mL}$ cycloheximide (Cyclo) on the accumulation of SRgp24. In the first three lanes, callus was grown in medium containing 200 mM NaCl for 3 weeks (NaCl/3wk) and then subcultured to fresh medium supplemented with either cycloheximide, mannitol, or control medium (minus NaCl). In the last five lanes, cells were grown for 3 weeks in control medium (minus NaCl) and then transferred into medium containing either 200 mM NaCl for 3 weeks (NaCl/3wk), 400 mM mannitol for 3 d (Mann/3d), 10^{-7} M ABA for 3 d (ABA/3d), 200 mM NaCl for 3 d (NaCl/3d), or control (minus NaCl) medium for 3 d (control/3d). Cells were harvested at the indicated times, and Ca^{2+} -extractable cell-wall proteins from each treatment were separated by SDS-PAGE and affino blotted with biotinylated Con A. Arrow indicates the position of SRgp24.

which exhibited the same osmotic potential as 200 mM NaCl. Lane Mann/3d in Figure 6 shows that the level of SRgp24 was reduced in callus grown in medium without salt containing 400 mM mannitol (compare lane NaCl/3wk → Control/3d with lane Mann/3d). The accumulation of SRgp24 was not totally suppressed by 400 mM mannitol, indicating that it was less responsive to 400 mM mannitol than to 200 mM NaCl. When callus was grown for 3 weeks in medium containing 200 mM NaCl and then transferred to medium without salt but containing 400 mM mannitol, the accumulation of SRgp24 continued to be suppressed as shown by the lack of reappearance of SRgp24 in lane NaCl/3wk → Mann/3d (Fig. 6).

ABA is known to alter the synthesis of several stress-related proteins (Skriver and Mundy, 1990), and lane ABA/3d in Figure 6 shows that the addition of 10^{-7} M ABA to medium without salt for 3 d did not decrease the synthesis of SRgp24 (compare lane NaCl/3wk → Control/3d with lane ABA/3d). These results suggest that ABA itself, at least at the concentration tested, has no influence on the accumulation of SRgp24. The protein synthesis inhibitor cycloheximide was

used to determine whether the synthesis of SRgp24 is the result of de novo protein synthesis. First, cells were grown in medium containing 200 mM NaCl for 3 weeks to deplete the level of SRgp24 and then were transferred to medium without salt but containing 0.75 μ g/mL cycloheximide for 3 d. Lane NaCl/3wk \rightarrow Cyclo/3d in Figure 6 shows that SRgp24 did not reappear to appreciable levels in the presence of cycloheximide. Although there is a weakly staining band that appears to be slightly smaller than 24 kD, this result suggests that the synthesis of SRgp24 detected in lane NaCl/3wk \rightarrow Control/3d is due to de novo protein synthesis.

Biochemical Characterization of SRgp24

SRgp24 was gel purified and subjected to Edman degradation to determine the amino acid sequence of the N terminus. An N-terminal sequence was obtained for the first 21 residues and is shown in Table I. The N-terminal sequence of SRgp24 revealed 55 to 60% identity with PR-5 protein. Among the list of PR-5 proteins, a 24-kD intercellular tobacco PR-5 protein and a 24-kD vacuolar salt-responsive tobacco PR-5 protein (osmotin) are noteworthy, and their sequences are shown for comparison.

Although SRgp24 and PR-5 proteins are similar at the N termini and have comparable M_r values, PR-5 proteins have never been reported to be glycoproteins, whereas SRgp24 shows high affinity for Con A, a lectin that binds to Man and Glc. To gain more information concerning the carbohydrate composition of SRgp24, enzymic digestion of the glycosyl group using Endo H was applied. This glycosidase cleaves in the core region of Asn-linked (N-linked) glycosyl groups. The cell-wall proteins extracted from callus grown on medium containing salt were resolved on 10% SDS-PAGE, transferred to nitrocellulose, and treated with either Endo H or buffer alone. Figure 7 shows that Endo H treatment removed the glycosyl moieties from the majority of cell-wall glycoproteins as judged by their ability to bind Con A. Unexpectedly, Endo H digestion did not affect Con A binding to SRgp24, suggesting that SRgp24 has a unique carbohydrate structure that is resistant to Endo H digestion. To confirm that SRgp24 is a glycoprotein, purified SRgp24 was subjected to carbohydrate compositional analysis. Results from these experiments indicated that Glc, GlcNAc, Man, and Gal residues were covalently attached to SRgp24.

Immunocytochemical Detection of SRgp24 in Mature Plants and Callus

Gel-purified SRgp24 was used to raise antiserum in rabbits, and the specificity of this antiserum was tested by immuno-

blotting. Figure 8A shows the results of an immunoblot against cell-wall proteins from both the control and salt-grown calli. It was evident that this antiserum recognized a protein band of 24 kD in the control callus but not in the salt-treated callus. The position of SRgp24 was further confirmed by comparing with Con A affinoblots (data not shown). A 24-kD polypeptide was also detected using anti-osmotin antibodies, indicating that SRgp24 is immunologically related to osmotin (Fig. 8B). A high molecular mass polypeptide isolated from callus grown in both medium containing and medium not containing salt also cross-reacts with the anti-SRgp24 antibodies. When preimmune serum was tested, this high molecular mass band was also detected on the immunoblot but not the band associated with SRgp24 (data not shown). This suggests that the high molecular mass band is the result of additional, nonspecific antibodies that already existed in the rabbit before the injection of our antigens. Although the higher band did not interfere with immunoblotting experiments, we affinity purified SRgp24-specific antibodies. After affinity purification, a single band associated with SRgp24 was detected on the immunoblot (data not shown).

To determine whether SRgp24 was present in mature plants, 5-week-old plants maintained in nutrient solution containing 20 mM NaCl were treated either with nutrient solution containing 200 mM NaCl for 1 week to produce plants grown under Na-excess conditions or with nutrient solution containing no salt for 1 week to produce plants grown under Na-limiting conditions. Ca^{2+} -extractable cell-wall proteins from leaves, roots, or stems of plants grown in medium without salt (-) or medium containing 200 mM NaCl (+) were separated by SDS-PAGE and subjected to immunoblot analysis using anti-SRgp24 serum. Figure 9 shows that SRgp24 was detected in much higher levels in the leaves of plants grown under Na-limiting conditions than in the leaves of plants grown under Na-excess conditions. Although difficult to ascertain after photographic reproduction, there was a small but detectable amount of SRgp24 present in stem tissue (Fig. 9) of plants grown under Na-limiting conditions as well. No SRgp24 was detected in roots of plants grown in medium without salt or medium containing 200 mM NaCl (Fig. 9) or in the stems or leaves of plants grown under Na-excess conditions. Tissue-printing experiments with leaves of plants grown under Na-limiting conditions indicated that essentially all of the leaf tissues (including epidermis, mesophyll parenchyma, and veins) accumulated significant levels of SRgp24. Under Na-excess conditions, however, only the leaf mesophyll cells accumulated SRgp24, and these levels were much lower than in plants grown under Na-deficient conditions (data not shown).

Table I. N-terminal amino acid sequence alignment of osmotin I (Singh et al., 1987a), acidic PR-5 (TPR) (Pierpoint et al., 1987), and SRgp24

X represents unidentified amino acids.

	10	20
Osmotin I	A T I E V R N N C P Y T V W A A S T P I G	
SRgp24	A T F Q V K N N X K F T V W A A A A R P G	
TPR	A T F D I V N Q C T Y T V W A A A S P G	

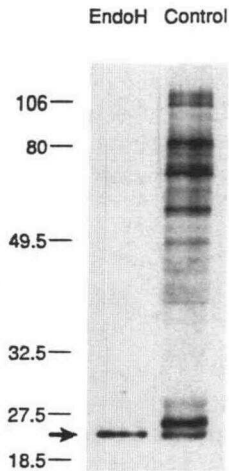


Figure 7. Endo H digestion of Ca^{2+} -extracted cell-wall glycoproteins. Cell-wall proteins obtained from control (minus NaCl) callus were separated on a 10% SDS-PAGE gel and then transferred to nitrocellulose. The nitrocellulose strips were incubated either with 60 munits of Endo H or with a water control (Control) for 48 h. After extensive washing, both strips were stained with Con A. The arrow indicates the position of SRgp24.

The cellular localization of SRgp24 in callus was also determined using affinity-purified antiserum against SRgp24. Figure 10A demonstrates that SRgp24 is preferentially associated with the cell-wall region of the callus grown in medium without salt, whereas only a few silver grains were detected in callus grown in medium containing 200 mM NaCl (Fig. 10B). These immunolocalization results are consistent with biochemical data demonstrating that SRgp24 is primarily associated with the cell wall. Examination of several anti-SRgp24 immunostained sections indicated that SRgp24 was not distributed uniformly in all cell types but was most

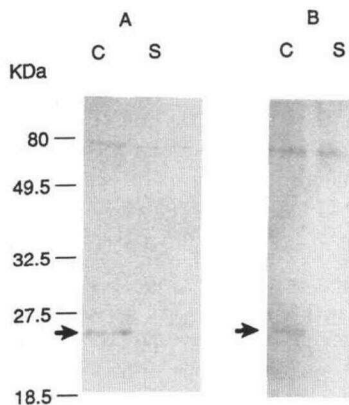


Figure 8. Immunoblots of proteins isolated from callus of *M. crystallinum* grown in either control medium for 3 weeks (C) or medium supplemented with 200 mM salt (S). After SDS-PAGE and transfer to nitrocellulose, the blots were reacted with either SRgp24 antiserum (A) or tobacco antiosmotin (B). Molecular mass of the pre-stained protein standards is indicated on the left. Arrows indicate the position of SRgp24.

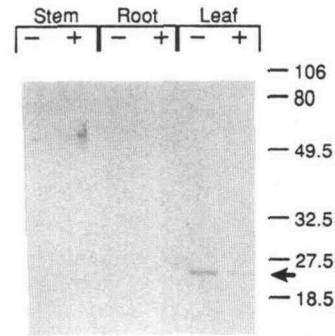


Figure 9. Immunoblot analysis of Ca^{2+} -extractable proteins from plants grown either under Na-limiting conditions (-) or Na-excess conditions (+). Protein (15 $\mu\text{g}/\text{lane}$) was separated on a 12% SDS-PAGE gel, transferred to nitrocellulose, and detected with anti-SRgp24 antiserum. SRgp24 accumulates to much higher levels in the leaves of plants grown under Na-limiting conditions than the leaves of plants grown under Na-excess conditions. SRgp24 was detected in small amounts in the stems of Na-deficient plants but was not detected in the roots. Arrow indicates the position of SRgp24.

concentrated in cells showing distinct, reticulated, secondary cell-wall thickenings characteristic of the tracheary elements of xylem.

DISCUSSION

In this report we describe the identification and characterization of a cell-wall glycoprotein (SRgp24) isolated from callus of *M. crystallinum*. SRgp24 is associated with several tissues in the leaves and stems of *M. crystallinum* plants as evidenced by immunoblotting and tissue-printing experiments. In both callus cultures and mature plants, the accumulation of SRgp24 is down-regulated in response to salt.

The N-terminal amino acid sequence of SRgp24 shares homology with PR-5 proteins. PR-5 proteins have both acidic and basic isoforms: the acidic forms are secreted to the intercellular space and the basic forms are sequestered in the vacuole (Bol et al., 1990). The similarities between SRgp24 and known PR-5 proteins may not only be in the amino acid sequences but in their function under stress conditions. An acidic 24-kD tobacco PR-5 protein accumulates in the intercellular space of tobacco mosaic virus-infected tobacco leaves (Cornelissen et al., 1986; Pierpoint et al., 1987). One significant difference between SRgp24 and PR-5 proteins is that SRgp24 is apparently glycosylated, whereas none of the PR-5 proteins, either acidic or basic forms, identified so far are glycoproteins (Bol et al., 1990).

SRgp24 is also similar to the basic PR-5 protein osmotin (Singh et al., 1987a) in that both are able to respond to a low water potential environment in the culture medium (Singh et al., 1987b). Unlike osmotin, which accumulates in the salt-adapted glycophytes (Singh et al., 1987a; King et al., 1988), the synthesis of SRgp24 is constitutive in cell cultures of ice plant and is suppressed by the presence of high salt. When cells were challenged with high salt, the level of SRgp24 decreased rapidly, as opposed to the cumulative accumulation pattern of osmotin, which requires an adaptation process

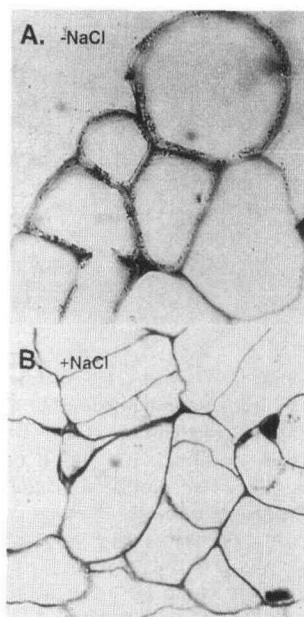


Figure 10. Determination of tissue distribution and cellular localization of SRgp24 in callus. A, Callus grown in medium without salt ($-NaCl$) was prepared for immunocytochemistry and immunodecorated with affinity-purified anti-SRgp24 antiserum. Note the association of silver grains with cells having thick cell walls. B, Callus grown in medium containing salt ($+NaCl$) was prepared for immunocytochemistry and immunodecorated with affinity-purified anti-SRgp24 antiserum. Note the general absence of thickened cell walls.

over several generations to reach maximal expression levels (Singh et al., 1985; LaRosa et al., 1989).

Atriplex nummularia is another halophyte that contains osmotin-like proteins that exhibit similar regulatory properties to SRgp24 (Casas et al., 1992). In the absence of salt, the constitutive synthesis of polypeptides immunologically related to tobacco osmotin was found both in cellular protein extracts and in proteins present in the culture medium of *A. nummularia* cells. Furthermore, the transcript levels of two osmotin-like genes isolated from *A. nummularia* cells decreased dramatically after exposure to salt (Casas et al., 1992). The different regulatory properties among the PR-5 family suggest that different forms of PR-5, or osmotin-like proteins, have separate functions. Indeed, polypeptides serologically related to SRgp24 were detected on immunoblots under several stress conditions, including aging, wounding, and treatment with chemical inducers (Yen, 1993). After the involvement of osmotin in osmotic adaptation in tobacco was suggested (LaRosa et al., 1989), several reports indicated that osmotin and osmotin-like proteins were induced during protoplast isolation (Grosset et al., 1990a, 1990b), in vitro flower formation (Neale et al., 1990; Richard et al., 1992), and pathogen infection (Pierpoint et al., 1987; Woloshuk et al., 1991). One study revealed that a PR-5 protein, AP24, exhibited antifungal activity and was highly homologous to osmotin (Woloshuk et al., 1991). Osmotin-like proteins, then, seem to be associated with general stress responses in plants (Woloshuk et al., 1991).

Cell-fractionation experiments performed with cell cultures of *M. crystallinum* indicated that SRgp24 was associated with the cell-wall fraction and with putative secretory vesicles of 1.12 to 1.15 g/cm³. To corroborate these findings, immunocytochemical studies were performed using affinity-purified antibodies against SRgp24. When thin sections of callus grown in medium without salt were reacted with this antibody, heavy staining in the cell-wall region of cells with thick cell walls was observed. Further anatomical investigations indicated that antibody labeling was preferentially associated with heavily lignified cells characteristic of tracheary elements.

Thomas and Bohnert (1993) have reported that a 24-kD polypeptide in *M. crystallinum* accumulates in response to salt and is recognized by antibodies to osmotin. This polypeptide, however, is not equivalent to SRgp24 as is evidenced by their opposite regulatory patterns in response to salt. It is perhaps important to note that SRgp24 was originally identified in putative secretory vesicles and is localized to the cell wall, whereas the polypeptide identified by Thomas and Bohnert (1993) is from a total protein extract. In experiments not presented, we also have observed the accumulation of a 24-kD polypeptide in response to salt from total protein extracts but have been unable to detect this polypeptide with SRgp24 antibody or tobacco osmotin antibody.

Euhalophytes, such as *M. crystallinum*, require NaCl for optimal growth (Flowers, 1985). The medium without salt used in our study may be abnormal or stressful, in that Na⁺ was omitted from the culture medium. Indeed, the highest growth rate observed in our culture occurred when 25 mM NaCl was supplied to the culture medium (Yen, 1993). However, the level of SRgp24 was similar in cultures grown in the absence of NaCl versus 25 mM NaCl (Fig. 4), which suggests that SRgp24 is not up-regulated by a salt deficiency.

In summary, SRgp24 with a Con A-binding capacity was found to be associated with the Ca²⁺-extractable cell-wall fraction in callus of *M. crystallinum*. SRgp24 is constitutively expressed in this halophyte in the absence of any adaptive processes. The salt response curve shows that synthesis of SRgp24 is suppressed in medium containing 100 to 200 mM NaCl. Time-course experiments and protein inhibitor treatments together reveal that the rapid reversibility of SRgp24 accumulation is the result of de novo protein synthesis. Salt and water-deficit stress both inhibit the synthesis of SRgp24, and ABA facilitated the suppression process.

ACKNOWLEDGMENTS

We would like to thank Dr. R.A. Bressan for the generous gift of tobacco osmotin antibody. Amino acid sequencing was performed in the Laboratory for Biotechnology and Bioanalysis at Washington State University. Microscopy and image analysis equipment was purchased with a grant from the National Science Foundation.

Received January 13, 1994; accepted April 14, 1994.

Copyright Clearance Center: 0032-0889/94/105/1179/09.

LITERATURE CITED

Bohnert HJ, Cushman JC, Michalowski CB, Rickers J, Meyer G, DeRocher EJ, Vernon DM, Krueger M, Vazquez-Moreno L,

- Velten J, Hofner R, Schmitt JM (1988) *Mesembryanthemum crystallinum*, a higher plant model for the study of environmentally induced changes in gene expression. *Plant Mol Biol Rep* 6: 10–28
- Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. *Annu Rev Phytopathol* 28: 113–138
- Bozarth CS, Mullet JE, Boyer JS (1987) Cell wall proteins at low water potentials. *Plant Physiol* 85: 261–267
- Casas AM, Nelson DE, Ragothama KG, Paino D'Urzo M, Singh NK, Bressan RA, Hasegawa PM (1992) Expression of osmotin-like genes in the halophyte *Atriplex nummularia* L. *Plant Physiol* 99: 329–337
- Chu C, Dai Z, Ku MSB, Edwards GE (1990) Induction of crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. *Plant Physiol* 93: 1253–1260
- Cornelissen BJC, Hooft van Huijsduijnen RAM, Bol JF (1986) A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. *Nature* 321: 531–532
- Cushman JC, DeRocher EJ, Bohnert HJ (1990) Gene expression during adaptation to salt stress. In F Katterman, ed, *Environmental Injury to Plants*. Academic Press, San Diego, CA, pp 173–203
- Esaka M, Hayakawa H, Hashimoto M, Matsubara N (1992) Specific and abundant secretion of a novel hydroxyproline-rich glycoprotein from salt-adapted winged bean cells. *Plant Physiol* 100: 1339–1345
- Faye L, Chrispeels MJ (1985) Characterization of N-linked oligosaccharides by affino blotting with concanavalin A-peroxidase and treatment of the blots with glycosidases. *Anal Biochem* 149: 218–224
- Flowers TJ (1985) Physiology of halophytes. *Plant Soil* 89: 41–56
- Grimes HD, Breidenbach RW (1987) Plant plasma membrane proteins. Immunological characterization of a major 75 kD protein group. *Plant Physiol* 85: 1048–1054
- Grosset J, Marty I, Chartier Y, Meyer Y (1990a) mRNAs newly synthesized by tobacco mesophyll protoplast are wound inducible. *Plant Mol Biol* 15: 485–496
- Grosset J, Meyer Y, Chartier Y, Kauffmann S, Legrand M, Fritig B (1990b) Tobacco mesophyll protoplast synthesize 1,3- β -glucanases, chitinases, and "osmotins" during in vitro culture. *Plant Physiol* 92: 520–527
- Heun AM, Gorham J, Lüttge U, Wyn Jones RG (1981) Changes of water-relation characteristics and levels of organic cytoplasmic solutes during salinity-induced transition of *Mesembryanthemum crystallinum* from C₃ photosynthesis to crassulacean acid metabolism. *Oecologia* 50: 66–72
- Hurkman WJ, Tanaka CK (1987) The effects of salt on the pattern of protein synthesis in barley roots. *Plant Physiol* 83: 517–521
- Hurkman WJ, Tanaka CK, DuPont FM (1988) The effects of salt stress on polypeptides in membrane fractions from barley roots. *Plant Physiol* 88: 1263–1273
- Iraki NM, Bressan RA, Carpita NC (1989) Extracellular polysaccharides and proteins of tobacco cell culture and changes in composition associated with growth-limiting adaptation to water and saline stress. *Plant Physiol* 91: 54–61
- Kim WK, Franceschi VR, Krishnan HB, Okita TW (1988) Formation of wheat protein bodies: involvement of the Golgi apparatus in gliadin transport. *Planta* 176: 173–182
- King GJ, Turner VA, Hussey CE Jr, Wurtele ES, Lee SM (1988) Isolation and characterization of a tomato cDNA clone which encodes for a salt-induced protein. *Plant Mol Biol* 10: 401–412
- Laemmli UK (1975) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277: 680–684
- LaRosa PC, Singh NK, Hasegawa PM, Bressan RA (1989) Stable NaCl tolerance is associated with enhanced accumulation of osmotin. *Plant Physiol* 91: 855–861
- McElwain EF, Bohnert HJ, Thomas JC (1992) Light mediates the induction of phosphoenolpyruvate carboxylase by NaCl and abscisic acid in *Mesembryanthemum crystallinum*. *Plant Physiol* 99: 1261–1264
- Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Meeks-Wagner DR, Peacock WJ, Dennis ES (1990) Chitinase, β -1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. *Plant Cell* 2: 673–684
- Paul MJ, Cockburn W (1989) Pinitol, a compatible solute in *Mesembryanthemum crystallinum* L. *J Exp Bot* 40: 1093–1098
- Pierpoint WS, Tatham AS, Papin DJC (1987) Identification of the virus-induced protein of tobacco leaves that resembles the sweet protein thaumatin. *Physiol Mol Plant Pathol* 31: 291–298
- Rabilloud T, Carpentier G, Tarroux P (1988) Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis* 9: 288–291
- Reviron M-P, Vartanian N, Stallantini M, Huet J-C, Pernollet J-C, de Vienne D (1992) Characterization of a novel protein induced by progressive or rapid drought and salinity in *Brassica napus* leaves. *Plant Physiol* 100: 1486–1493
- Richard L, Arro M, Hoebeke J, Meeks-Wagner DR, Tran Thanh Van K (1992) Immunological evidence of thaumatin-like proteins during tobacco floral differentiation. *Plant Physiol* 98: 337–342
- Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfunkoch E, Regnier FE, Bressan RA (1987a) Characterization of osmotin, a thaumatin-like protein associated with osmotic adaptation in plant cells. *Plant Physiol* 85: 529–536
- Singh NK, Handa AK, Hasegawa PM, Bressan RA (1985) Proteins associated with adaptation of cultured tobacco cells to NaCl. *Plant Physiol* 79: 126–137
- Singh NK, LaRosa PC, Handa AK, Hasegawa PM, Bressan RA (1987b) Hormonal regulation of protein synthesis associated with salt tolerance in tobacco cells. *Proc Natl Acad Sci USA* 84: 739–743
- Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 2: 503–512
- Smith DE, Fisher PA (1984) Identification, developmental regulation, and response to heat shock of two isogenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J Cell Biol* 99: 20–28
- Thomas JC, Bohnert HJ (1993) Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum*. *Plant Physiol* 103: 1299–1304
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350–4354
- Tranbarger TJ, Franceschi VR, Hildebrand DF, Grimes HD (1991) The soybean 94 kD vegetative storage protein is a lipoxygenase that is localized in paraveinal mesophyll cell vacuoles. *Plant Cell* 3: 973–987
- Treichel S (1986) The influence of NaCl on Δ -pyrroline-5-carboxylate reductase in proline-accumulating cell suspension cultures of *Mesembryanthemum nodiflorum* and other halophytes. *Physiol Plant* 67: 173–181
- Winicov I, Button JD (1991) Accumulation of photosynthesis gene transcripts in response to sodium chloride by salt-tolerant alfalfa cells. *Planta* 183: 478–483
- Winter K (1985) Crassulacean acid metabolism. In J Barber, NR Baker, eds, *Photosynthetic Mechanisms and the Environment*. Elsevier, Amsterdam, The Netherlands, pp 329–387
- Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, van den Elzen PJM, Cornelissen JC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *Plant Cell* 3: 619–628
- Yen HE (1993) Biochemical and molecular characterization of a salt-responsive glycoprotein in the halophyte *Mesembryanthemum crystallinum*. PhD thesis. Washington State University, Pullman, WA
- Zhao Z, Heyser JW, Bohnert HJ (1989) Gene expression in suspension culture cells of the halophyte *Distichlis spicata* during adaptation to high salt. *Plant Cell Physiol* 30: 861–867