To the Graduate Council:

I am submitting herewith a dissertation written by Dale Lynn Vogelien entitled "Physiological Characterization of NaCl-Tolerant Mutants of the Fern *Ceratopteris richardii*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Leslie G. Hickok, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

PHYSIOLOGICAL CHARACTERIZATION OF NACL-TOLERANT MUTANTS OF THE FERN *CERATOPTERIS RICHARDII*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Dale Lynn Vogelien

December 1993

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ACKNOWLEDGEMENTS

Several people have helped me generously and in various ways during the course of my PhD. program. Certainly, many thanks go to my committee members, Robert (Oz) Augé, Donald Dougall, Les Hickok and Otto Schwartz, for their guidance, suggestions and, above all, their cooperation (in times of immediate need). In particular, I am extremely indebted to Les Hickok who served as my Mentor. I thank Dave Ringelberg and Steve Nold of Dr. David White's laboratory at the Center for Environmental Biotechnology for their invaluable assistance in the examination of lipid composition. Many thanks to Thomas Warne for his counsel and friendship. Finally, I would like to thank my family and friends, whose names are too numerous to mention, for giving me support and encouragement throughout my graduate career at the University of Tennessee.

ABSTRACT

The fern *Ceratopteris richardii* has been used successfully as a model system to select for single gene nuclear mutations that confer tolerance to NaCl. In this dissertation project two unlinked mutations, *stl1* and *stl2*, were selected for use in comparative studies with the wild type in an attempt to ascertain the physiological basis of tolerance associated with these mutations.

Initial studies were designed to address the kinds of stress imposed by excess NaCl, namely osmotic and ionic stress. Cross-tolerance tests comparing the growth response of *stl1* gametophytes with wild type to various osmotic and ionic stress agents indicated that this mutation is associated with a specific tolerance to Na^+. In addition, *stl1* displayed similar water relations and organic and inorganic solute profiles as the wild type in response to NaCl stress. Similar studies with *stl2* gametophytes indicated that tolerance conferred by the *stl2* mutation is largely associated with the ability to respond to ionic stress imposed by NaCl. In addition, *stl2* gametophytes displayed a high level of tolerance to Na^+ and Mg^{2+} salts, suggesting a nonspecific tolerance for cations and anions. While similar organic solute profiles were found between *stl2* and the wild type, analysis of tissue ion content revealed that *stl2* was associated with selective accumulation of K^+ and reduced accumulation of Na^+ during NaCl stress, resulting in higher K^+/Na^+ ratios. Additional studies showed supplementation of Ca^{2+} to the external medium can lessen the inhibitory effect of NaCl on wild type and *stl1* gametophytic growth , but had

no effect on *stl2* gametophytes. The positive effect of Ca^{2+} on growth during salinity stress in the wild type and *stl1* genotypes was found to correlate with maintenance of higher K^+ and reduced Na^+ levels. *stl2* gametophytes were also found to be highly sensitive to the level of K^+ in the culture medium, with reduced levels of K^+ resulting in enhanced gametophytic growth. Thus, it appears that *stl2* gametophytes suffer from K^+ toxicity under normal culture conditions. Lastly, similar lipid composition was found between genotypes in the absence and presence of NaCl stress, thus discounting the possible involvement of a lipid-based mechanism of tolerance.

In accordance with the cumulative results obtained for each mutant genotype and information obtained from the literature, hypotheses regarding the physiological basis of tolerance to NaCl were proposed. The *stl1* mutation was suggested to be associated with enhanced compartmentation of Na^+ within the cell, perhaps within the vacuole. It was proposed that the *stl2* mutation affects a K^+/cation influx channel located in the plasma membrane. In wild type cells selectivity of the channel for K^+ and K^+ uptake is influenced by extracellular Ca^{2+} binding to the channel protein. When Ca^{2+} is bound to the protein, the channel is selective for K^+ over other cations and K^+ uptake is enhanced. Removal or replacement of Ca^{2+} results in the loss of channel selectivity for K^+, and consequently the movement of other monovalent cations through the channel increases. It is proposed that the mutant (*stl2*) protein possesses a higher affinity for Ca^{2+}. This altered Ca^{2+} binding results in enhanced K^+ uptake in the absence of NaCl stress and maintains channel selectivity for K^+ during salinity stress. Alterantively, *stl2* may no longer be sensitive to Ca^{2+}.

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PART 1

INTRODUCTION

1. INTRODUCTION TO THE PROBLEM

The accumulation of salts in agricultural soil has been and continues to be a major problem facing agriculture. Salinization of crop land primarily results in a reduction in crop productivity (Maas, 1984). On a global scale it is estimated that one third of all irrigated land has been affected by soil salinity (Epstein, 1980). While a number of salts can contribute to soil salinity, the toxic effects of Na^+ and Cl^- are considered the most important (Epstein and Rains, 1987). A saline soil is one that contains approximately 2560 mg/L total dissolved salts in a soil extract (Lewis, 1984), which translates into a concentration of 44 mM NaCl. Most crop plants are glycophytes and are sensitive to salinities of 3000 ppm (51 mM NaCl), and most cannot survive more than 5000 ppm (86 mM NaCl) (Maas, 1984; Toenniessen, 1984).

Conventional means of dealing with soil salinity include careful water and land management. Practices such as controlling the chemical composition of irrigation water, ensuring adequate drainage, leaching salts out of the root zone and chemical treatment of soil to correct soil toxicities and nutrient deficiencies can be used to control the spread of this problem (Oster et al., 1984). These solutions, while perhaps the best, are long-term and usually expensive, and consequently are not often feasible for or practiced by developing countries.

Breeding crop plants with enhanced salt tolerance is another strategy to deal with soil salinity that has been pursued in conjunction with good land and water management. Efforts to breed for salt tolerance have resulted in the development of

some crop varieties with enhanced tolerance to NaCl. For example, more tolerant lines of alfalfa, barley, tomato and wheat have been selected (Epstein et al., 1980; Shannon, 1984). Progress with this approach has been slower than anticipated due to a combination of factors, which include (i) incomplete knowledge of the effects of salinity on plants (ii) the interacting nature of ionic and osmotic properties of salts on plants (iii) ineffective selection methods (iv) changes in salt tolerance with ontogeny and (v) the lack of knowledge about the genes that affect salt tolerance (Shannon, 1984 and refs. therein). In addition, this approach is dependent upon sufficient genetic variation in salt tolerance within the existing germplasm, and some crop plants have shown only minor intraspecific differences (Shannon, 1984 and refs. therein).

Cell culture is a major research strategy currently being used towards the development of plants with increased tolerance to salinity. While being of potential value to breeders as a source of variation (spontaneous and induced), it has also been sought as a tool to obtain information on the physiological mechanisms associated with salinity tolerance. The advantages, disadvantages and potential of this approach have been discussed (Hanson, 1984; Stavarek and Rains, 1984; Epstein and Rains, 1987; Dracup 1991). Briefly, advantages include (i) ability to control environment and nutrient conditions, (ii) ability to manipulate large numbers of cells, (iii) increased potential for the selection of salt-tolerant variants and (iv) physiological characterizations at the cellular level. Disadvantages include the uncertain expression of the isolated character at the whole plant level and, in some species, the difficulty of regeneration of cell variants to whole plants. Numerous plant cell lines have been

selected for tolerance to salinity, and in most examples tolerance was not documented in the whole plant (Epstein and Rains, 1987 and refs. therein). In addition, this approach has had only limited success in developing stable regenerated plants whose inheritance patterns are clearly understood (Nabors et al., 1980; Bressan et al., 1987: Epstein and Rains, 1987 and refs. therein; Winicov, 1991).

Genetic engineering is a promising new approach that could have tremendous potential in developing plants possessing enhanced tolerance to NaCl. The prospects for genetic engineering of salt-tolerant crops have been reviewed (Hanson, 1984; Epstein and Rains, 1987; Cushman et al., 1990). In order for this approach to be effective some knowledge is necessary regarding the of the physiological mechanisms and genetic components associated with salt tolerance. While the technology for introduction and expression of foreign DNA has progressed, our understanding of tissue-specific and developmental stage-specific gene expression is limited (Ramagopal, 1987; Bohnert et al., 1989; Claes et al., 1990; Cushman et al., 1990). And perhaps more importantly, there is to date no knowledge of what genes to introduce or how to control the expression of those genes as needed.

Extending our knowledge of the physiological mechanisms associated with salinity tolerance is of fundamental importance to developing plants with enhanced salinity tolerance. A number of reviews provide comparative physiological analyses of glycophytes and halophytes and their adaptive responses(s) in the face of salt stress (Flowers et al., 1977; Greenway and Munns, 1980; Yeo, 1983; Rhodes, 1987; Flowers and Yeo, 1986; Munns and Termatt, 1986). Although a wide variety of

physiological manifestations have been reported, it is clear that ion relations play an important role in the overall effect of salinity on both glycophytes and halophytes (Flowers and Yeo, 1986; Cheeseman, 1988). In general, most halophytes respond to salinity by taking up Na^+ and Cl^- and accumulating these ions in the vacuoles of leaf cells, while osmotic adjustment of the cytoplasm is achieved by the accumulation of "compatible" organic solutes. Glycophytes typically restrict the net transport of Na^+ from the root to the shoot. Sensitivity in these plants is thought to result from (i) inability to adjust osmotically or (ii) inability to control ion uptake and/or compartmentation.

While significant progress has been made in identifying physiological responses associated with salt stress, less is known regarding the genetic components contributing to a tolerant phenotype. Most studies involve comparisons of different taxa or varieties, thus making it difficult to identify specific genetic differences associated with the salt stress response. Several studies provide information regarding regulation at the molecular level during adaptation of individual genotypes (Hurkman and Tanaka, 1987; Ramagopal, 1987; Bohnert et al., 1989; Hurkman et al., 1989; Cushman et al., 1990; Claes et al., 1990). However, this approach has not resulted in the identification of genetic differences between salt sensitive and tolerant genotypes.

2. RATIONAL: WHY USE *CERATOPTERIS*?

Isogenic lines that are identical in all features except the ability to tolerate increasing concentrations of salt would be a valuable tool for integrated genetic and physiological studies. It is in this respect that the fern *Ceratopteris richardii* has been useful. Features of the *Ceratopteris* life cycle are well documented (Hickok et al., 1987). These features have been exploited in the development of an effective and efficient haploid selection system for the isolation of a wide variety of mutations (Hickok et al., 1987). This system has an advantage over other plant systems in that both dominant and recessive mutations affecting specific traits can be quickly and easily selected and characterized.

Recently two single gene nuclear mutations associated with salt tolerance in gametophytes of the fern were isolated (Warne and Hickok, 1987). Both mutations conferred low levels of NaCl tolerance ($\leq$ 100 mM NaCl) to gametophytes. Subsequent mutagenesis of one of these strains, containing the mutation *stl1*, resulted in the isolation and identification of an additional nuclear mutation, *stl2*, conferring a high level of tolerance ($>$ 225 mM NaCl) to gametophytes (Hickok et al., 1991).

Other examples of single gene mutations associated with salt tolerance are limited. Able (1969) reported a recessive single gene mutation responsible for Cl- exclusion in a variety of soybean. Kueh and Bright (1982) identified proline accumulating mutants of barley that were tolerant to low ($<$ 100 mM) salt concentrations. More recently, three single gene mutants of *Nicotiana plumbaginifolia* were isolated by selecting

protoplast-derived colonies resistant to NaCl, KCl and PEG (Sumaryati et al., 1992). The physiological basis of tolerance in all three mutants was found to be proline accumulation, with the mutants accumulating higher levels of proline than the wild type on basal and salinized media (Sumaryati et al., 1992). Saleki et al. (1993) report the isolation of three recessive mutations conferring tolerance (175 - 225 mM NaCl) to germinating seeds of *Arabidopsis thaliana* var Columbia. In the same study germinating seeds were found to accumulate higher levels of Na^+ and K^+ in the presence of elevated levels of NaCl.

Single gene mutants provide a means of studying the contribution of individual genetic components in the expression of the physiologically and genetically complex salt tolerant phenotype. The *stl1* and *stl2* mutations have been genetically characterized and shown to be unlinked single gene nuclear mutations, but the physiological basis of tolerance has yet to be determined.

3. OBJECTIVES

Physiological characterizations of the *stl1* and *stl2* mutations have thus far involved a study of their level of tolerance to NaCl. With increasing NaCl, both spore germination and gametophyte growth of the mutants are greater than the wild type (Warne and Hickok, 1987; Hickok et al., 1991). Tolerance was also observed in the sporophytic generation, however a much lower tolerance (60 mM NaCl) was observed in mutant homozygote sporophytes than in gametophytes (Hickok et al., 1991). In addition, growth enhancement was observed in *stl1* even in the absence of NaCl, while *stl2* showed restricted growth relative to the wild type in the absence of NaCl. The *stl2* mutation also showed enhanced tolerance to increasing levels of hydroxyproline, relative to the wild type and *stl1* (Hickok, unpublished).

The principal goal of this study was to provide basic information regarding the physiological basis of tolerance associated with the *stl1* and *stl2* mutations in the gametophyte stage of development. Initial studies were designed to address the kinds of stress imparted by NaCl. These include osmotic stress and ionic stress, the latter resulting from the specific toxic effects of accumulated Na+ and/or Cl- or interference with internal solute balance (Rains, 1979). Specific objectives included determining if tolerance is (i) associated with osmotic or ionic stess imposed by NaCl, (ii) specific for Na^+ and/or Cl^-, (iii) associated with an enhanced ability to adjust osmotically (iv) associated with an enhanced ability to control ion uptake and/or compartmentation. When it was subsequently established that the *stl2* mutation

involved altered Na^+ and K^+ accumulation during salinity stress, Na^+/Ca^+ and Na^+/K^+ interactions were examined to gain a better understanding of the role of these three ions in the tolerant response. Lastly, membrane lipid composition was examined to determine if differences in composition were correlated with salinity tolerance imparted by *stl1* and *stl2*.

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PART 2

SOLUTE ANALYSIS AND WATER RELATIONS OF GAMETOPHYTE MUTANTS TOLERANT TO NACL IN THE FERN *CERATOPTERIS RICHARDII*

Vogelien DL, Hickok LG, Augé RM, Stodola A, Hendrix D (1993) Solute analysis and water relations of gametophyte mutants tolerant to NaCl in the fern *Ceratopteris richardii*. Plant, Cell and Environ (in press).

1. INTRODUCTION

The mechanisms enabling plants to achieve enhanced tolerance to salt are as yet not completely understood. Available information clearly shows that salt stress responses are complex, both at the cellular and organismal levels (Epstein and Rains, 1987; Cheeseman, 1988; Claes et al., 1990). Such complexity, coupled with the quantitative nature of the trait, suggests that the salt tolerant phenotype involves the interplay of a number of genes (Shannon, 1984; Tal, 1984; Cushman et al., 1989).

A number of reviews provide comparative physiological analyses of glycophytes and halophytes and their adaptive response(s) in the face of salt stress (Flowers et al., 1977; Greenway and Munns, 1980; Yeo, 1983; Rhodes, 1987). Although a wide variety of physiological manifestations have been reported, it is clear that ion relations play an important role in the overall effect of salinity on both glycophytes and halophytes (Flowers and Yeo, 1986; Cheeseman, 1988). In general, most halophytes respond to salinity by taking up Na^+ and Cl^- and accumulating these ions in the vacuoles of leaf cells. Osmotic adjustment of the cytoplasm is achieved by the accumulation of "compatible" organic solutes, such as proline, glycine betaine, organic acids and sugar alcohols. Many glycophytes typically respond to low salt concentrations (below 100 mol m^{-3}) (mol m^{-3} = mM) by restricting the net transport of Na^+ from the root to the shoot and may be limited in their ability to adjust osmotically. Some salt-sensitive glycophytes (e.g., fruit trees) are unable to control ion uptake and compartmentation and thus suffer from the effects of ion toxicity.

While significant progress has been made in identifying physiological responses associated with salt stress, less is known regarding the genetic components contributing to a tolerant phenotype. Most studies involve comparisons of different taxa or varieties, thus making it difficult to identify specific genetic differences associated with the salt stress response. In vitro plant cell and tissue culture has been used to generate salt tolerant varieties (Stavarek and Rains, 1984; Bressan et al., 1987), but this approach has had only limited success in developing stable regenerated plants whose inheritance patterns are clearly understood (Nabors et al., 1980; Bressan et al., 1987; Dracup, 1991). Other studies have provided information regarding regulation at the molecular level during adaptation of individual genotypes (Hurkman and Tanaka, 1987; Hurkman et al., 1989; Cushman et al., 1989; Claes et al., 1990). However, this approach has not resulted in identifying individual genetic differences between salt sensitive and tolerant genotypes.

The ideal experimental plant material for integrated physiological and genetic studies would be isogenic strains differing solely in the ability to tolerate increasing concentrations of salt (Shannon, 1984). However, examples of single gene mutations associated with salt tolerance are limited. Abel (1969) reported a recessive single gene mutation that confers salt sensitivity in a variety of soybean by altering the transport of Cl^- ions from root to shoot. Kueh and Bright (1982) identified proline accumulating mutants of barley that were tolerant to low (< 100 mol m^{-3}) salt concentrations. More recently, Warne and Hickok (1987) described the isolation of two single gene nuclear mutations associated with salt tolerance in gametophytes of

the fern *Ceratopteris richardii*. These mutations were found to confer low levels of NaCl tolerance to gametophytes. Subsequent mutagenesis of one of these strains, containing the mutation stl1, resulted in the isolation and identification of an additional nuclear mutation, stl2, conferring a high level of tolerance (> 275 mol m^{-3} NaCl) to gametophytes (Hickok et al., 1991).

The stl1 and stl2 mutations have thus far been characterized only by their level of tolerance to NaCl. With increasing external NaCl concentration, both spore germination and gametophytic growth of the mutants are greater than the wild type. Growth enhancement is observed in stl1 even in the absence of NaCl, while stl2 and the double mutant show restricted growth relative to the wild type in the absence of NaCl. In addition, stl2 shows enhanced tolerance to the proline analogue hydroxyproline (unpublished data). One possible mechanism resulting in tolerance to hydroxyproline involves the overproduction of proline, which could attenuate the effect of hydroxyproline on proteins and cell metabolism. The present study examines a number of physiological and metabolic characteristics in these genotypes under stressed and unstressed conditions. The levels of several organic solutes (amino acids, betaine, sugars and organic acids) and major ions (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-) are compared, as well as the ability of each strain to adjust osmotically under salt stress.

2. MATERIALS AND METHODS

Culture

Spores of *Ceratopteris richardii* Brongn., strain Hn, served as the wild type (W.T.) spore source in these studies (Hickok et al., 1987). Strain N10, a weakly salt tolerant strain containing the mutation stl1, was derived from the wild type by mutagenesis and selection as previously described (Warne and Hickok, 1987). Strain 10α23, highly salt tolerant and containing the stl1 mutation and additional stl2 mutation, was derived from N10 spores by similar mutagenesis and selection (Hickok et al., 1991). Strain N23, containing the stl2 mutation, was obtained during the genetic analysis of 10α23 by backcrossing with the wild type (Hickok et al., 1991).

Gametophyte tissue for analysis of organic solutes was obtained by sowing surface sterilized spores onto nutrient medium containing 0, 60, and 140 mol m^{-3} NaCl following standard procedures described by Warne and Hickok (1987). To facilitate harvesting, gametophyte tissue used in ion and pressure-volume analyses was obtained from liquid cultures established by inoculating spores into liquid nutrient medium at a sowing density of 6 mg spores/ 200 cm^3 medium. Liquid cultures were maintained on a gyrator shaker at 100 rpm. All cultures were maintained under constant conditions at 28 $\pm$ 2 °C and approximately 85 μmol m^{-2} s^{-1} PAR. Tissue was harvested for analytical use at 21 days following initiation of culture. Culture method (solid vs. liquid culture media) does not affect the growth response of any of the genotypes to salinity stress. Growth of wild type and N10 gametophytes is similarly reduced by 60

mol m^{-3} NaCl (approximately 20% and 15%, respectively) when grown on solid or in liquid culture media. Neither gametophyte size or development of strain N23 is affected by 60 mol m^{-3} NaCl stress under either culture method.

Analysis of Free Amino Acids

Amino acids were extracted using a procedure modified from Rhodes et al. (1987). Gametophyte tissue was harvested and immediately extracted by homogenizing with methanol (approximately 0.4 g fresh weight / 5 cm^3 methanol). Tissue was extracted once more with 5 cm^3 methanol and the methanol extracts combined. Extracts were stored at 4 °C for 48 h, at which time a known amount of *l*-norleucine was added to serve as an internal standard (100 - 250 nmol). Following the addition of the standard, 5 cm^3 $CHCl_3$ and 6 cm^3 distilled H_2O were added, and the extracts were allowed to phase separate. The upper aqueous phase was evaporated to dryness under a stream of dry air. The residue was redissolved in 2 cm^3 distilled H_2O and applied to a 1 x 2 cm Dowex-50-H^+ column. Columns were washed with 10 cm^3 distilled H_2O and amino acids were eluted with 6 cm^3 6 kmol m^{-3} NH_4OH. The NH_4OH eluates were divided into two equal volumes and evaporated to dryness under a stream of dry air. Amino acid analyses were performed using Waters Pico-Tag reverse phase pre-column PTC-AA derivatization as described by Bidlingmeyer (1984), except that a 4.6 mm x 25 cm column was used.

Analysis of Betaines

Gametophyte tissue, approximately 0.2 g fresh weight (gfw), was quickly placed in 2 cm^3 of ice-cold methanol:chloroform:water (MCW) (12:5:3, v/v/v) and stored at -40 °C until ready to proceed. Samples were homogenized, centrifuged to remove cell material, and extracted twice more with 2 cm^3 ice-cold MCW (12:5:3, v/v/v). The pooled supernatants were extracted with 2.5 cm^3 CW (2:3, v/v) overnight at 4 °C. The upper aqueous layer was collected and evaporated to dryness under a stream of dry air. The residue was redissolved in 1 cm^3 distilled H_2O prior to measurement of quaternary ammonium compounds. Betaine and choline were determined by the method of Storey and Wynn (1977). Glycine betaine and choline were used as standards.

Analysis of Soluble Sugars and Organic Acids

Gametophyte tissue, approximately 50 mg, was quickly placed in 1.5 cm^3 80% ice-cold ethanol and stored at -20 °C prior to analysis. Tissue was subsequently washed 3-5 times with 2 cm^3 80% ethanol for 15 min at 80 °C and the supernatents pooled. It was assumed that carbohydrate extraction was complete when the supernatant was no longer green. The alcohol extracts were then centrifuged through a bed of activated charcoal in microcentrifugation tubes to remove substances which might interfere with enzymatic analyses (Hendrix and Peelen, 1987). Samples of ethanol extracts were pipetted into microtitration plate wells, the ethanol evaporated by incubation at 50 °C, and the residue remaining redissolved in 20 mm^3 distilled

H_2O. The microtitration plates were then covered and allowed to equilibrate for 1 hr. Glucose, fructose, sucrose and malate levels were determined with a commercial colorimetric analysis procedure (Sigma Chemical Co., Kit 115-A). Glucose in sample residues was enzymatically converted to glucose-6-phosphate and quantified by measuring the reduction of iodonitrotetrazolium violet (INT) in the presence of glucose-6-dehydrogenase. Fructose was converted enzymatically to fructose-6-phosphate and subsequently determined as glucose-6-phosphate following addition of phosphoglucoisomerase (Sigma Chemical Co.) to sample wells and assaying as above. Sucrose in the ethanolic residue was determined by similarly assaying for glucose and fructose after hydrolysis with invertase (Sigma Chemical Co.). Malate in the residue was determined in a similar fashion from the reduction of INT color reagent using malate dehydrogenase (Sigma Chemical Co.). Absorbance for all microplate assays was measured using an ELISA plate reader at 492 nm. HPLC analyses of ethanol extracts confirmed the results obtained by enzymatic analysis and rule out the possibility that unidentified sugars and organic acids other than those described accumulate to any significant levels in unstressed and stressed tissue.

Ion Analysis

Gametophytes were harvested by filtering onto miracloth(TM) using a Buchner funnel with vacuum. Collected gametophytes were washed four times with 100 cm^3 of a mannitol solution that was isotonic to the liquid culture medium. After fresh weight determination, gametophytes were frozen in liquid N_2, ground to a fine

powder, lyophilized, and stored at -20 °C. For cation analyses approximately 100 mg dried ground tissue was thoroughly soaked in 10 cm^3 HNO_3. Five cm^3 60% $HClO_4$ was added and the extract heated on a hot plate until the HNO_3 was almost evaporated. The sample was allowed to cool, at which time 10 cm^3 of 6 kmol m^{-3} HCl was added, followed by a final dilution to 100 cm^3 with distilled H_2O. Major cation levels (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were measured by ICP emission spectroscopy. Cl^- was extracted by incubating approximately 50 mg dried ground tissue in 5 cm^3 distilled H_2O at 85 °C for 1 h. Samples were then homogenized using a Virtishear(TM), centrifuged to remove plant material, and extracted twice more with 5 cm^3 distilled H_2O at 85 °C for 30 min. The supernatents were pooled and filtered using a 0.45 μm acrodisc filter unit. Cl^- contents were determined by ion chromatography.

Water Relations

Osmotic potential (Ψ_π) of culture media was measured with a Wescor 5500x vapor pressure osmometer (Wescor, Logan, UT), corrected to ambient laboratory temperature (24 °C). Water relations of gametophytes were characterized with pressure-volume (PV) analysis (Tyree and Jarvis, 1982), using thermocouple psychrometry to estimate gametophyte water potential (Ψ). Gametophyte cultures were poured into a vacuum filter funnel onto ashless filter paper and quickly rinsed with distilled H_2O. Surface water was then removed with vacuum. Although surface water probably could not be removed entirely without damaging or dehydrating

gametophyte protoplasm, vacuum filtration proved to be an effective method for rinsing NaCl and removing surface water from the quite hydrophobic gametophyte surfaces. No water was observed on gametophyte surfaces under microscopic examination following filtration. Following filtration, filter papers with gametophytes were immediately placed in covered petri dishes inside a humid chamber, where samples (0.1 to 0.2 gfw) were removed from dishes and inserted into SC-10 thermocouple psychrometers with sample changers connected to NT-3 nanovoltmeter thermometers (Decagon Devices, Pullman, WA). Sample manipulations were performed in an enclosed, humid chamber to minimize evaporative water loss from tissues. Vapor equilibrium of samples with the gas phase within the psychrometer generally occurred within 10 min, at which time μV readings were recorded. Samples were removed from psychrometers and allowed to dry between successive Ψ measurements. Psychrometers were calibrated with a graded series of NaCl solutions and standard psychrometer operating procedures were observed (Briscoe 1984). PV parameters were computed as described previously (Augé, 1991). Bulk gametophyte Ψ_π and turgor potential (Ψ_p) were calculated between hydration at harvest (100% gametophyte relative water content (RWC)) and about 40% gametophyte RWC. Values for Ψ_p were computed as the difference between Ψ and Ψ_π at any particular RWC. PV plots were generated for samples from four replicate flasks of each genotype at 0 mol m^{-3} and 60 mol m^{-3} NaCl, for a total of 24 plots. The mean standard error represents the mean of the standard errors for all data points in a figure and was calculated by taking the square root of the error mean square from the

analysis of variance and dividing it by the square root of the number of observations in a mean (Steel and Torrie, 1980).

3. RESULTS

In all studies, strains were examined under stressed and unstressed conditions. A NaCl concentration was chosen that results in slight reduction in wild type gametophyte growth (Hickok et al., 1991). At 60 mol m^{-3} NaCl the wild type and strain N10 show an approximate 20% and 15% reduction in gametophyte growth, respectively, while no effect on growth is observed in strains N23 and 10α23. Comparisons between the wild type and NaCl tolerant strains were made at this concentration.

Organic Solute Levels

The levels of several low molecular weight organic compounds were examined. In most cases differences between strains in response to NaCl stress (60 mol m^{-3}) were minimal. Changes in free amino acid pools of the wild type and strain 10α23 are shown in Table 1. The wild type and 10α23 had similar free amino acid composition under unstressed conditions. A two-fold increase in total free amino acids was observed at 60 mol m^{-3} NaCl in both strains, and again the free amino acid compositions were similar. The increase was due to an accumulation of the majority of amino acids. The singular notable difference observed at 60 mol m^{-3} NaCl in free amino acid composition between the wild type and 10α23 was the greater accumulation of GLN in 10α23. GLN, GLU and SER were the major components of the free amino acid pool in unstressed cultures of both the wild type and 10α23.

Table 1. Intracellular Pools of Amino Acids and Betaine in the Wild Type and Strain 10α23 Under Stressed and Unstressed Conditions.

	wild type		10α23		
	NaCl concentration(mol m^{-3})				
solute	0	60	0	60	140
ASP	[a]5.8	5.8	5.1	4.3	3.2
GLU	14.6	14.4	17.0	14.5	7.3
ASN	3.1	5.8	3.5	4.1	2.6
SER	16.0	11.1	16.3	10.3	5.7
GLN	37.9	37.6	38.2	48.9	45.7
GLY	3.5	2.5	3.4	1.5	1.2
HIS	0.3	[c]ND	0.5	0.3	0.3
ARG	0.1	0.2	ND	ND	ND
GABA	2.6	2.3	2.2	1.6	1.1
THR	3.1	3.2	2.6	1.7	0.8
ALA	5.7	3.3	6.0	3.1	1.5
PRO	0.7	9.8	0.6	7.1	28.9
BUT	ND	ND	ND	ND	ND
TYR	0.2	0.2	0.1	0.1	0.03
VAL	1.5	1.1	1.3	0.8	0.5
MET	1.2	0.4	0.2	0.1	0.04
ILE	0.8	0.4	0.6	0.2	0.1
LEU	0.5	0.4	0.2	0.1	0.1
PHE	2.1	1.2	2.2	1.2	0.7
LYS	0.3	0.3	0.1	0.1	0.1
total (μmol/gfw)	7.80	15.37	8.48	19.08	50.58
betaine (μmol/gfw)	[b]0.78	0.73	0.76	0.80	1.95

[a]Each value represents the average of two independent analyses and is provided as a percent of the total free amino acid pool.
[b]Each value represents the average of two independent samples.
[c]Not detected.

Although none of these amino acids increased significantly as a percent of the total pool in the wild type at 60 mol m^{-3} NaCl, GLN levels increased 10% above that found in unstressed 10α23 tissue. Increases in proline content were greater than that of any other amino acid at 60 mol m^{-3} NaCl (12-fold increase), however this increase was detected in both the wild type and 10α23. To examine further the contribution of GLN and PRO to the amino acid pool in 10α23 during NaCl stress, free amino acid levels were examined in 10α23 at 140 mol m^{-3} NaCl stress. This NaCl concentration achieves a similar 20% reduction in gametophyte size in 10α23 (and N23), but is lethal to the wild type. The level of total free amino acids continued to increase in 10α23 at this NaCl concentration. Only proline became an increasingly larger percent of the total amino acid pool, resulting in an approximate 48-fold increase in proline levels above the control (0 mol m^{-3} NaCl). The level of GLN was similar to that observed for tissue exposed to 60 mol m^{-3} NaCl.

Betaine levels for the wild type and 10α23 are also given in Table 1. Similar levels were detected in both strains in unstressed conditions and remain unchanged in response to 60 mol m^{-3} NaCl stress. A two-fold increase in the level of betaine was observed for 10α23 at 140 mol m^{-3} NaCl treatment.

Other organic solutes examined include various soluble carbohydrates and organic acids (Table 2). Sucrose was the major sugar present in all genotypes under both conditions, accompanied with lesser amounts of glucose and fructose. Malate was the only organic acid found in significant levels. Similar levels of glucose were detected

Table 2. Concentration of Organic Solutes in the Wild Type and NaCl-Tolerant Strains Under Stressed and Unstressed Conditions. Each value represents the mean of five replicate samples. Means with the same letter within a column are not significantly different as determined using Duncan's Multiple Range Test (N= 5, alpha= 0.05).

		Solute Concentration (millimoles/ kg plant water)			
strain	[NaCl]	glucose	fructose	sucrose	malate
W.T.	0	1.13^{AB}	0.58^{B}	1.44^{B}	0.21^{C}
	60	0.90^{B}	0.38^{B}	4.56^{A}	1.09^{B}
N10	0	1.39^{A}	1.05^{A}	2.12^{B}	0.68^{B}
	60	0.84^{B}	0.24^{B}	4.08^{A}	0.90^{B}
N23	0	0.82^{B}	0.29^{B}	1.72^{B}	1.09^{B}
	60	0.79^{B}	0.21^{B}	3.86^{A}	1.84^{A}

in all genotypes in unstressed conditions and remained unchanged in response to NaCl stress. Fructose levels were similar in the wild type and strain N23 under both conditions. Slightly higher constitutive levels of fructose were detected in strain N10, but a level similar to those found in the wild type and N23 was detected at 60 mol m^{-3} NaCl stress. Similar constitutive levels of sucrose were detected in the three genotypes and roughly doubled in all strains in response to 60 mol m^{-3} NaCl. Strains N10 and N23 had slightly higher levels of malate in the absence of NaCl stress than the wild type. During 60 mol m^{-3} NaCl stress the wild type and N10 accumulate similar levels of malate, while N23 continues to accumulate slightly higher levels. Although sucrose and malate levels increase in all strains during 60 mol m^{-3} NaCl stress their individual contribution to Ψ_π is relatively low in all genotypes (less than 3% for each). HPLC analysis of whole tissue extracts confirm that glucose, fructose, sucrose and malate are the major organic sugars and acids present. Consequently, it is unlikely that unidentified sugars and organic acids other than these accumulate to any significant levels in unstressed and stressed tissues.

Inorganic Solute Levels

The levels of several major cations and Cl^- were monitored in the wild type and strains N10 and N23 under stressed and unstressed conditions to examine the possibility that the enhanced tolerance to NaCl was associated with altered ion accumulation (Table 3). Similar ion compositions were observed in the wild type and N10 in the absence of NaCl stress, while N23 showed slightly higher constitutive

Table 3. Concentrations of Inorganic Solutes in the Wild Type and NaCl-Tolerant Strains Under Stressed and Unstressed Conditions. Each value represents the mean of three independent analyses. Means with the same letter within a column are not significantly different as determined using Duncan's Multiple Range Test (N= 3, alpha= 0.05).

		Ion Concentration (millimoles/ kg plant water)					
strain	[NaCl]	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	K^+/Na^+
W.T.	0	0.22^{D}	55.44^{C}	2.31^{C}	3.95^{B}	18.40^{C}	
	60	46.99^{A}	57.44^{C}	4.10^{A}	6.95^{A}	40.76^{AB}	1.1^{B}
N10	0	0.30^{D}	57.99^{C}	2.09^{BC}	3.72^{B}	22.04^{C}	
	60	32.88^{B}	55.66^{C}	2.62^{BC}	5.01^{B}	33.38^{B}	1.7^{B}
N23	0	0.34^{D}	81.99^{B}	2.89^{B}	4.38^{B}	23.64^{C}	
	60	24.08^{C}	99.59^{A}	2.21^{C}	3.46^{B}	46.36^{A}	4.0^{A}

levels of K^+. Substantial changes and differences in ion levels were observed in response to NaCl stress. Cl^- levels roughly doubled in all strains, while Ca^{2+} and Mg^{2+} levels changed relatively little. A large increase in the level of Na^+ was detected in all strains, but both N10 and N23 accumulated substantially less Na^+ than the wild type. In addition, N23 showed a slight increase in K^+ content in response to NaCl, while essentially no change was detected in N10 or the wild type. A K^+/Na^+ ratio close to 1 is observed in the wild type, while this ratio increases in strains N10 and N23 to 1.7 and 4.0, respectively. The increase in the K^+/Na^+ ratio correlates with the level of NaCl tolerance in these strains.

Water Relations

Analysis of variance indicated no significant main effect of genotype in any of the water relations characters depicted in Fig. 1. Turgor at harvest ranged from 0.6 to 0.8 MPa in gametophytes grown at 0 mol m^{-3} and 60 mol m^{-3} NaCl (Fig. 1A). Ψ at harvest ranged from -0.09 to -0.15 MPa in gametophytes grown at 0 mol m^{-3} NaCl and was slightly lower, -0.2 to -0.24 MPa, in gametophytes grown at 60 mol m^{-3} NaCl (Fig. 1B). 60 mol m^{-3} NaCl would be expected to lower the Ψ of the external medium by 0.29 MPa, and this is similar to that observed, for the Ψ of the external medium at 0 and 60 mol m^{-3} NaCl was -0.05 and -0.29 MPa, respectively. The observed drop in Ψ for gametophyte tissue at 60 mol m^{-3} NaCl, which should be in equilibrium with the external medium, is only around 0.1 MPa. This difference between expected and observed values may be due to an overestimation of Ψ for

Figure 1. Ψ_p (A), Ψ (B) and Ψ_π at harvest and the turgor loss point (C) in the wild type and NaCl tolerant strains exposed to 0 and 60 mol m^{-3} NaCl stress for 21 days. Measurements were made by PV analysis using thermocouple psychrometry. Data are provided as the mean of 4 measurements. The standard errors of the mean are 0.12 (Ψ_p), 0.05 (Ψ), 0.22 (Ψ_π at turgor loss) and 0.12 (Ψ_π at harvest).

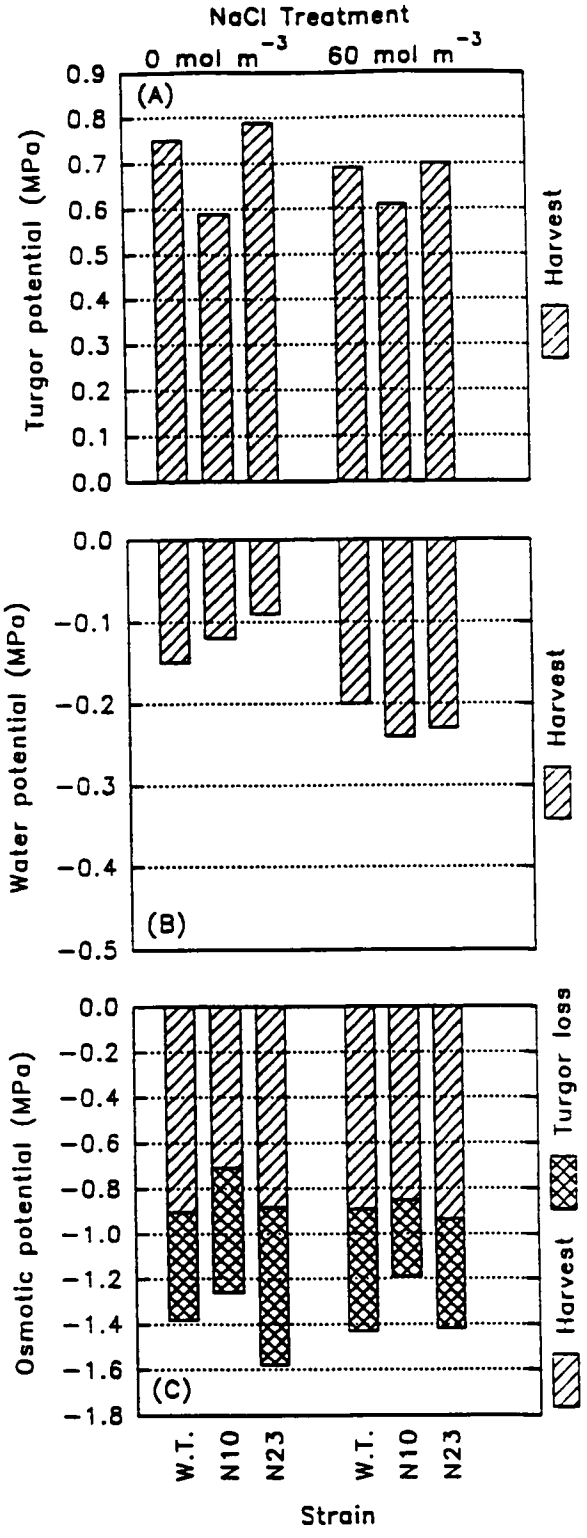
NaCl Treatment
0 mol m -3
60 mol m -3
(A)
Turgor potential (MPa)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Harvest
(B)
Water potential (MPa)
0.0
−0.1
−0.2
−0.3
−0.4
−0.5
Harvest
(C)
Osmotic potential (MPa)
0.0
−0.2
−0.4
−0.6
−0.8
−1.0
−1.2
−1.4
−1.6
−1.8
Harvest
Turgor loss
W.T.
N10
N23
W.T.
N10
N23
Strain

Figure 1

gametophyte tissue in the absence of salt stress, thus resulting in a more negative value. Support for this possible explanation lies in high level of variation for Ψ measurements at this treatment (SE of 0.05). Ψ_π at harvest remained near -0.9 MPa in N23 and the wild type, regardless of salt treatment (Fig. 1C). Ψ_π at harvest was 0.14 MPa lower in N10 gametophytes grown at 60 mol m^{-3} than those at 0 mol m^{-3} NaCl. Ψ_π at turgor loss was unaffected by NaCl treatment within each genotype.

Water content at harvest was similar among genotypes at each salt concentration (95% at 0 mol m^{-3} and 93% at 60 mol m^{-3}). Symplastic water percentages, 77% to 80% at 0 mol m^{-3}, were 11%, 18% and 7% lower at 60 mol m^{-3} in the wild type, N10 and N23.

The various organic and inorganic solutes that have been examined account for about 50% of the Ψ_π measured at harvest. The inability to account completely for the Ψ_π of gametophyte tissue may be due to the presence of unidentified solutes. However, due to the lack of significant differences in any water relations characters or major low molecular weight carbohydrates and organic acids, it seems unlikely that the genotypes differ in the ability to accumulate organic compounds to be used for osmotic adjustment of the cytoplasm.

The maximum contribution to Ψ_π in the absence of salinity stress was made by K^+ and Cl^- (all genotypes), contributing approximately 15% and 6%, respectively, of the osmotic potential of the wild type and strain N10, and 23% and 7% in strain N23. During 60 mM NaCl stress K^+, Na^+, and Cl^- were the major contributors to osmotic potential. Amino acids contributed approximately 2% and 5% to osmotic potential in

the absence and presence of 60 mM NaCl stress, respectively.

4. DISCUSSION

All strains exhibited major changes in total amino acid, soluble sugar, organic acid, and tissue ion levels in response to the 60 mol m^{-3} NaCl treatment. Only the wild type and strain N10 exhibit a reduction in growth at this concentration (Warne & Hickok, 1987), while no visible effect on gametophyte size or development is observed in the highly salt tolerant N23 single mutant (stl2) and 10α23 double mutant (stl1 stl2) strains (Hickok et al., 1991).

Differences in organic solutes between strains in response to 60 mol m^{-3} NaCl stress were minimal. A previous study had shown strain N23 to also posses enhanced tolerance to hydroxyproline (unpublished data), suggesting that tolerance to NaCl by this strain may be associated with proline overproduction. In this study, no difference in proline levels was found in a comparision between the wild type and double mutant. In addition, little difference in soluble sugars and organic acid content was detected in these genotypes. Strain N10 accumulated slightly higher constitutive levels of fructose (0.5 - 0.7 mol m^{-3}), however the significance of this difference in the adjustment of these strains to salinity is unclear. In addition, N23 has a slightly higher constitutive level of malate than the wild type and N10. Since the increase is small, approximately 1 mol m^{-3}, it is unlikely to be important in terms of osmotic adjustment. Alternatively, the higher constitutive level of malate in this strain may be an indirect effect of higher constitutive levels of K^+. Several forms of ion interaction have been suggested to be involved in the regulation of malate synthesis, such as the

control of malate synthesis by means of cytosolic pH (which presumably changes during the exchange of K^+ for metabolic H^+) or the release of feedback inhibition on PEP carboxylase due to the accumulation of K-malate in the vacuole (Osmond, 1976).

The organic solutes selected for comparisons in this study are commonly implicated to play a role in osmotic adjustment in a wide variety of plants (Flowers et al., 1977; Greenway and Munns, 1980). Glucose, fructose, sucrose and the cyclitol, pinitol, were the prevalent low molecular weight carbohydrates in the mangrove fern *Acrostichum speciosum* (Popp, 1984). With the exception of pinitol, these same carbohydrates were prevalent in *Pteris tremula*, a nonmangrove fern (Popp, 1984). Pinitol levels were examined in *Ceratopteris* gametophytes, and were found to be present in very low levels (D. Hendrix, unpublished data).

Lack of significant differences in water relations between genotypes agreed with the lack of any major differences in organic solute levels or total ion accumulation between genotypes in the absence or presence of NaCl stress. Although differences in various water relations characteristics between salt sensitive and salt tolerant plants are typical (Greenway and Munns, 1980; Yeo, 1983), other examples have documented minimal differences in water relations and ion accumulation (total or individual) when exposed to salinity stress (Kingsbury et al., 1984).

The single notable difference between the wild type and strain N10 was the slightly lower level of Na^+ accumulated by N10 at 60 mol m^{-3} NaCl stress. The stl1 mutation confers a low level of salt tolerance to gametophytes, thus it is likely that any difference in physiological or metabolic processes may also be small. Further, 60

mol m^{-3} NaCl stress results in nearly similar reductions of gametophyte growth in these strains. Comparison at a slightly higher NaCl concentration, one which would produce the greatest difference in gametophyte growth between the wild type and stl1, may be more effective in making physiological comparisons.

In contrast to organic solute and water relations profiles, ion analyses indicated some major differences between the wild type and N23. Substantial differences were evident with respect to Na^+ and K^+ accumulation. N23 contained higher constitutive levels of K^+ than did the wild type. Under conditions of 60 mol m^{-3} stress this strain continued to selectively accumulate K^+. Greater accumulation of K^+ and reduced accumulation of Na^+ results in a significantly higher K^+/Na^+ ratio (4.0 compared to 1.1 in the wild type). Since differences in both K^+ and Na^+ accumulation are associated with the stl2 mutation, higher K^+/Na^+ ratios may be the result of an enhanced ability to selectively take up K^+ and/or an enhanced ability to exclude Na^+, either by directly limiting Na^+ uptake or through a more active Na^+ extrusion. The involvement of such activities in plant responses to NaCl stress has been discussed by others (Greenway and Munns, 1980; Jeschke, 1984; Yang et al., 1990; Watad et al., 1991). Presumably, higher K^+/Na^+ ratios imply that a plant is better able to deal with ionic stress imposed NaCl, by reducing the potential toxic affect of Na^+ in the cytoplasm and/or maintaining the balance of other essential nutrient ions, such as K^+.

Salt tolerance in glycophytes has frequently been associated with the ability to limit the accumulation of Na^+ and maintenance of high K^+/Na^+ ratios in the shoots (Greenway and Munns, 1980; Gorham et al., 1985; Gorham et al., 1987; Yang et al.,

1990). The salt sensitive cell line of *Citrus aurantium* exhibited a greater loss of K^+ than the more tolerant selected lines during exposure of cells to increasing NaCl concentrations (Ben-Hayyim et al., 1985), resulting in a lower K^+/Na^+ ratio in the sensitive line relative to the tolerant lines. Ben-Hayyim et al. (1985) suggested that enhanced growth of the tolerant cells was related to the ability to retain higher levels of internal K^+.

Few examples of single gene mutations involved in the regulation of ion uptake or transport in higher plants are known. Perhaps the best known examples are the genes involved in Cl^- transport in soybean (Abel, 1969) and Na^+ accumulation in pepper (Tal and Benzioni, 1977; Benzioni and Tal, 1978). As documented here, stl2 is similarly associated with altered K^+ and Na^+ accumulation. In addition, a high level of tolerance to salt is conferred to gametophytes (Hickok et al., 1991). Because the haploid gametophyte is small in size, generally a few mm^2 in area, and growth is two dimensional, resulting in a structure that is a single cell layer in thickness, it is likely that stl2 is associated with a cellular response to salt stress. This suggestion is supported by the limited tolerance observed in homozygous mutant (slt2) sporophytes at 60 mol m^{-3} NaCl relative to wild type sporophytes (Hickok et al., 1991). Thus, stl2 provides an opportunity to study the role of a single gene in cellular regulation of ion accumulation during salinity stress. Continued selections for enhanced tolerance in sporophytes, using stl2 as the background genotype, may allow a better understanding of the role of a cellular process in an integrated whole plant response.

Acknowledgements

I would like to thank Robert Augé for performing the measurements of water potential and its components, Donald Hendrix for his contribution to the carbohydrate and organic acid analyses, Charles Murphy for overseeing the operation of the HPLC in the amino acid analysis, and Cindy Stiles for overseeing the operation of the ICAP AA. This work was supported by National Science Foundation grant No. DCB-8803620 to L.G.H. and USDA grant No. 92-37100-7673 to L.G.H. and R.M.A.

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PART 3

RESPONSE OF WILD TYPE AND NACL-TOLERANT MUTANT STRAINS OF *CERATOPTERIS RICHARDII* TO OSMOTIC AND IONIC STRESS

Vogelien DL, Hickok LG (1993) Response of wild type and NaCl-tolerant mutant strains of *Ceratopteris richardii* to osmotic and ionic stress. J Plant Physiol (submitted)

1. INTRODUCTION

Considerable progress has been made in the general understanding of the physiology of plant-salt interactions. A number of reviews are available that provide comparative physiological analyses of glycophytes and halophytes and their adaptive responses during salt stress (Flowers et al., 1977; Greenway and Munns, 1980; Flowers and Yeo, 1986; Rhodes, 1987; Cheeseman, 1988; Flowers and Yeo 1989). Salinity stress consists of osmotic and ionic effects (Rains, 1979), and it is thought that, when confronted with excess salinity, glycophytes suffer osmotic stress, due to an inability to osmoregulate, or ionic stress, resulting from inadequate control of ion uptake or compartmentation (Greenway and Munns, 1980). Halophytes succeed in avoiding these stresses in a variety of ways (Flowers et al., 1977; Greenway and Munns, 1980). Yet in spite of the extensive diversity in response types, the genetic mechanisms resulting in tolerance are not entirely understood. It has been acknowledged that isogenic lines will be a valuable tool for investigating controlling mechanisms and possible pleiotropic effects (Shannon, 1984; Tal, 1984).

A variety of ions can contribute to soil salinity (Epstein and Rains, 1987), however Na^+ and Cl^- are the most prevalent and considered to be the most important with respect to having toxic effects on plants (Flowers and Yeo, 1986). Consequently, most selections and physiological studies have used NaCl as the stress agent in studies on salinity. Some comparative studies of sensitive and tolerant cell lines and plants have used cross-tolerance tests to examine the effect of various

osmotic and ionic stress agents on growth and/or solute accumulation (Kochba et al., 1982; Stavarek and Rains, 1984; Ben-Hayyim et al., 1985; Ben-Hayyim et al., 1987; Hassan and Wilkins, 1988; Sumaryati et al., 1992; Saleki et al., 1993). Studies of this nature provide information regarding the contribution of osmotic or ionic effects in the salinity response. Cross-tolerance to a variety of stress agents may suggest a common factor or mechanism involved in tolerance. In addition, comparisons between various salts may reveal information on the sensitivity and tolerance to specific ions (Ben-Hayyim et al., 1985; Eshel, 1985; Kingsbury and Epstein, 1986; Hassan and Wilkins, 1988; Saleki et al., 1993).

In an attempt to approach the problem of salinity tolerance from a genetic perspective, we have isolated two unlinked single gene mutations that confer enhanced tolerance to NaCl in the fern *Ceratopteris richardii* (Warne and Hickok, 1987; Hickok et al., 1991). These mutations, designated *stl1* and *stl2*, confer, respectively, a low ($\leq$ 100 mM NaCl) and high ($>$ 225 mM NaCl) level of tolerance, with both spore germination and gametophyte growth of the mutants being greater than the wild type (Warne and Hickok, 1987; Hickok et al. 1991). The *stl2* mutation also shows enhanced tolerance to hydroxyproline, relative to the wild type and *stl1*, suggesting that this mutation might be associated with proline overproduction or accumulation. However, a comparison of water relations and the levels of various organic compounds revealed no differences between wild type and mutant genotypes in the absence or presence of moderate NaCl (60 mM) stress (Part 2). These findings eliminated the possibility that *stl1* or *stl2* possess an enhanced ability to accumulate an

organic compound(s) that could be used in osmotic adjustment of the cytoplasm. In the same study it was shown that the *stl1* mutant accumulated similar levels of Na^+ and Cl^- as well as other major ions as the wild type in the absence and presence of moderate (60 mM) NaCl stress, while the *stl2* mutant displayed higher constitutive levels of K^+, and accumulated higher K^+ and reduced Na^+ levels during 60 mM NaCl stress. Taken together, these findings suggest a differential response of the wild type and mutant strains to ionic stress imposed by NaCl.

Here we examine the effects of various ionic agents on gametophyte growth in an attempt to establish if tolerance imparted by the *stl1* and *stl2* mutations is specific for NaCl (Na^+ and/or Cl^-), or a general tolerance for cations and/or anions. Growth responses of wild type and mutant strains to isosmotic concentrations of osmotic stress agents (mannitol and melibiose) are also examined to substantiate that the *stl1* and *stl2* mutations confer tolerance to ionic stress imposed by NaCl. Ion accumulation in each genotype as a function of NaCl concentration and time of exposure to moderate (60 mM) NaCl stress is also examined to address the possible role of altered ion accumulation in the tolerant response of these mutants.

2. MATERIALS AND METHODS

Plant Material

Spores of *C. richardii* Brongn., strain Hn, served as the wild type spore source in these studies (Hickok et al., 1987). Strain *stl1*, a weakly salt tolerant strain, was derived from the wild type by mutagenesis and selection as previously described (Warne and Hickok, 1987). A highly salt tolerant strain, containing the *stl1* mutation and an additional (*stl2)* mutation, was derived from *stl1* spores by similar mutagenesis and selection (Hickok et al., 1991). The *stl2* genotype was obtained during the genetic analysis of the double mutant (*stl1 stl2*) by backcrossing with the wild type (Hickok et al., 1991).

Cross Tolerance Experiments

Strains were initiated by sowing disinfected spores onto agar-solidified nutrient medium lacking the stress agent following standard procedures described by Warne and Hickok (1987). Just prior to meristem development (approximately 6 days from soaking of spores), gametophytes were transferred to medium supplemented with a stress agent. Growth responses to mannitol and melibiose were made over a range of concentrations that were isosmotic to 50, 75, 100, 125, 150, 175 and 200 mM NaCl. An approximate osmotic potential was calculated for culture media containing a given NaCl concentration using the van't Hoff relationship: $\Psi_\pi = -CiRT$ (C = molality of the solution, i = ionic dissociation factor, R = gas constant, T = temperature in

°K). A concentration of mannitol or melibiose was used that made the same estimated contribution to media Ψ_π. The specificity of tolerance for a particular ion(s) was tested by replacing Na^+ with equivalent molar concentrations of K^+, Mg^{2+}, and Cl^- with SO_4^{2-} using the salts Na_2SO_4, KCl, K_2SO_4, $MgCl_2$ and $MgSO_4$. Comparative studies of gametophyte growth of strains in response to a particular treatment were performed by measuring areas of the essentially two-dimensional gametophytes. Gametophytes were removed from treatment 9 days after transfer and mounted on slides in Hoyer's medium (Beeks, 1955) mixed with 0.5% acetocarmine. Areas were measured with the aid of a dissecting microscope using the computer-interfaced BioQuant(TM) Image Analysis System. Each treatment consisted of 30 gametophytes.

NaCl Treatments for Ion Analysis

Gametophyte tissue used in ion analyses was obtained from liquid cultures established by inoculating disinfected spores into liquid basal medium at a sowing density of 6 mg spores/ 200 ml medium (Part 2). Tissue ion content was examined as a function of NaCl concentration and length of exposure to 60 mM NaCl. For the former study NaCl was added at 18 days following initiation of culture in 30 mM increments every 12 h until the desired final concentration was acheived (30, 60, 90, 120 and 150 mM). NaCl was added to cultures in the form of a sterile stock solution (1.4 M NaCl in nutrient media). To examine the effect of exposure time to NaCl on tissue ion content, 60 mM NaCl (added in 30 mM increments over 24 h) was added

to cultures upon initiation, after 1 week, 2 weeks and 18 days of culture. Control flasks received equivalent volumes of basal medium. Liquid cultures were maintained on a gyrator shaker at 100 rpm under constant conditions at 28 $\pm$ 2 °C and approximately 85 μmol m^{-2} s^{-1} PAR. Tissue was harvested for analytical use at 21 days following initiation of culture.

Cation Analysis

Gametophytes were harvested by filtering onto miracloth(TM) using a Buchner funnel with vacuum. Collected gametophytes were washed four times with 100 ml isotonic solution of mannitol. After fresh weight determination, gametophytes were frozen in liquid N_2, ground to a fine powder, lyophilized and stored under desiccant at room temperature. Approximately 200 mg dried ground tissue was dry ashed by heating at 450 °C for 4 h. Ashed tissue samples were taken up in 10 ml 2N HCl. Major cation levels (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were measured by ICAP atomic absorption spectroscopy (Thermo Jarrell Ash ICAP 61).

3. RESULTS

In order to determine if the *stl1* and *stl2* mutations confer tolerance to the osmotic or ionic components of salinity, the growth responses of the wild type and mutant strains to isosmotic levels of osmotic and ionic stress were examined. Mannitol and PEG are osmotica commonly used to generate water, or osmotic, stress. Previous studies found PEG to be highly toxic to *Ceratopteris* gametophytes (unpublished data). Since mannitol has been reported to penetrate some cells, thereby reducing the osmotic potential difference across the plasma membrane, an additional osmoticum, melibiose, was also utilized (Dracup et al., 1986). The growth responses of the three genotypes to increasing concentrations of mannitol and melibiose are shown in Figure 1. Differences between wild type and mutant strains during increasing levels of osmotic stress generated by mannitol are very small. Melibiose at concentrations of 350 mM and 400 mM results in complete inhibition of growth in all genotypes, while the same levels of mannitol result in significantly reduced growth, but not complete growth inhibition. These differences in growth responses between isosmotic levels of mannitol and melibiose may be an indication that mannitol is slowly being metabolized by gametophytes. Regardless, both *stl1* and *stl2* show a low level of tolerance to mannitol and melibiose, relative to the wild type. While the relative level of tolerance to melibiose is slightly greater than that for mannitol, particularly for *stl2*, it is still considered a low level of tolerance. For comparison, the growth responses of the wild type and mutant strains to increasing concentrations of NaCl are

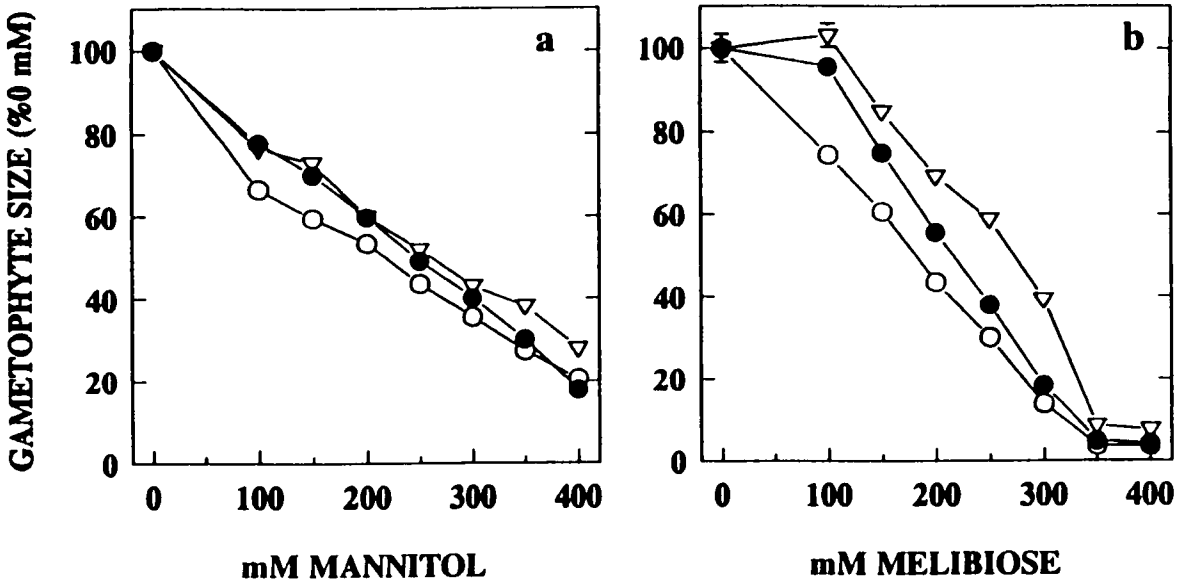

Figure 1. Growth Responses of the Wild Type, *stl1* and *stl2* Gametophytes to Mannitol (a) and Melibiose (b). Comparisons were made over a range of concentrations that were isosmotic to 0, 50, 75, 100, 125, 150, 175, and 200 mM NaCl. Gametophytes were examined nine days after transfer to medium supplemented with the stress agent. Data are provided as the mean $\pm$ standard error of 30 measurements. In most cases the standard error is smaller than the symbols. o, wild type; •, *stl1*; ▽, *stl2*

provided in Figure 2a. The growth curves presented in this figure are typical for these genotypes when challenged with increasing levels of NaCl, with *stl1* gametophytes showing a low level of tolerance ($\leq$ 125 mM NaCl), while a high level of tolerance is demonstrated by *stl2* ($>$ 200 mM NaCl). Comparisons of growth responses to mannitol, melibiose and NaCl are facilitated by the use of isosmotic concentrations between individual tests. A similar low level of tolerance to mannitol, melibiose and NaCl was observed for *stl1*. However, *stl2* shows a much higher level of tolerance to isosmotic concentrations of NaCl.

The specificity of tolerance for NaCl was examined by replacing Na^+ with equimolar concentrations of K^+ and Mg^+, and Cl^- with SO_4^{2-}. The growth responses of wild type, *stl1* and *stl2* gametophytes to increasing concentrations of NaCl, Na_2SO_4, $MgCl_2$, $MgSO_4$, KCl, K_2SO_4 are shown in Figure 2. The wild type was relatively sensitive to all salts tested. *stl1* gametophytes show a similar low level of tolerance to NaCl and Na_2SO_4 (Figure 2a-b), but were as sensitive as the wild type to other salts examined. In addition to NaCl, *stl2* shows high levels tolerance to Na_2SO_4, $MgCl_2$ and $MgSO_4$ (Figure 2a-d). One interesting feature of *stl2* gametophyte growth responses to ionic stress agents is the slight growth enhancement observed at low levels of NaCl, $MgCl_2$ and $MgSO_4$. All concentrations of KCl and K_2SO_4 examined were extremely toxic to the wild type and mutant strains (Figure 2e and f).

In a previous study the levels of major cations, Cl^- and various organic solutes

Figure 2. Growth Responses of the Wild Type, *stl1* and *stl2* Gametophytes to Sodium (a, b), Magnesium (c, d) and Potassium (e, f) Salts. Comparisons were made over a range of concentrations that were equimolar to 0, 75, 100, 125, 150, 175, and 200 mM Na^+ and Cl^-. Gametophytes were examined nine days after transfer to medium supplemented with the stress agent. Data are provided as the mean $\pm$ standard error of 30 measurements. In most cases the standard error is smaller than the symbols. o, wild type; •, *stl1*; ▽, *stl2*

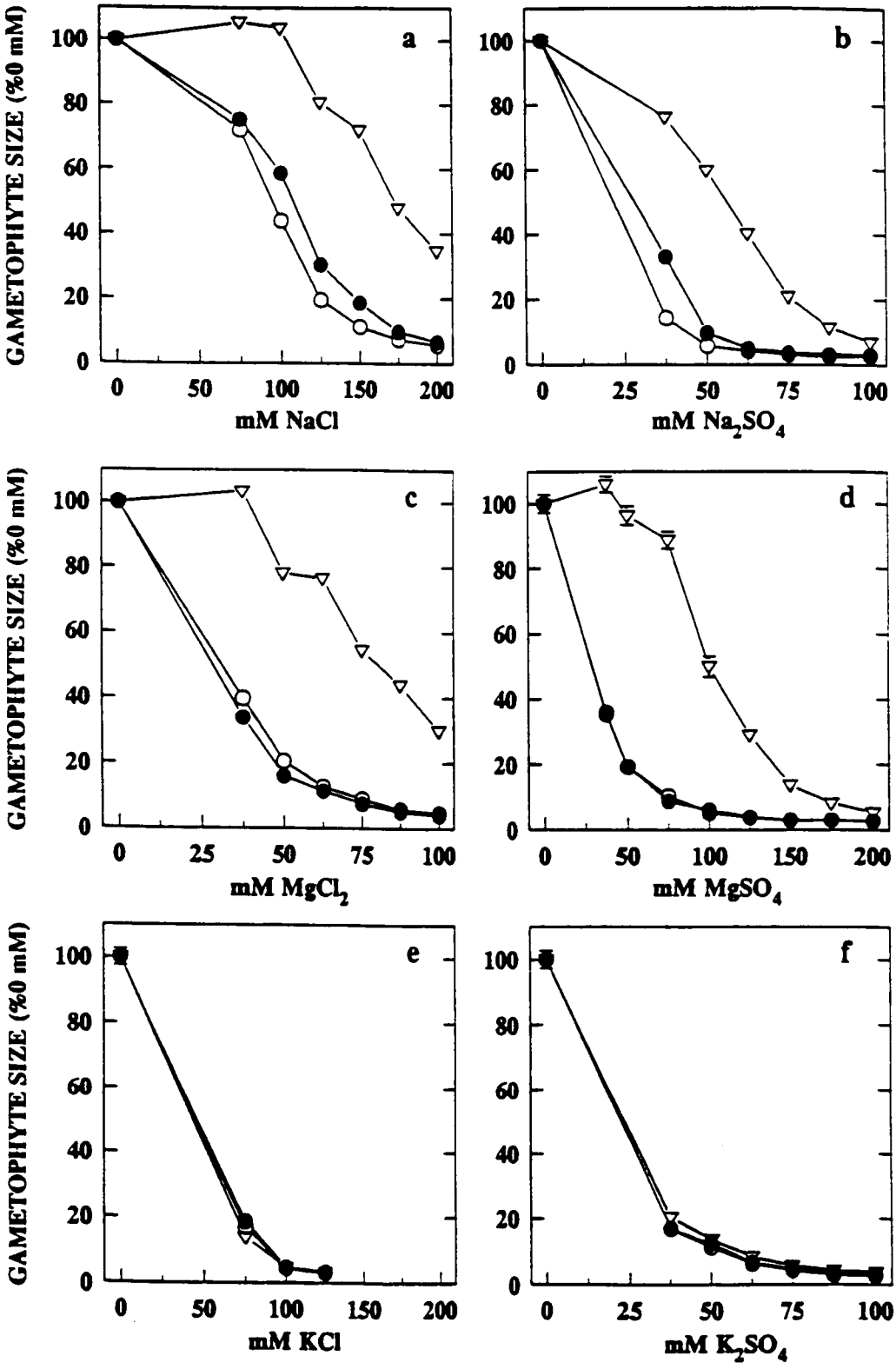
GAMETOPHYTE SIZE (%0 mM)
a
b
c
d
e
f
mM NaCl
mM Na_2SO_4
mM $MgCl_2$
mM $MgSO_4$
mM KCl
mM K_2SO_4

Figure 2

were examined in these genotypes in the absence and presence of 60 mM NaCl (Part 2). While no substantial differences were detected between the wild type and *stl1*, alterations in both Na^+ and K^+ levels were noted in *stl2*. To gain a better understanding of the possible role of Na^+ and K^+ in the salinity response of *stl1* and *stl2*, Na^+ and K^+ content in gametophytes were monitored over a range (0 - 150 mM) of NaCl concentrations (Figure 3). Similar levels of these ions were detected in all strains in the absence of NaCl stress (approximately 46 mM K^+ and 2 mM Na^+). At NaCl concentrations above 60 mM, *stl2* gametophytes maintain higher levels of K^+ and lower levels of Na^+ relative to the wild type and *stl1*. In the presence of 150 mM NaCl stress, *stl2* gametophytes contain a 49% higher level of K^+ and a 35% lower level of Na^+ than the wild type. There is essentially no difference between the wild type and *stl1* in K^+ and Na^+ content in response to increasing levels of NaCl stress. Figure 4 provides the corresponding K^+/Na^+ ratios for the data presented in Figure 3. A similar K^+/Na^+ ratio is observed for the wild type, *stl1* and *stl2* in the absence of NaCl stress ($K^+/Na^+ \approx 23$). A 50 % drop in this ratio is detected for all strains at 30 mM NaCl stress, and it continues to decline as the level of NaCl stress increases. As indicated by the differences in K^+ and Na^+ levels shown in Figure 3, significantly greater K^+/Na^+ ratios are observed for *stl2* than the wild type at the higher levels of NaCl stress. At 150 mM NaCl a ratio of 4 is found for *stl2*, as compared to 1.7 for the wild type. Comparable K^+/Na^+ ratios were found for the wild type and *stl1* at all levels of NaCl stress.

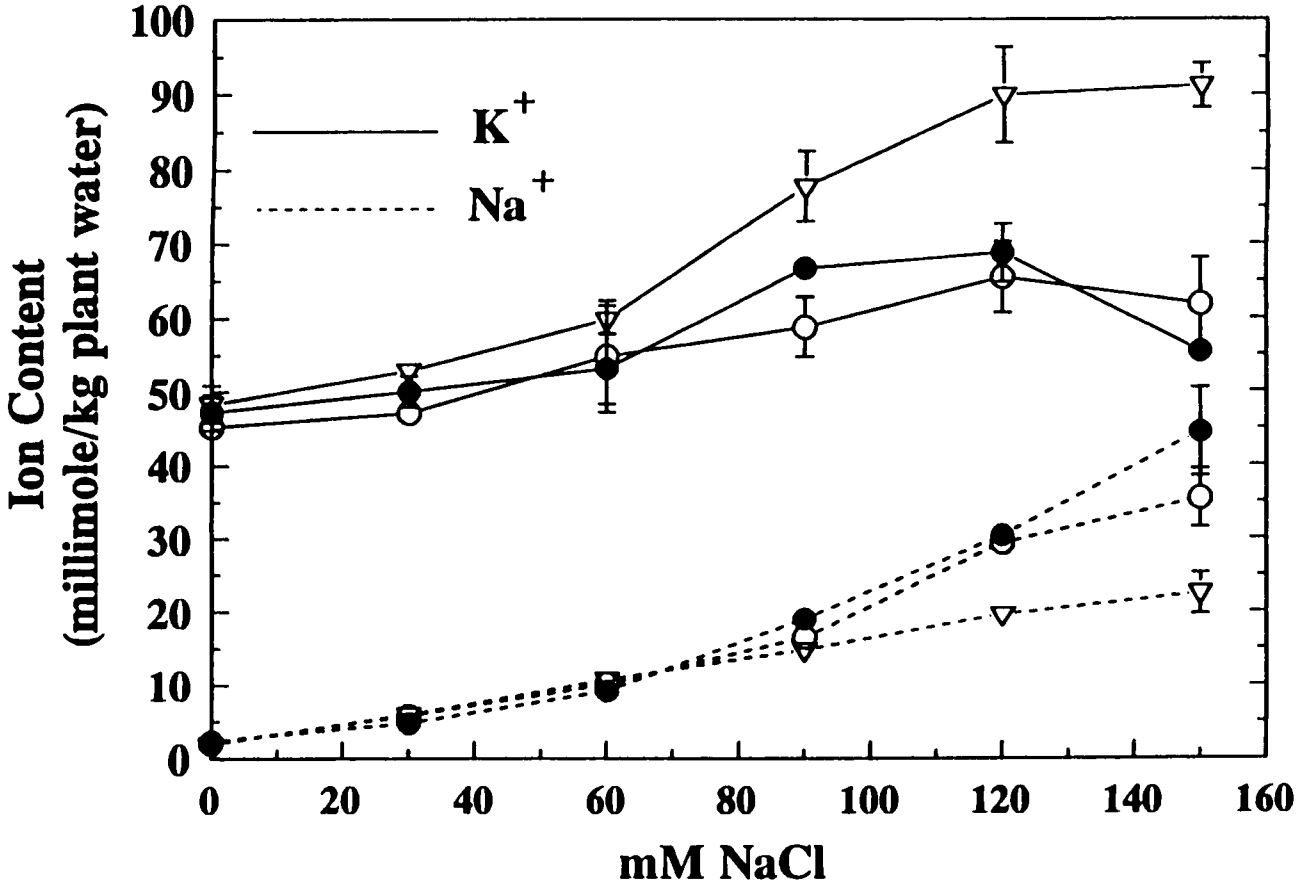

Figure 3. K^+ and Na^+ Content of Wild Type, *stl1* and *stl2* Gametophyte Tissue During Increasing Levels of NaCl Stress. Gametophytes (18 days from sowing) were exposed to NaCl for 72 hours, and subsequently harvested. Data are provided as the mean ± standard error of three independent samples. In some cases the standard error is smaller than the symbols. o, wild type; •, *stl1*; ▿, *stl2*

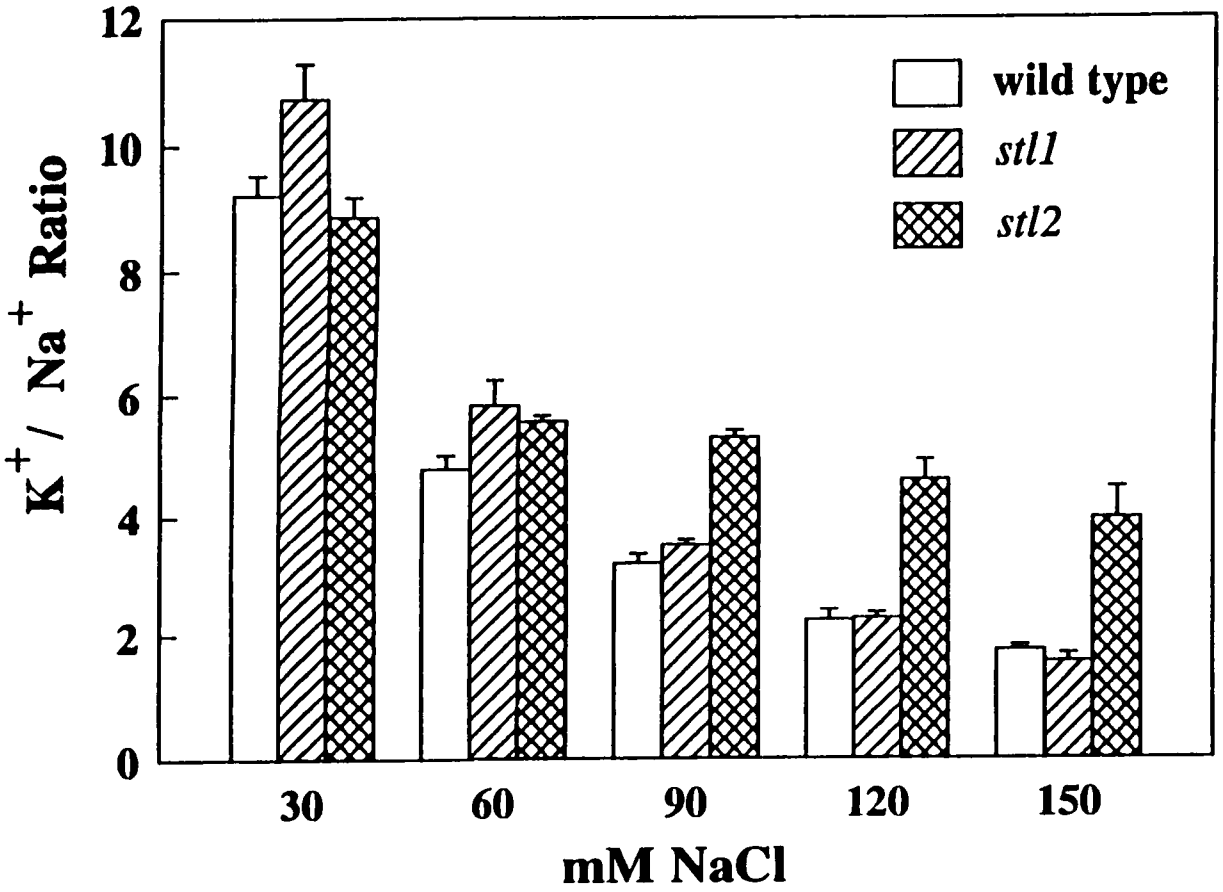

Figure 4. K^+/Na^+ Ratios in the Wild Type and Mutant Strains During Increasing Levels of NaCl Stress. Gametophytes (18 days from sowing) were exposed to NaCl for 72 hours, and subsequently harvested. Data represent the mean $\pm$ standard error of three independent analyses.

Previous studies (Part 2) involved constant exposure to NaCl (21 days), with spores being inoculated into medium containing 60 mM NaCl. Substantial differences in K^+ and Na^+ content between the wild type and *stl2* were detected at this level of NaCl stress. In the above analysis examining tissue ion content as a function of NaCl concentration, a 3 day exposure to NaCl was employed in order that higher NaCl concentrations (above 60 mM) could be tested (gametophytes that were 18 days old received NaCl treatment for 3 days). In this case a difference in Na^+ and K^+ content was not detected until 90 mM NaCl stress. To resolve this discrepancy between studies, Na^+ and K^+ content was examined as a function of the length of exposure time to 60 mM NaCl. A 14 day or 21 day (continuous) exposure to NaCl resulted in detectable differences between the wild type and *stl2* in K^+ and Na^+ content (data not shown). However, exposure to 60 mM NaCl for 3 days or 7 days prior to harvest did not generate significant differences in ion profiles, even though growth of the wild type was affected while that of *stl2* was not. Thus, a considerable duration of exposure to NaCl is necessary to result in substantial differences in Na^+ and K^+ content.

4. DISCUSSION

A principal objective of this study was to determine if the *stl1* and *stl2* mutations conferred tolerance to ionic or osmotic stress, both of which are imposed by excess salinity, and if tolerance was specific for Na^+ and/or Cl^-. Although it is difficult to clearly separate osmotic effects from specific ion effects (Harms and Oertli, 1985; Kingsbury and Epstein, 1986), attempts to do so employ isosmotic levels of osmotic stress agents and ionic stress agents (Kochba et al., 1982; Sharma et al., 1984; Ben-Hayyim et al., 1985; Ben-Hayyim et al., 1987; Hassan and Wilkins, 1988; Sumaryati et al., 1992; Saleki et al, 1993). Work by Cramer et al. (1986) indicated that addition of NaCl, or potentially any salt, to a complex nutrient solution can change the concentration and activity of the ions added and present through ion pairing and precipitation. With this in mind, the approach taken in these studies was to survey the effects of isosmotic levels of osmotic and NaCl stress or, when assessing the effects of various salts, replace Na^+ and Cl^- with equimolar concentrations of cations and anions, and compare the relative responses of *stl1* and *stl2* to that of the wild type for a given stress agent.

There are clear differences between the three genotypes in response to various stress agents. *stl2* gametophytes showed a much higher level of tolerance to NaCl than to isosmotic levels of osmotic stress, indicating that this strain is largely tolerant to ionic stress imposed by NaCl. In addition, tolerance imparted by this mutation does not appear to be specific for Na^+ or Cl^-, for *stl2* was shown to confer a similar

high level of tolerance to NaCl, Na_2SO_4, $MgCl_2$ and $MgSO_4$. The results from cross-tolerance tests indicated that *stl1* confers a similar low level of tolerance to isosmotic levels of osmotic stress agents and NaCl. While this result could be interpreted to suggest that *stl1* is tolerant to osmotic stress imposed by NaCl, cross-tolerance tests employing various ionic stress agents indicate a specificity of tolerance to Na^+.

During increasing levels of NaCl stress *stl2* maintains higher levels of K^+ and lower levels of Na^+, relative to the wild type. In addition to a greater increase in Na^+ content during increasing levels of NaCl stress, the wild type experiences only a slight increase in K^+ content. This would suggest that sensitivity to NaCl stress involves, at least in part, Na^+ toxicity. The altered accumulation of K^+ and Na^+ in *stl2* results in higher K^+/Na^+ ratios in gametophytes, thus the *stl2* mutation presumably confers tolerance to NaCl by maintenance of more favorable K^+ - Na^+ relations during salinity stress. Ion content was not monitored during comparable levels of Na_2SO_4, $MgCl_2$, $MgSO_4$ or osmotic stress. Thus, the nature of the tolerance to these stress agents is not known. Considering that *stl2* is a single gene nuclear mutation, these tolerant responses must share some common factor or mechanism. Additional tolerance to nonionizing compounds, such as mannitol, melibiose and hydroxyproline (Hickok, unpublished), further implicates a factor that exerts a general, or nonspecific, effect on membrane transport.

Tolerance to osmotic, or water stress, is often associated with the accumulation of solutes, either organic compounds or salt ions, which can be subcellularly compartmentalized and osmotically balanced through the accumulation of organic

solutes in the cytoplasm (Greenway and Munns, 1980). Previous studies did not reveal any differences in the accumulation of various organic solutes or water relations between the wild type and *stl1* or *stl2* during moderate NaCl stress (Part 2), thus discounting the possibility that either mutation ultimately results in the enhanced ability to use organic compounds for osmoregulation of the cytoplasm. Previous (Part 2) and present examinations of ion content during increasing levels of NaCl stress also revealed no differential accumulation of Na^+, Cl^-, K^+ or other major cations in *stl1*. Taken together, the available information suggests the *stl1* mutation may be associated with a more effective compartmentation of Na^+ during low levels of NaCl stress, perhaps within the vacuole.

Although organic and inorganic solute profiles from *stl1* were not examined during comparable levels of osmotic stress generated with non-ionizing agents, it is plausible that wild type gametophytes respond to osmotic stress (in general) in the same manner, by accumulating ions, some of which (eg. Na^+) are toxic when accumulated in the cytoplasm and need to be subcellularly compartmentalized. There is no appreciable amount of Na^+ in liquid basal medium (0.2 mM), however fairly significant levels (22 mM) are present as a contaminant in the agar used to solidify the nutrient medium for use in growth studies (Difco Laboratories, Bacto Agar 1040 Elemental Analysis). It is not known how much of this Na^+ source is actually available to the growing plants. Perhaps during osmotic stress generated by mannitol and melibiose some portion of this Na^+ source is available for use, in addition to other inorganic solutes, in osmotic adjustment. Alternatively, the possibility exists

that differences in organic and/or inorganic solute profiles do exist between *stl1* and the wild type, yet the differences are too small to be detected as a significant difference when solute profiles are compared.

It was somewhat surprising that potassium salts were extremely toxic to all genotypes, more so than comparable concentrations of NaCl. K^+ is an essential nutrient ion, serving roles in metabolism and turgor maintenance. While some studies report KCl or K_2SO_4 to have a similar effect on growth as NaCl (Kochba et al., 1982; Harms and Oertli, 1985; Sumaryati et al., 1992), others note that potassium salts are as toxic, or more so, than sodium salts (Ben-Hayyim et al., 1985; Eshel, 1985; Hassan and Wilkins, 1988; Cramer et al., 1990). While selectivity for K^+ uptake over Na^+ is an important factor in tolerance to NaCl stress (Greenway and Munns, 1980; Lauchli and Stelter, 1982; Wrona and Epstein, 1985), inadequate regulation of K^+ uptake during an abundance of the ion would be fatal.

The data in Figure 3 indicate that wild type and *stl2* gametophytes contain comparable levels of K^+ in the absence of NaCl stress. While *stl2* gametophytes were consistently found to contain slightly higher levels of K^+ than the wild type, they were not significantly (statistically) different. A previous study showed the *stl2* mutant to possess significantly higher constitutive levels of this ion (82 mM), compared to wild type tissue (55 mM) (Part 2). A difference in K^+ levels between these two genotypes of this magnitude has yet to be confirmed in subsequent studies examining tissue ion content. Thus, we can only conclude that *stl2* is associated with substantially higher K^+ levels when challenged with NaCl stress.

Tolerance to NaCl is known to differ with physiological stages of development (Shannon, 1984 and refs therein; Saleki et al., 1993). The *stl1* and *stl2* mutations confer tolerance to NaCl during spore germination and gametophyte growth (Warne and Hickok, 1987; Hickok et al., 1991), while a much lower level of tolerance is observed in mutant homozygous sporophytes (Hickok et al., 1991). Physiological studies have thus far involved comparisons of the gametophyte stage of development, and it has been suggested that these mutations result in a cellular mechanism of tolerance (Part 2)). Understanding the nature of tolerance imparted by *stl1* and *stl2* will be useful in future comparisons examining the role of these mutations in other physiological stages of development during salinity stress.

In summary, the *stl2* mutation largely confers tolerance to ionic stress imposed by NaCl, and tolerance does not appear to be specific for Na^+ or Cl^-. Tolerance to increasing levels of NaCl stress is associated with higher K^+ levels and reduced Na^+ levels, resulting in higher K^+/Na^+ ratios. *stl1* possesses a low level of tolerance to osmotic and ionic stress agents, and tolerance may be specific for Na^+. Evaluation of all findings suggests that this mutation is associated with enhanced, yet limited, compartmentation of Na^+.

Acknowledgements

I would like to thank Cindy Stiles for overseeing the operation of the ICAP AA for ion analyses. This work was supported by NRI Competetive Grants Program/ USDA grant No. 92-3700-7673.

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PART 4

NA^+/CA^{2+} AND NA^+/K^+ INTERACTIONS IN NACL-TOLERANT MUTANTS WITH ALTERED ION RELATIONS

Vogelien DL, Hickok LG, Warne TR (1993) Na^+/Ca^{2+} and Na^+/K^+ interactions in NaCl-tolerant mutants with altered ion relations. Plant Physiol (in progress)

1. INTRODUCTION

In an attempt to approach the problem of salinity tolerance from both a genetic and physiological perspective, we have used the fern *Ceratopteris richardii* as a model system to select unlinked single gene mutations that confer tolerance to NaCl in germinating spores and gametophytes (Warne and Hickok, 1987; Hickok et al., 1991). One of these mutations, designated *stl1*, confers a low level of tolerance to NaCl ($\leq$ 125 mM NaCl) (Warne and Hickok, 1987). Comparative physiological studies with wild type gametophytes indicated that tolerance conferred by this mutation is specific for Na^+ (Part 3), and, since no difference in the level of accumulation of this ion or other major ions and organic solutes was found (Part 2), we suggested that *stl1* is associated with enhanced compartmentation of Na^+ within the cell. The *stl2* mutation, conferring a high level of tolerance to NaCl (> 225 mM NaCl) (Hickok et al., 1991), is associated with reduced Na^+ and greater K^+ accumulation, relative to the wild type genotype, during increasing levels of NaCl stress (Part 2 and 3).

Considerable effort has been made to understand the interaction between Na^+ and Ca^{2+} during salinity stress (Lauchli and Schubert,1989; Lauchli, 1990; Rengel, 1992). Ca^{2+} has been shown to lessen the adverse effects of NaCl on growth in plants (LaHaye and Epstein, 1969; LaHaye and Epstein, 1971; Norlyn and Epstein, 1984; Cramer et al., 1986; Kurth et al., 1986; Kent and Lauchli, 1985; Subbarao et al., 1990) and cultured plant cells (Ben-Hayyim and Kochba, 1983; Ben-Hayyim et al.,

1987). Some of the reported effects of supplemental Ca^{2+} during NaCl stress include maintenance of K^+/Na^+ selectivity (Kent and Lauchli, 1985; Cramer et al., 1987), reduced Na^+ uptake (Epstein, 1961; Cramer et al., 1987; Cramer et al., 1989; Subbarao, 1990), reduced leakage of cytosolic K^+ from cells (Cramer et al., 1985) and maintenance of ion uptake and transport regulation, particularly K^+ transport (Epstein, 1961; Lauchli and Epstein, 1970; Kent and Lauchli, 1985; Nakamura et al., 1990; Subbarao, 1990). Any one of these effects essentially results in more favorable K^+-Na^+ relations in cells and tissues during salinity stress. Yet, the exact role of Ca^{2+} during salinity stress is not known. There is evidence that Na^+ causes displacement of Ca^{2+} form the plasma membrane, that this displacement correlates with increased K^+ efflux, and that these effects can be relieved by increasing external Ca^{2+} concentration (Cramer et al., 1985; Lynch et al., 1987). These findings have led to the proposal that the positive effect of Ca^{2+} on growth is closely related to effects on membrane integrity and function and ion transport in roots and shoots (Lauchli and Schubert, 1989). In addition, it has been suggested that NaCl alters cell Ca^{2+} homeostasis by inhibiting Ca^{2+} influx and increasing Ca^{2+} efflux (Cramer et al., 1987; Lynch and Lauchli, 1988). These and other findings have led to the proposal that changes in Ca^{2+} fluxes at the plasma membrane are the primary response of NaCl stress, triggering a chain of events resulting in various damaging effects (Lynch and Lauchli, 1988; Lynch et al., 1989; Rengel, 1992).

In some plants K^+/Na^+ selectivity during NaCl stress appears to include efficient competition of K^+ against Na^+ during influx at the plasmalemma (Rains and Epstein,

1967; Jeschke and Nassery, 1981; Jeschke, 1984). It has been suggested that K^+ application should reduce the damaging effects of salinity on plant growth (Ben-Hayyim et al., 1987). While some plants respond favorably to K^+ fertilization (Jeschke and Nassery, 1981), supplemental K^+ has had no effect, or even a negative effect, on salinity tolerance in others (Lauter et al, 1988; Bar-Tal et al., 1991), despite its effect on increasing K^+ content in the plant and reducing the Na^+/K^+ ratio in plant tissue (Bar-Tal et al., 1991).

In this study we examine various ion relations in order to better define the role of K^+, Na^+, and Mg^{2+} and Ca^{2+} in the salinity response of the wild type and mutant strains, thus further defining the mechanisms of tolerance associated with the *stl1* and *stl2* mutations. To examine the possibility that Ca^{2+} is involved in the salinity response of either of these mutations, the effect of external Ca^{2+} concentration on growth and ion content during NaCl stress was compared to that of the wild type. In addition, because the *stl2* mutation also confers a similar high level of tolerance to Mg^{2+} salts (Part 3), the effect of elevated external levels of Ca^{2+} on Mg^{2+} toxicity in the three genotypes was examined. Lastly, the role of K^+ in the tolerance response of *stl1* and *stl2* was explored by examining the effect of K^+ concentration in the external medium on growth and K^+ and Na^+ content during salinity stress.

2. MATERIALS AND METHODS

Plant Material

Spores of the diploid (n = 39) homosporous fern, *Ceratopteris richardii* Brongn., from the following strains were used in this study: the wild type strain (Hickok et al., 1987), strain N10, containing the *stl1* mutation (Warne and Hickok, 1987), and strain N23, containing the *stl2* mutation (Hickok et al., 1991). The *stl1* and *stl2* mutations confer a low ($\leq$125 mM) and high (>200 mM) level of tolerance to NaCl, respectively. Both NaCl tolerant strains were derived from the wild type by X-ray mutagenesis and selection, with genetic analysis confirming them to be unlinked single gene nuclear mutations.

Gametophyte Culture and Growth Assessment

Gametophyte cultures were axenically established by sowing surface sterilized spores (Warne et al., 1986) onto 1% agar-solidified basal inorganic medium consisting of Parkers's macronutrients and Thompson's micronutrients (Klekowski, 1969), pH 6.0. Immature premeristematic gametophytes (obtained approximately 6 days from sowing of spores) were subsequently transferred to basal medium that was supplemented with a particular Ca^{2+}/Na^{+}, Ca^{2+}/Mg^{2+} or K^{+}/Na^{+} treatment (described below). All cultures (before and during salt treatments) were maintained under constant conditions of 28 $\pm$ 2 °C and approximately 85 μmole m^{-2} s^{-1} PAR. The effect of individual treatments on gametophyte growth was assessed by measuring

areas of the essentially two-dimensional gametophytes. Growth was determined at 9 days following transfer to supplemented media by mounting gametophytes on slides in Hoyer's medium (Beeks, 1955) mixed with 0.5% acetocarmine and determining individual gametophyte area using a computer-interfaced image analysis system (BioQuant IV™, R & M Biometrics, Inc.). In all studies, each treatment consisted of a minimum of 20 (to as many as 30) gametophytes. Values presented represent the mean and standard error of the mean.

Calcium concentrations of 0.1, 0.2 (level in basal medium), 1 and 2 mM were examined in combination with increasing concentrations of NaCl (0 - 225 mM). 1 mM calcium was found to achieve the greatest level of relief to the toxic effect of NaCl on growth in the wild type, thus this calcium level was used in subsequent studies examining the effect of supplemental calcium on ion content during NaCl stress. Concentrations of 0.2 and 1 mM calcium were examined in combination with 0, 25, 50, 75 and 100 mM $MgCl_2$. In both sets of experiments calcium was added in the form of $CaSO_4$. To examine the effect of external levels of potassium on the growth responses of the three genotypes during salinity stress, NaH_2PO_4 was substituted for KH_2PO_4 in the basal medium, and potassium, in the form of K_2SO_4, was added to give final potassium concentrations of 0, 1.85, 3.7 (concentration in basal medium), 7.4, 18.5, and 37 mM. Each potassium concentration was examined in combination with 0, 75, 150, and 225 mM NaCl.

To facilitate harvesting of gametophyte tissue for use in ion analyses, gametophytes were cultured in liquid medium at a sowing density of 6 mg spores/200

ml medium. Calcium and potassium levels were modified as described above. Liquid cultures were maintained on a gyrator shaker at 100 rpm under the same culture conditions given above. NaCl treatments began 18 days following initiation of culture, and were applied in 35 mM increments every 12 h until the desired final concentration was achieved. NaCl was applied in the form of a concentrated stock solution made with complete nutrient media with calcium and potassium concentration modified as necessary. Tissue was harvested at day 21 of the culture period, thus resulting in a three day exposure period to NaCl stress.

Cation Analysis

Gametophyte tissue was harvested by filtering the liquid culture onto miracloth(TM) using a mild vacuum and rinsing four times with 100 ml of an isotonic solution of mannitol. Approximately 100 mg of ground dried tissue was dry ashed by heating at 450 °C for 4 h. Ashed tissue was taken up in 10 ml 2N HCl. Major cation levels (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were measured by ICAP atomic absorption spectroscopy (Thermo Jarrell Ash ICAP 61). Ion content is expressed as millimole/ kg plant water. Tissue water content was calculated for each salt treatment using the formula: [(fresh wt. - dry wt.)/ fresh wt.] x 100. Each treatment was performed in triplicate, and data are provided as the mean $\pm$ standard error of the mean.

3. RESULTS

Ca^{2+} Effects on Salinity Responses

The effect of supplemental Ca^{2+} (1 mM) on gametophyte growth of the wild type and mutant strains during increasing levels of NaCl stress is illustrated in Figure 1. Growth at 1 mM Ca^{2+} has been compared to growth at 0.2 mM Ca^{2+}, the level of Ca^{2+} in basal/normal culutre medium. The data provided represent gametophyte growth at 1 mM Ca^{2+} as a % of control growth, or growth at 0.2 mM Ca^{2+}. During NaCl stress supplemental Ca^{2+} resulted in a significant enhancement in wild type and *stl1* gametophyte growth (e.g. a 223 and 240% growth enhancement for the wild type and *stl1*, respectively, at 175 mM NaCl), while the growth response of *stl2* gametophytes was similar to that at 0.2 mM, with only a small enhancement in growth observed (124% growth enhancement at 175 mM NaCl). In addition, growth enhancement detected in the wild type and *stl1* due to supplemental calcium is accompanied by a decrease in the level of tissue necrosis and chlorosis that normally occurs in these gametophytes during high levels of NaCl stress. Figure 1 presents the % growth enhancement or inhibition due to 1 mM Ca^{2+}. Presented in Figure 6 (Appendix) are the absolute gametophytic growth responses of these genotypes for 0.2 and 1 mM Ca^{2+} treatments during increasing levels of NaCl stress.

Ca^{2+} concentrations of 0.1 and 2 mM were also tested in combination with increasing levels of NaCl stress (data not shown). The growth responses of each of

Figure 1. The Effect of Supplemental Calcium on Wild Type and Mutant Gametophyte Growth During Increasing Levels of NaCl Stress. Growth in the presence of 1 mM Ca^{2+} is presented as a % of growth at 0.2 mM Ca^{2+} (concentration in basal culture media) for individual NaCl concentrations. Values represent the mean $\pm$ SEM of 20 measurements. In most cases the error bars are smaller than the symbols. Absolute gametophytic growth (area in mm^2) for 0, 75, 100, 125, 150 175, 200 and 225 mM NaCl treatments at 0.2 mM Ca^{2+} are: (wild type) 4.4, 3.2, 2.3, 1.4, 0.83, 0.56, 0.43. and 0.28 mm^2; (*stl1*) 6.0, 3.9, 2.6, 1.4, 0.91, 0.57, 0.45, 0.28 mm^2; (*stl2*) 2.4, 2.0, 1.5, 1.4, 1.2, 0.90, 0.83, 0.60 mm^2. Complete absolute growth responses are presented in Figure 6 (Appendix) for each genotype at 0.2 and 1 mM Ca^{2+}.

gametophyte area
(% of 0.2 mM calcium)
300
250
200
150
100
50
0
0
50
100
150
200
250
mM NaCl
wild type
stl1
stl2

Figure 1

the genotypes when supplied with 0.1 mM Ca^{2+} were similar to those observed with 0.2 mM Ca^{2+}, thus employment of this lower level of Ca^{2+} did not result in a greater sensitivity to NaCl stress in any of the genotypes. Also, an external Ca^{2+} concentration of 2 mM did not result in greater growth enhancements in any of the genotypes. Maximum amelioration of the toxic effects of NaCl on growth of the wild type (and *stl1*) was achieved with 1 mM Ca^{2+}. Further, the response of the wild type, with respect to growth and tissue necrosis, when supplied with 1 mM Ca^{2+} resembled the growth response of *stl2* under normal (0.2 mM) Ca^{2+} levels (Figure 2). Comparison of absolute gametophyte size at 0.2 and 1 mM Ca^{2+} treatments in the absence of NaCl stress indicates that 1 mM Ca^{2+} does not result in significantly greater growth in any of the three genotypes (see Figure 6 and Figure 7 for 0 mM NaCl and $MgCl_2$, respectively, Appendix). Thus, 0.2 mM Ca^{2+} is not growth limiting for any of the genotypes in the absence of salt stress.

It was of interest to determine if the positive effect of supplemental Ca^{2+} on wild type growth correlated with altered Na^+ - K^+ relations, such as those previously noted for *stl2* (Part 3). Tissue ion content was determined from gametophytes grown under basal (0.2 mM) and elevated (1 mM) levels of Ca^{2+} in the absence and presence of 175 mM NaCl stress. The results, shown in Table 1, indicate that this was indeed the case. During 175 mM NaCl stress, wild type gametophytes supplied with 1 mM Ca^{2+} maintained higher levels of K^+ and lower levels of Na^+ than similarly stressed gametophytes supplied with 0.2 mM Ca^{2+}. The response of *stl1* is similar to that of

Figure 2. Growth Response of the Wild Type and *stl2* During Increasing Levels of NaCl Stress Supplemented with 0.2 and 1 mM Ca^{2+}. Data represent the mean $\pm$ SEM of 20 measurements. Error bars are smaller than the symbols. Absolute gametophytic growth (area in mm^2) for 0 mM NaCl at 0.2 mM Ca^{2+} is 4.4 and 2.4 mM^2 for wild type and *stl2* gametophytes, respectively. Complete absolute growth responses are presented in Figure 6 (Appendix) for each genotype at 0.2 and 1 mM Ca^{2+}.

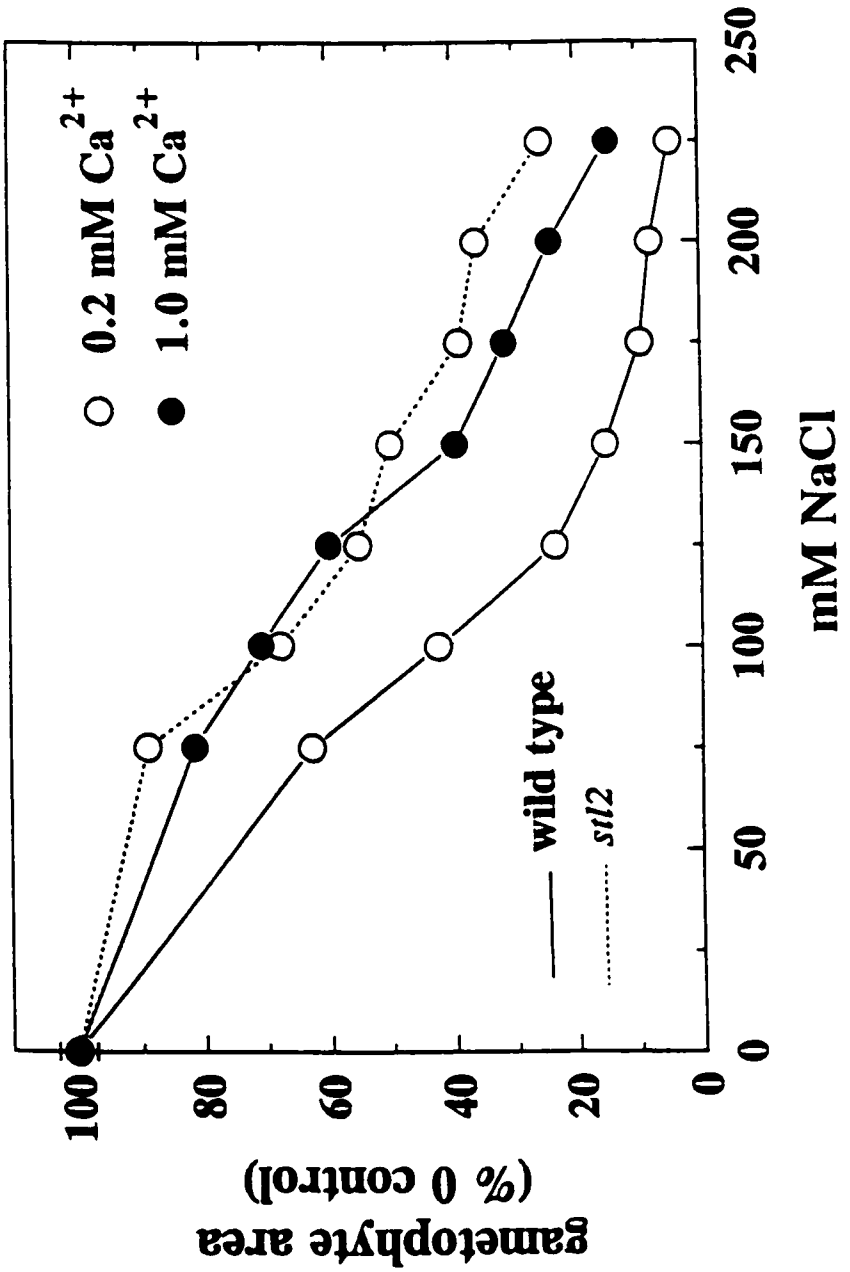
gametophyte area
(% 0 control)
100
80
60
40
20
0
0
50
100
150
200
250
mM NaCl
0.2 mM Ca2+
1.0 mM Ca2+
wild type
stl2

Figure 2

Table 1. The Effect of Supplemental Calcium (1 mM) on Tissue Ion Content of Wild Type and Mutant Strains When Challenged with a High Level of NaCl Stress. Values represent the mean ± SEM of three independent replicates.

	0 mM NaCl		175 mM NaCl	
	mM Ca^{2+}		mM Ca^{2+}	
	0.2[a]	1	0.2	1
millimole K^+/ kg plant water				
WT	47.3 ± 1.9	51.4 ± 0.6	45.5 ± 5.1	92.2 ± 4.6
stl1	49.7 ± 1.3	46.1 ± 3.3	51.59 ± 0.6	80.6 ± 11.7
stl2	54.3 ± 4.0	49.8 ± 1.5	71.6 ± 5.7	115.8 ± 8.4
millimole Na^+/ kg plant water				
WT	2.1 ± 0.1	2.3 ± 0.6	50.8 ± 2.5	19.3 ± 0.9
stl1	2.0 ± 0.4	2.6 ± 0.6	51.3 ± 1.3	20.8 ± 2.7
stl2	2.0 ± 0.4	1.9 ± 0.5	29.8 ± 1.4	15.5 ± 2.2
K^+/Na^+				
WT	22.7 ± 0.8	25.4 ± 5.4	0.9 ± 0.2	4.8 ± 0.1
stl1	26.3 ± 4.6	18.8 ± 3.9	1.0 ± 0.03	3.9 ± 0.2
stl2	28.7 ± 5.1	30.1 ± 8.7	2.5 ± 0.5	7.6 ± 0.5

[a] The level of Ca^{2+} in basal culture medium.

the wild type, with supplemental Ca^{2+} resulting in presumably more favorable K^+ andNa^+ relations during NaCl stress. Interestingly, while 1 mM Ca^{2+} did not substantially affect *stl2* gametophyte growth during NaCl stress, a significant change in K^+ and Na^+ content was detected.

Supplemental (1 mM) Ca^{2+} was also able to alleviate $MgCl_2$ toxicity in wild type and *stl1* gametophytes, as shown in Figure 3. Again, 1 mM Ca^{2+} did not significantly alter the gametophytic growth response of *stl2* during increasing levels of $MgCl_2$ stress. Previous studies showed wild type and *stl1* gametophytes to be extremely sensitive to magnesium salts, with concentrations of 50 mM $MgCl_2$ or $MgSO_4$ resulting in an 80% reduction in gametophyte size and higher levels resulting in complete growth inhibition (under basal culture conditions) (Part 3). Increasing the external Ca^{2+} concentration from 0.2 mM to 1 mM resulted in a nearly 300% enhancement in gametophyte size of both wild type and *stl1* at 50 mM $MgCl_2$. The effect of 0.2 and 1 mM Ca^{2+} on absolute gametophytic growth of the three genotypes is presented in Figure 7 (Appendix).

K^+ Effects on Salinity Responses

Similar studies were performed examining the effect of external levels of K^+ on the salinity response in these genotypes. Unlike Ca^{2+}, elevated K^+ concentrations in the medium did not alleviate the inhibitory effect of sodium chloride on growth in the wild type or mutant strains (Figure 4). The level of K^+ in basal medium is 3.7 mM, and is subsequently referred to a contol levels. As in previous figures, the effect of

Figure 3. The Effect of Supplemental Calcium on Wild Type and Mutant Gametophyte Growth During Increasing Levels of $MgCl_2$ Stress. Growth in the presence of 1 mM Ca^{2+} is presented as a % of growth at 0.2 mM Ca^{2+} (concentration in basal culture media) for individual $MgCl_2$ concentrations. Values represent the mean $\pm$ SEM of 20 measurements. In most cases the error bars are smaller than the symbols. Absolute gametophytic growth (area in mm^2) for 0, 25, 50, 75 and 100 mM $MgCl_2$ treatments at 0.2 mM Ca^{2+} are: (wild type) 3.9, 3.9, 0.93, 0.40, 0.30 mm^2; (*stl1*) 4.8, 4.8, 1.1, 0.45 and 0.28 mm^2; (*stl2*) 2.8, 3.5, 2.2, 1.9 and 1.24 mm^2. Complete absolute growth responses are presented in Figure 7 (Appendix) for each genotype at 0.2 and 1 mM Ca^{2+}.

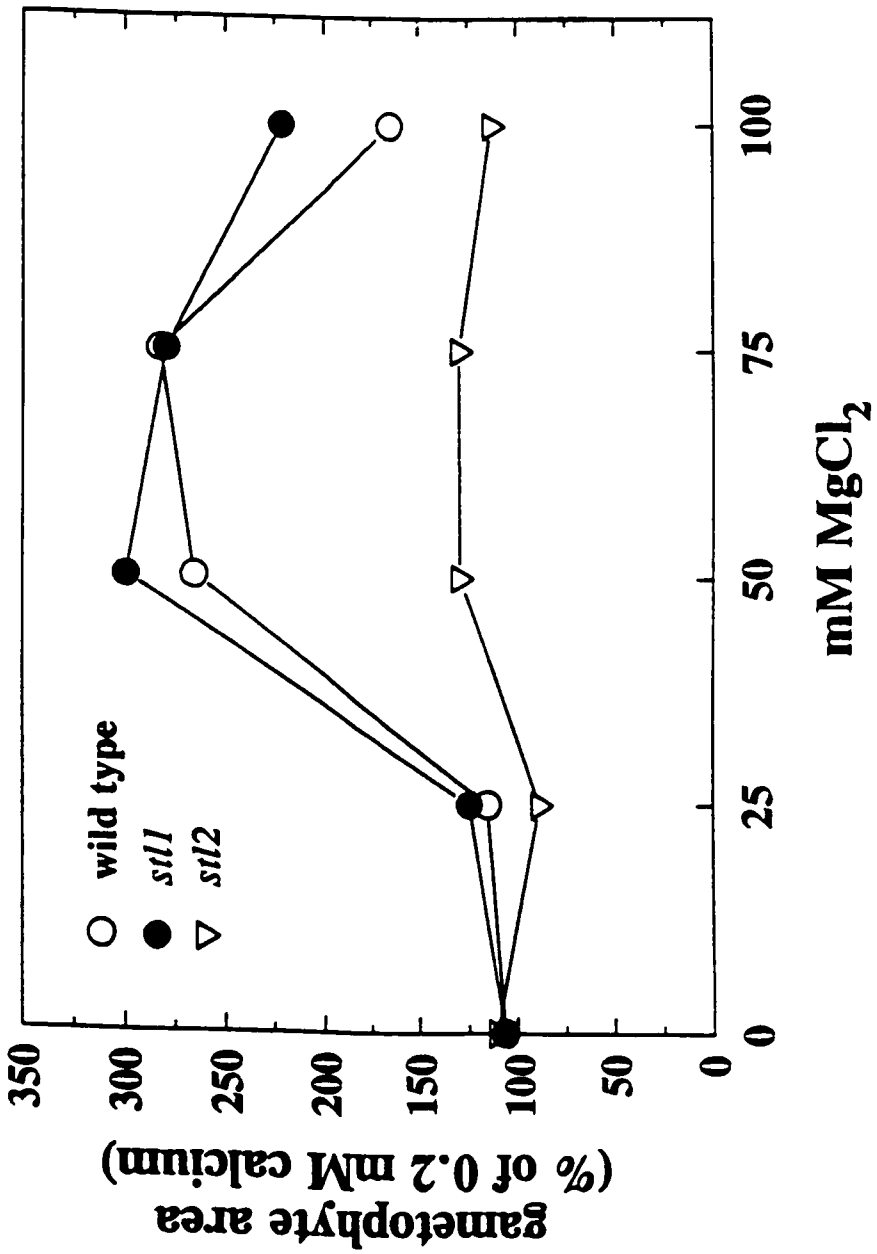
gametophyte area
(% of 0.2 mM calcium)
350
300
250
200
150
100
50
0
0
25
50
75
100
mM $MgCl_2$
wild type
stl1
stl2

Figure 3

Figure 4. The Effect of K^+ Concentration in the Medium on Gametophytic Growth During NaCl Stress. The growth response for individual K^+ concentrations is presented as a % of growth at 3.7 mM K^+ (concentration in basal culture media) for individual NaCl concentrations. Values represent the mean $\pm$ SEM of 20 measurements. In most cases the error bars are smaller than the symbols. Absolute gametophytic growth (area in mm^2) for 0, 75, 150 and 225 mM NaCl treatments at 3.7 mM K^+ are: (wild type) 3.6, 2.8, 0.49 and 0.25 mm^2; (*stl1*) 3.3, 2.8, 0.57 and 0.26 mm^2; (*stl2*) 2.9, 2.8, 1.1 and 0.5 mm^2. Complete absolute growth responses for each genotype are presented in Figure 8 (Appendix). These data have also been graphed as a function of K^+ concentration for 0, 75 and 150 mM NaCl (Figure 9, Appendix).

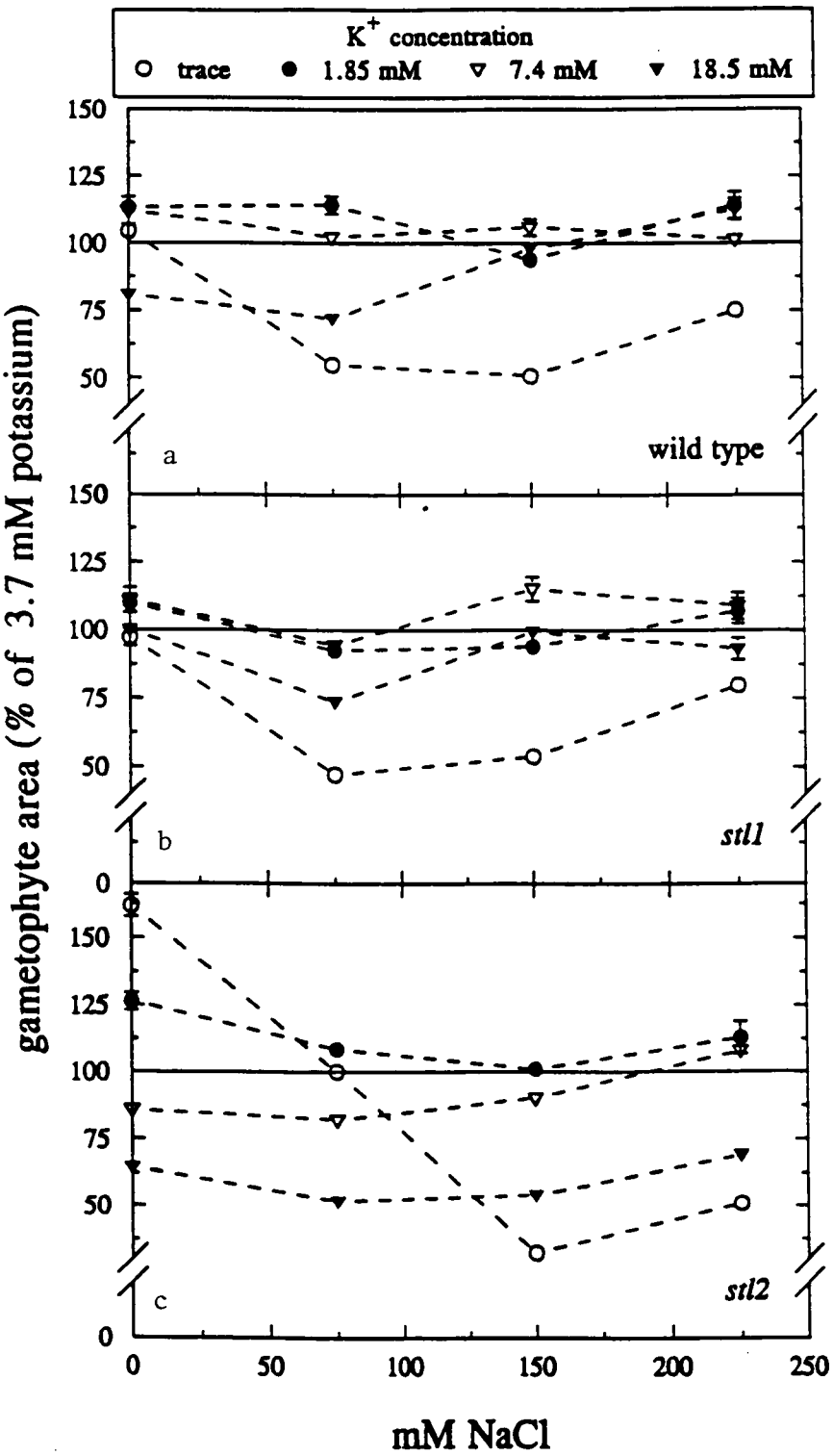
K+ concentration
trace
1.85 mM
7.4 mM
18.5 mM
gametophyte area (% of 3.7 mM potassium)
a
wild type
b
stl1
c
stl2
mM NaCl

Figure 4

individual externallly supplied K^+ concentrations on gametophytic growth is expressed as a % of growth observed under control conditions. K^+ concentrations that are 1/2 (1.85 mM) and 2 (7.4 mM) times that present in basal media have very little effect on the tolerant response of the wild type, *stl1* or *stl2*, as reflected by the small deviation of these growth curves (dashed lines) from that of the control (solid line in Figure 4a-c). Trace levels of K^+ (K^+ present as a contaminant in agar, etc.) resulted in greater sensitivity to NaCl stress in the wild type and *stl1* at all levels of NaCl stress. For example, an approximate 50% reduction in gametophytic growth is noted in these genotypes supplied with trace levels of K^+ in combination with 75 mM NaCl. In contrast, at this same K/Na treatment *stl2* gametophytes are equivalent in size to those of the control (eg. receiving 3.7 mM K^+ in combination with 75 mM NaCl). However, this relative tolerance is not observed at the higher NaCl concentrations examined. The most interesting observation from this series of tests involves the growth response of *stl2* to trace K^+ levels. In the absence of NaCl stress a growth increase of 161% over that of the control was detected. Further, as external K^+ concentration increased, gametophyte size decreased (0 mM NaCl). This observation was particularly interesting in that *stl2* gametophytes under normal culture conditions are 40% smaller in size than wild type gametophytes. Absolute gametophytic growth for each genotype is presented in Figures 8 and 9 in the Appendix.

Because *stl2* appeared to be very sensitive to external K^+ concentrations in the absence of NaCl stress, the effect of reduced levels of K^+ on gametophytic growth was examined in greater detail. The data presented in Figure 5 represent absolute

Figure 5. Gametophyte Growth of the Wild Type and *stl2* Grown Under Normal and Reduced Levels of Potassium. Values represent the mean ± SEM of 30 individual measurements.

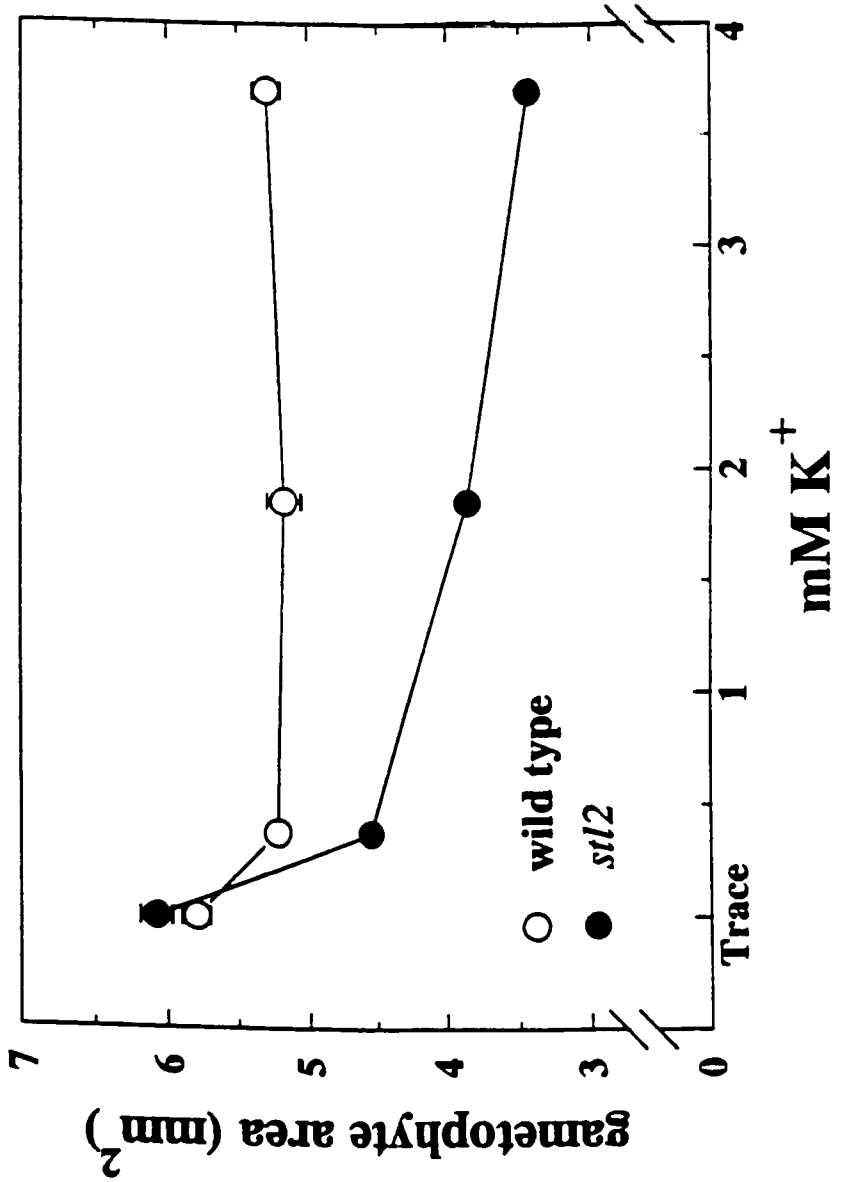

Figure 5

growth in terms of gametophyte area. While wild type gametophytic growth is only slightly affected by lower K^+ concentrations, *stl2* gametophyte growth increases as the K^+ concentration in the medium decreases. Under trace levels of K^+, *stl2* gametophytes are roughly equivalent in size to wild type gametophytes.

Tissue ion content under control (3.7 mM) and reduced K^+ (0.37 mM) levels in the presence and absence of 175 mM NaCl stress was determined, and the results are presented in Table 2. Similar levels of K^+ and Na^+ are found in *stl2* under both K^+ concentrations in the absence of NaCl, and these levels are comparable to those found for the wild type. During 175 mM NaCl stress *stl2* gametophytes supplied with 0.37 mM K^+ show severely reduced levels of K^+ and higher levels of Na^+, relative to levels found for this genotype with 3.7 mM K^+. This loss in ability to control K^+ and Na^+ levels during NaCl stress with reduced K^+ is in agreement with the growth response of *stl2* noted in Figure 4 (trace K^+/ 150 mM NaCl).

Table 2. The Effect of Reduced Potassium Concentration on Tissue Ion Content of Wild Type and *stl2* Gametophytes

	0 mM NaCl		175 mM NaCl	
	mM K^+		mM K^+	
	0.37	3.7[a]	0.37	3.7
millimole K^+/ kg plant water				
WT	47.3 ± 0.9	48.4 ± 0.6	40.5 ± 0.9	66.6 ± 1.0
stl2	52.1 ± 0.2	54.9 ± 0.6	14.2 ± 0.7	105.9 ± 0.5
millimole Na^+/ kg plant water				
WT	3.6 ± 0.4	5.2 ± 0.4	66.3 ± 0.9	63.0 ± 1.0
stl2	3.3 ± 0.1	5.6 ± 0.2	78.4 ± 1.6	39.7 ± 2.2
K^+/Na^+				
WT	13.5 ± 1.5	9.5 ± 1.0	0.6 ± 0.01	1.1 ± 0.02
stl2	15.8 ± 0.5	9.8 ± 0.5	0.2 ± 0.01	2.7 ± 0.1

[a] The level of K^+ in basal culture medium.

4. DISCUSSION

Under NaCl and $MgCl_2$ stress the wild type and *stl1* showed similar responses to supplemental Ca^{2+} and K^+. Previous studies led to the suggestion that *stl1* was associated with enhanced compartmentation of Na^+ within the cell (Part 3). The studies reported here give no further insight as to the mechanism of tolerance in *stl1*.

Elevated external levels of Ca^{2+} clearly alleviated the toxic effect of NaCl and $MgCl_2$ on growth of wild type gametophytes, yet no substantial effect on the growth response of *stl2* gametophytes was detected. This differential response of *stl2* gametophytes to supplemental Ca^{2+} suggests that Ca^{2+} may be involved in the salinity response of this mutation. The positive effect of supplemental Ca^{2+} on wild type growth during NaCl stress correlated with higher K^+ levels, reduced Na^+ levels and a higher K^+/Na^+ ratio, essentially resulting in ion relations similar to those of *stl2* gametophytes during NaCl stress (at basal levels of Ca^{2+}). Interestingly, although higher levels of K^+ and reduced levels Na^+ were also observed in *stl2* with supplemental Ca^{2+}, this was less pronounced than the wild type and was not correlated with a substantial effect on growth during NaCl stress. One possible explanation may be that an even greater drop in Na^+ content is required to result in a greater growth enhancement of *stl2* gametophytes. Na^+ levels were still 7-fold higher than those noted for *stl2* in the absence of NaCl stress. Alternatively, Ca^{2+} may not be directly involved in the function of *stl2*. For instance, Ca^{2+} may affect some other cellular process, presumably a membrane transport process, that achieves the same

end result on K^+ and Na^+ levels. This is conceivable when one considers the numerous transport processes that could be involved in the transport of K^+ and Na^+ across the plasma membrane (pumps, channels, carriers) and the probability that these processes are coordinated to provide ionic and charge homeostasis.

stl2 gametophytes are clearly sensitive to external K^+ concentration. The difference in gametophyte size between *stl2* and the wild type on basal medium and the increase in growth achieved by *stl2* gametophytes on medium with reduced levels of K^+ suggests that *stl2* gametophytes suffer from K^+ toxicity when grown on normal culture media. This toxicity may be caused by a greater rate of K^+ uptake from the medium. If this is the case, higher constitutive levels of K^+ might be expected. However, data in Tables 1 and 2 do not show this to be so, in that *stl2*, at best, may be associated with only slightly higher constitutive levels of K^+. Because cells presumably have means of controlling K^+ homeostasis, and some components involved in this control would undoubtedly involve the expenditure of cellular energy, the smaller size of *stl2* gametophytes under normal culture conditions may be due to a drain on ATP pools that is necessary to maintain acceptable cytoplasmic levels of K^+. Thus, in this case, higher constitutive levels would not be detected as long as sufficient energy is available for regulation of K^+ homeostasis. Lastly, in the presence of reduced levels of K^+ *stl2* gametophytes lose the ability to tolerate high levels NaCl stress, exhibiting a phenotype that is similar to that of the wild type.

Considering the small size of gametophytes, their essentially two-dimensional shape, and the involvement of altered K^+ - Na^+ relations in the salinity response of

stl2 gametophytes, it is very likely that the *stl2* mutation is associated with a cellular process which involves altered ion transport at the plasma membrane. The limited tolerance observed in homozygous mutant sporophytes at 60 mM NaCl supports the suggestion of the involvement of a cellular process (Hickok et al., 1991). While lipid and protein composition of a membrane can affect both passive (Douglas and Walker, 1984; Hirayama and Mihara, 1987) and active membrane transport processes (Serrano et al., 1988; Cooke and Burden, 1990; Kasamo, 1990), the possibility that *stl2* in some way resulted in altered membrane lipid composition, which in turn would effect membrane permeability and/or the activity of membrane proteins, was discounted when no significant differences in lipid composition (including levels of major classes, sterol species composition, and fatty acid profiles of polar lipids) could be detected between this strain and the wild type (Part 5).

Consideration has subsequently been given to membrane proteins whose activity, if altered, could account for the available results. Likely candidates include cation channels (K^+ or nonspecific), a H^+-ATPase, and K^+/H^+ and Na^+/H^+ antiporters. Na^+/K^+ and K^+/H^+ pumps (ATPases), K^+/H^+ and Na^+/H^+ symporters and Na^+ channels, although demonstrated in bacterial, fungal and animal systems, have not yet been identified in higher plants.

The observed differences in K^+ content of *stl2* gametophytes under NaCl stress and the apparent sensitivity of this genotype to external K^+ could involve K^+ channels whose selectivity and/or activity has been altered. Most studies of K^+ channels in higher plants involve specialized cells of particular tissues, such as stomatal guard

cells (Schroeder et al., 1987; Schroeder, 1988; Schroeder and Hagiwara, 1989; Blatt, 1991; Schroeder and Fang, 1991) and pulvinar cells (Moran et al., 1988; Moran, 1990). Using patch clamp techniques, two classes of K^+ channels, inward and outward rectifying voltage-dependent channels, have been identified in the plasma membrane of these two cell types. In guard cells, elevated (micromolar) levels of cytosolic Ca^{2+} have been implicated in the regulation of both channel types, inactivating inward rectifying K^+ channels and triggering an anion efflux leading to depolarization and opening of K^+ efflux channels. There are numerous reports that K^+ channels having properties both similar to and differing from those found in stomatal guard cells and pulvinar cells are present in other types of higher plant cells (Tazawa et al., 1987 and refs. therein; Hedrich and Schroeder, 1989 and refs. therein; Van Duijn et al., 1993 and refs. therein).

A plasma membrane H^+-ATPase is another candidate to be seriously considered as the membrane protein affected by the *stl2* mutation. The electrical and pH gradients generated by H^+-ATPases are thought to provide the driving force for the transport and cotransport of various nutrients. Consequently, altered activity of this membrane protein would likely effect the transport of a variety of solutes, and could conceivably result in the altered K^+ - Na^+ relations observed for *stl2*. A number of extracellular signals have been shown to affect the activities of H^+- ATPases (Hedrich and Schroeder, 1989 and refs. therein; Zocchi, 1990 and refs. therein), however Ca^{2+} is not among them. Possible modulation of the activity of this protein by protein kinases has been proposed, and the initial activation of the protein kinases is

suggested to involve cytosolic Ca^{2+} (Zocchi, 1989). If the *stl2* mutation did affect a H^+-ATPase, altered accumulation of other nutrient ions, sugars or amino acids might also be expected, particularly in absence of NaCl stress. However, no substantial differences in the levels of major ions, free amino acids or sugars were noted (Part 2 and 3). In addition, a more active ATPase would not explain the enhanced growth of *stl2* gametophytes that was observed on medium containing reduced levels of K^+. The fact that *stl2* gametophytes are smaller in size under normal culture conditions in the absence of any NaCl stress suggests that the mutation is constitutive, and not induced by exposure to NaCl stress. Almost identical levels of K^+ were noted in wild type gametophytes under normal and reduced levels of external K^+ (no NaCl), indicating that sufficient K^+ is available for growth. Thus, similar demands on metabolic energy would be expected during growth normal and reduced levels of K^+.

Taking into consideration all available information, we advance the following model to explain the possible mechanism of action of *stl2*. It is proposed that the *stl2* mutation affects a K^+/cation channel located in the plasma membrane that is involved in K^+ influx. In wild type cells, cation selectivity of this channel and K^+ uptake are is influenced by external Ca^{2+} binding to the channel protein. When Ca^{2+} is bound to the channel protein the channel is selective for K^+ over Na^+. Increased K^+ uptake may be the direct result of the altered selectivity of the channel or due to a change in normal channel gating also caused by Ca^{2+} binding. Removal or replacement of Ca^{2+} results in the loss of selectivity for K^+ and the subsequent movement of other monovalent cations through the channel. It is proposed that the *stl2* mutation results

in a higher affinity of this channel protein for Ca^{2+}, such that removal or replacement of this ion is more difficult. In the absence of NaCl stress the altered Ca^{2+} binding results in greater K^+ uptake. Tighter binding of Ca^{2+} to the mutant channel protein would, in effect, maintain channel selectivity for K^+ during salinity stress.

There are reports demonstrating that Ca^{2+} regulates K^+ channels in cells. Ketchem and Poole (1991) describe a voltage-dependent K^+ efflux channel from corn protoplasts that is both activated and inactivated, depending on Ca^{2+} concentration, by cytosolic Ca^{2+}, which in turn is controlled by Ca^{2+} influx. Cytoplasmic Ca^{2+} has been found to inhibit inward-rectifying K^+ channels in guard cells (Schroeder and Hagiwara, 1989). In certain animal cells a voltage-dependent K^+ channel has been identified for which external Ca^{2+} is essential for normal channel gating (closure) and maintenance of channel selectivity for K^+ (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990).

Similarities in basic channel properties (e.g.; gating, cation specificity) from different cells suggests that these ion channels may share general mechanisms for ion transport across the plasma membrane of plants. However, diversity in the functions of cells and the many roles of K^+ in physiological and biochemical processes also suggests that differences in channel properties and regulation are likely to exist. For example, patch clamp techniques led to the identification of an outward rectifying cation channel in protoplasts from wheat roots that was shown to be highly selective to K^+, two to four times more so than the analogous K^+ channel of guard cells (Schachtman et al., 1991). In this case Ca^{2+} did not alter selectivity of the channel

for K^+ (vs Na^+). Data from one study involving pulvinar protoplasts indicated that membrane hyperpolarization resulting from activation of the H^+-ATPase was not the sole factor controlling inward K^+ fluxes (Kim et al., 1992), but there was no indication as to what other mechanism(s) might be involved.

The proposed model is attractive in that it can explain available results. Tolerance to Na^+, and the resulting higher K^+/Na^+ ratio, is explained by the channel's ability to maintain selectivity for K^+ over Na^+ during NaCl stress. In addition, greater K^+ uptake may promote Na^+ or cation exclusion by modulating membrane potential (Tyerman, 1992), thus promoting tolerance to NaCl and other salts by restricting uptake of the salt. Limited tolerance to osmotic stress agents (Part 3) may be due to the use of available (or more easily obtainable) K^+ in osmotic adjustment of the cytoplasm. Growth sensitivity of this mutant to basal levels of K^+ would be the result of increased uptake of K^+, presumably due to greater availability. Enhanced K^+ uptake in the absence of NaCl stress would likely result in a need for the cell to expend energy in an effort to maintain acceptable cytoplasmic levels of K^+ and electrical homeostasis.

The proposed model also accounts for the observed tolerance of *stl2* gametophytes to magnesium salts. As noted earlier, tolerance may involve the ability of K^+ to modulate membrane potential in such a way that uptake of Mg^{2+} is not favored. Alternatively, Mg^{2+} may displace Ca^{2+} from the channel protein in a manner similar to that suggested for Na^+ (Lauchli and Schubert, 1989), and consequently affect channel selectivity and activity. In addition, it is also possible that Mg^{2+} may

effectively block this K^+/cation channel in the wild type, but be less effective in this action in *stl2*. While Mg^{2+} blockage of plasma membrane K^+ or cation channels has not yet been demonstrated in plant cells, closure of one kind of K^+ channel (inward rectifying) in animal systems has been shown to be enhanced by blockage with intracellular Mg^{2+} (Matsuda, 1988). Cross-tolerance tests indicated that low levels of Na^+ and Mg^{2+} salts result in a small enhancement in growth of *stl2* gametophytes (Part 3). In light of the proposed model, low levels of Na^+ and Mg^{2+} might relieve K^+ toxicity in *stl2* gametophytic tissue by causing a low level of Ca^{2+} displacement, thus resulting in a slight reduction in K^+ uptake.

Admittedly, the available results do not dismiss the possibility that the *stl2* mutation is associated with a membrane protein other than a K^+ channel, such as a H^+-ATPase or a K^+/H^+ antiporter. However, the proposed model is the simplest in design that could be constructed to best account for all available observations. Clearly, electrophysiological studies are needed to specifically look at cation channel proteins in the wild type and *stl2*, identifying channel types and examining channel(s) selectivity to various cations and the effect of Ca^{2+} on selectivity and activity. Comparative studies involving K^+ uptake and efflux are also necessary. Some of these studies are currently in progress and will undoubtedly aid in deciding the validity of the proposed mechanism of action of the *stl2* mutation. Initial results from short term $^{86}Rb^+$ uptake and efflux studies indicate that *stl2* possesses a higher rate of both uptake and efflux relative to the wild type and maintains higher uptake rates of $^{86}Rb^+$ in response to increasing extracellular levels of Na^+ (Warne and Hickok,

unpublished). Differences between these genotypes have also been detected in K^+-stimulated H^+-extrusion and membrane potential (Kinraide, Warne and Hickok, unpublished).

Acknowledgements

I would like to thank Cindy Styles for overseing the operation of the ICAP AA for ion analyses. This work was supported by NRI Competetive Grants Program/ USDA grant No. 92-3700-7673.

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APPENDIX

Figure 6. Absolute gametophytic growth (mm^2) of wild type and mutant genotypes for 0.2 and 1 mM Ca^{2+} treatments during increasing levels of NaCl stress. Values represent the mean $\pm$ SEM of 20 measurements. In all cases the error bars are smaller than the symbols. The data presented in this figure were used to generate Figure 1, in which the effect of 1 mM Ca^{2+} on growth is presented as a % of growth at 0.2 mM Ca^{2+} (concentration in basal medium) at individual NaCl concentrations for each genotype (e.g % growth enhancement or inhibition), and Figure 2, in which wild type and *stl2* gametophytic growth responses to increasing NaCl stress are presented as a % of control (0 mM NaCl) growth for a given Ca^{2+} treatment.

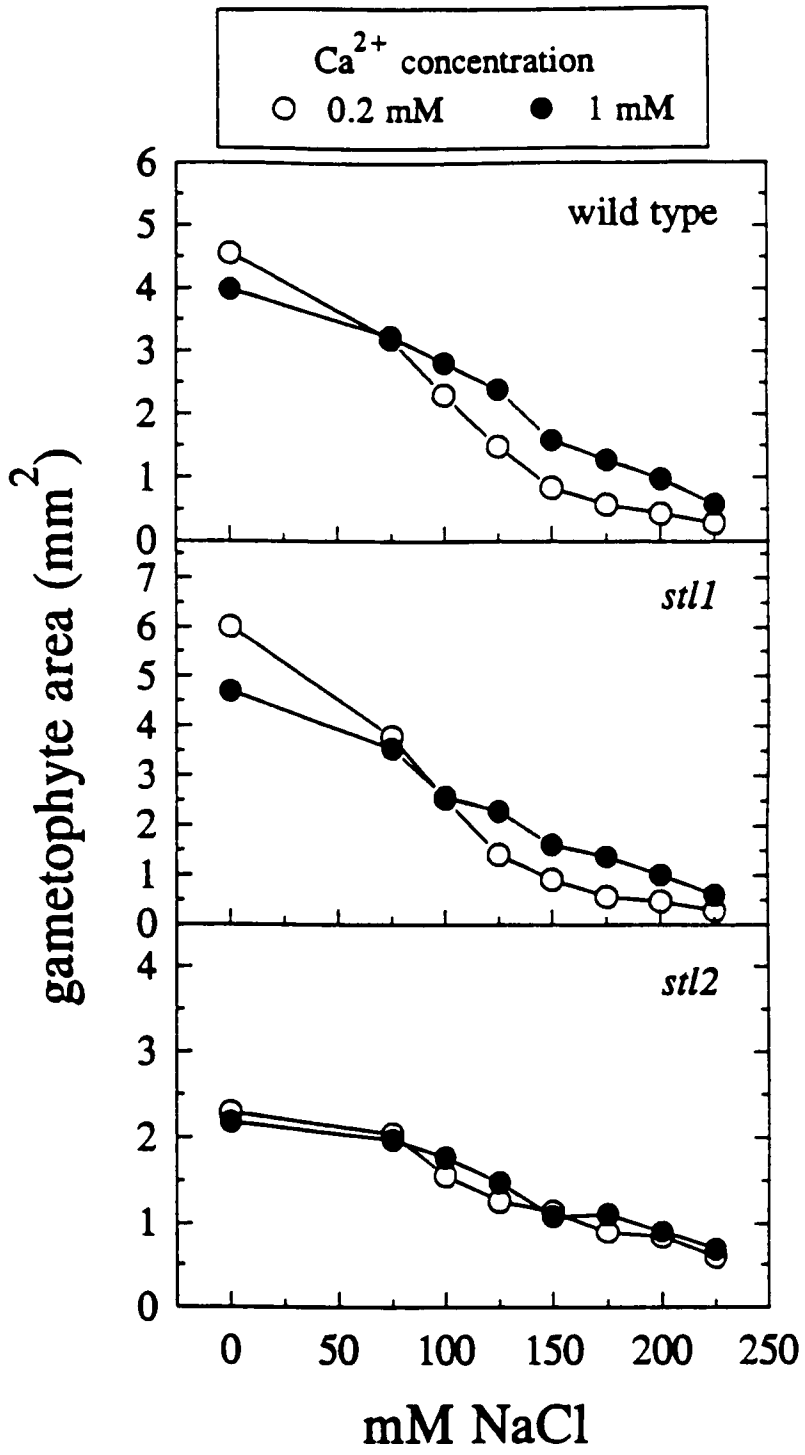
Ca2+ concentration
0.2 mM
1 mM
wild type
stl1
stl2
gametophyte area (mm2)
mM NaCl
0
50
100
150
200
250

Figure 6

Figure 7. Absolute gametophytic growth (mm^2) of wild type and mutant genotypes for 0.2 and 1 mM Ca^{2+} treatments during increasing levels of $MgCl_2$ stress. Values represent the mean $\pm$ SEM of 20 measurements. In all cases the error bars are smaller than the symbols. The data presented in this figure were used to generate Figure 3, in which the effect of 1 mM Ca^{2+} on growth is presented as a % of growth at 0.2 mM Ca^{2+} (concentration in basal medium) at individual NaCl concentrations for each genotype (e.g % growth enhancement or inhibition).

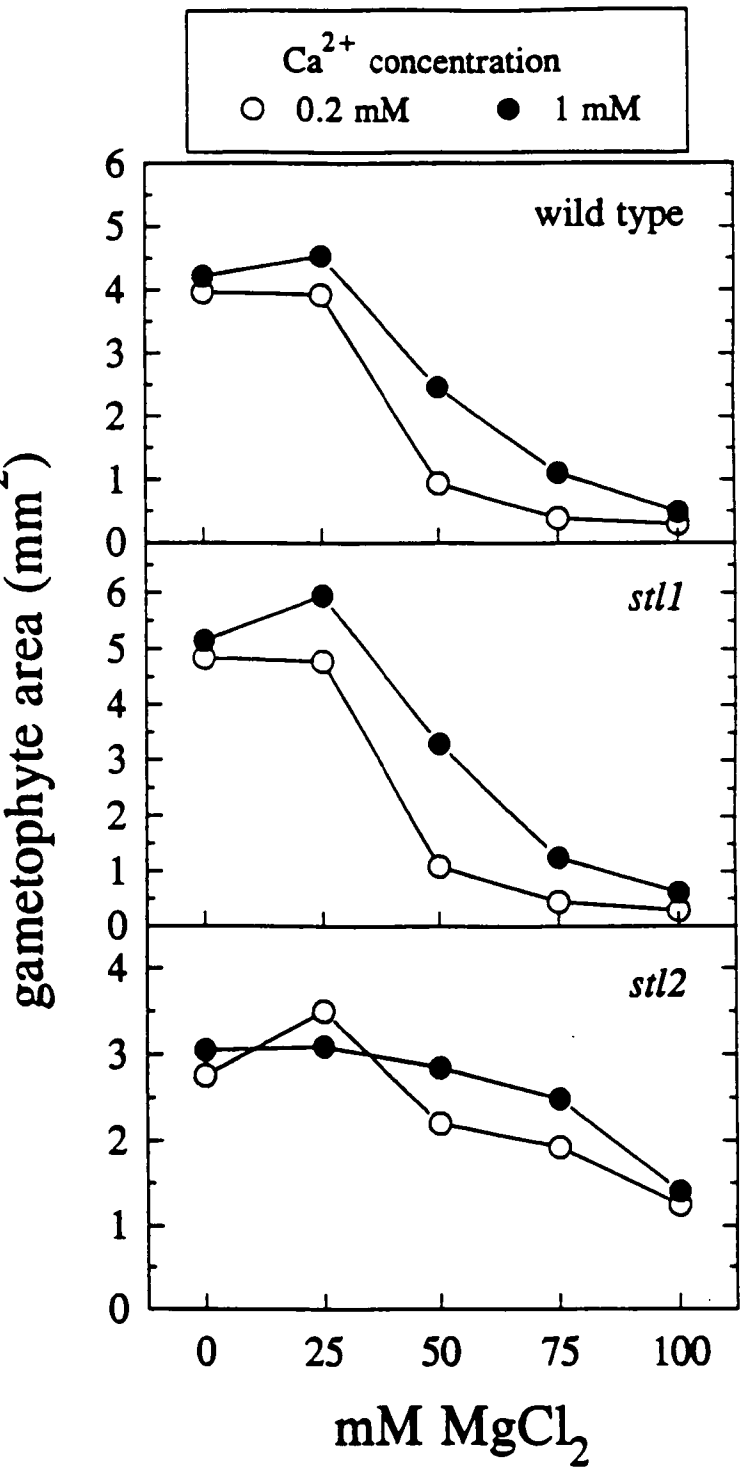
Ca2+ concentration
0.2 mM
1 mM
wild type
stl1
stl2
gametophyte area (mm2)
mM MgCl2
0
25
50
75
100

Figure 7

Figure 8. Absolute gametophytic growth (mm^2) of wild type and mutant genotypes for various external levels of K^+ during increasing levels of NaCl stress. Values represent the mean $\pm$ SEM of 20 measurements. In most cases the error bars are smaller than the symbols. The data presented in this figure were used to generate Figure 4, in which the effect of various levels of externally supplied K^+ on growth is presented as a % of growth at 3.7 mM K^+ (concentration in basal medium) at individual NaCl concentrations for each genotype (e.g % enhancement or inhibition).

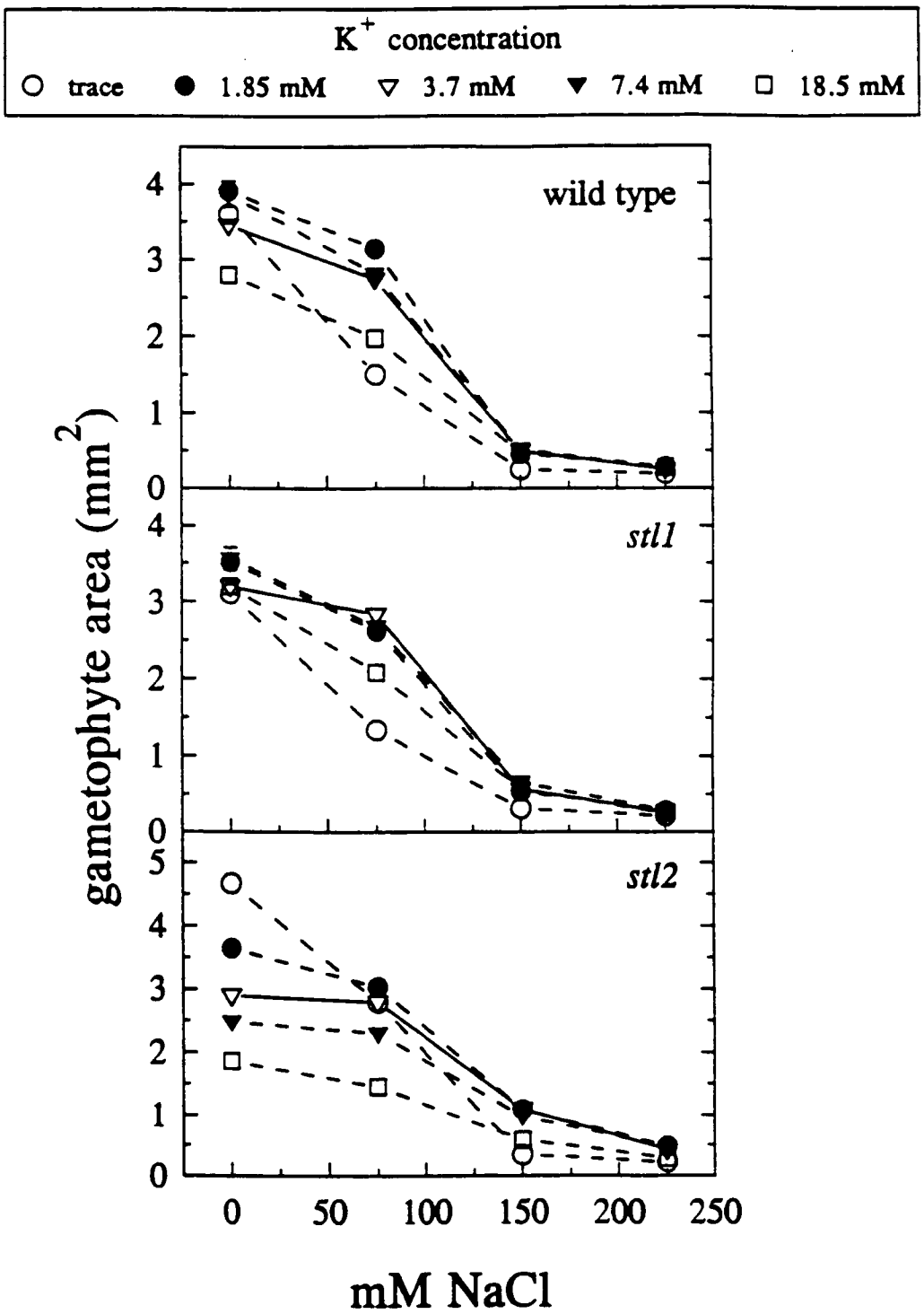
K+ concentration
trace
1.85 mM
3.7 mM
7.4 mM
18.5 mM
wild type
stl1
stl2
gametophyte area (mm2)
mM NaCl
0
50
100
150
200
250

Figure 8

Figure 9. Absolute gametophyte growth (mm^2) of wild type and mutant genotypes as a function of external K^+ concentration for 0, 75 and 150 mM NaCl stress. Values represent the mean $\pm$ SEM of 20 measurements. In most cases the error bars are smaller than the symbols. These data are the same values used to generate Figure 8, the difference being that gametophyte area for selected NaCl treatments is presented a function of K^+ concentration.

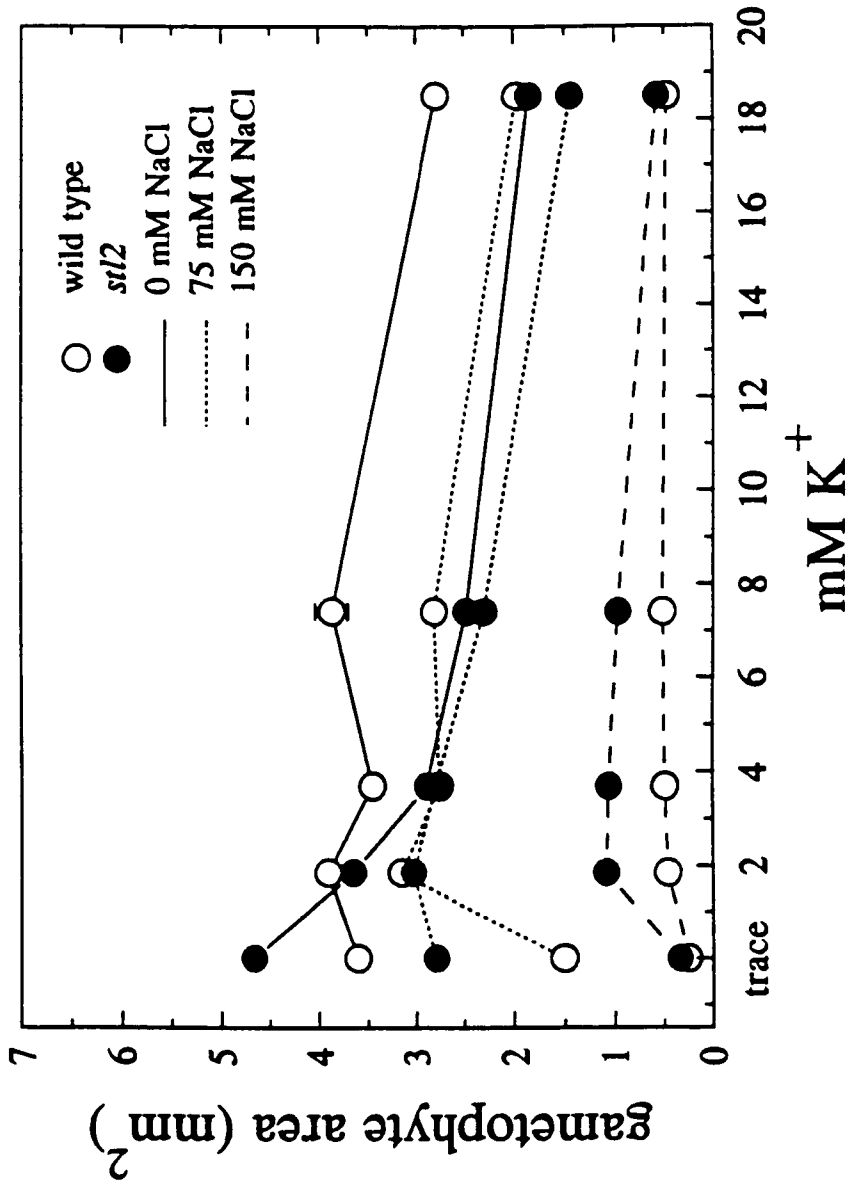
wild type
stl2
0 mM NaCl
75 mM NaCl
150 mM NaCl
gametophyte area (mm2)
0
1
2
3
4
5
6
7
trace
2
4
6
8
10
12
14
16
18
20
mM K+

Figure 9

PART 5

COMPARISON OF LIPID COMPOSITION IN THE WILD TYPE, *STL1*, AND *STL2* GENOTYPES

1. INTRODUCTION

Two mutations have been isolated that confer tolerance to NaCl in gametophytes of the fern *Ceratopteris richardii*. The *stl1* mutation confers a low level of tolerance to gametophytes ($\leq$ 100 mM NaCl) (Warne and Hickok, 1987), while the *stl2* mutation confers tolerance to NaCl concentrations of 225 mM and above (Hickok et al., 1991). Previous studies showed that, relative to the wild type and *stl1*, *stl2* gametophytes accumulated higher levels of K^+ and reduced levels of Na^+ during salinity stress (Part 2 and 3). This altered accumulation of K^+ and Na^+ resulted in a higher K^+/Na^+ ratio, thus presumably maintaining more favorable K^+-Na^+ relations in this genotype during salinity stress. Because the *stl2* mutation appears to involve altered ion uptake during salinity stress, the involvement of an ion transport process(es) at the plasma membrane is implicated.

Membrane lipid composition strongly influences membrane stability, fluidity and permeability properties (Kuiper, 1984; Kuiper, 1985; Hale and Orcutt, 1987). The major lipid components of plant membranes are phospholipids (PL), glycolipids (GL), and sterols. Sterols tend to reduce membrane fluidity and permeability (Kuiper, 1984; Kuiper, 1985; Hale and Orcutt, 1987). Glycolipids enhance the permeability properties of membranes (Kuiper, 1984; Hirayama and Mihara, 1987). Phospholipids control membrane stability and fluidity via Ca^{2+} cross-linking potential with anionic head groups of phospholipids (Landau and Leshem, 1988). Acyl groups of polar lipids influence membrane fluidity, with longer and more unsaturated fatty acids

generally resulting in greater membrane fluidity (Kuiper, 1984; Hale and Orcutt, 1987). Membrane fluidity and permeability properties can significantly affect transport processes of a membrane. Both passive (Douglas and Walker, 1984; Hirayama and Mihara, 1987) and active ion uptake (Serrano et al., 1988; Cooke and Burden, 1990; Kasamo, 1990) are influenced by membrane lipid composition.

To explore the possible role of lipid composition in a membrane-based mechanism of salinity tolerance lipid composition has been examined in numerous plants. A survey of plants that differ in salt tolerance reported that an increase in the ratio of GL/PL in roots correlated with increasing salt tolerance (Hirayama and Mihara, 1987). A similar increase in this ratio was observed in response to external salinity in the halophyte *Atriplex gmelinic*, and it was further demonstrated that the increased ratio of GL/PL in root membranes resulted in membranes being more permeable to Na^+ and Cl^- (Hirayama and Mihara, 1987). There is also evidence for sterol involvement in membrane-based salt tolerance mechanisms (Kuiper 1984, Kuiper, 1985 and refs. therein; Douglas and Walker, 1984; Douglas and Sykes, 1985; Blitts and Gallagher, 1990 a and b). Higher sterol/PL ratios are associated with the more tolerant plant species and are maintained or elevated under saline conditions. In addition, changes in sterol species composition, as reflected in a change in the relative proportions of individual sterols, have been correlated with chloride exclusion ability in varieties of *Citrus* (Douglas and Walker, 1983; Douglas and Sykes, 1985). An increase in plasma membrane fatty acid saturation has also been observed in a highly salt tolerant alga, *Dunaliella salina*, (Peeler et al., 1989) and in sugar beet (Stuiver et

al., 1981) in response to NaCl.

In this study the PL, GL and sterol content, sterol species composition and fatty acid profiles of polar lipids were examined in order to determine if differences in lipid composition were correlated with the salinity tolerance imparted by *stl1* and *stl2*. Comparisons were made between wild type, *stl1* and *stl2* genotypes in the absence and presence of a moderate salt stress (60 mM NaCl).

2. MATERIALS AND METHODS

Culture

Gametophyte tissue was obtained by sowing surface sterilized spores onto nutrient medium containing 0 and 60 mM NaCl following standard procedures described by Warne and Hickok (1987). Cultures were grown under constant conditions at 28 $\pm$ 2 °C and approximately 85 μmol m^{-2} s^{-1} PAR. Tissue was harvested for analytical use at 21 days following initiation of culture.

Lipid Extraction

Gametophyte tissue (100 mg fresh weight) was extracted using the single-phase chloroform:methanol:water extraction system of Bligh and Dyer (1959) as modified by White et al. (1979). Tissue was extracted for 4 hours at room temperature. Extracts were phase-separated by addition of equal volumes of chloroform and water to provide a final solvent volume ratio of 1:1:0.9 for chloroform:methanol:water. Phase-separation was allowed to continue overnight (approximately 18 hours). The organic phase was reduced to dryness under vacuum (< 37 °C) with a rotary evaporator. The residue was transferred to test tubes with 3 x 2 ml chloroform washes and the solvent subsequently evaporated using a stream of nitrogen gas (< 37 °C). Dried extracts were stored under nitrogen at -20 °C until lipid class separation.

Lipid Isolation

Total lipid was resuspended in 1 ml hexane:chloroform (4:1, v/v). 250 μl was retained for sterol analysis and the remaining sample fractionated into general lipid classed using silicic acid column chromatography. Chromatography columns were constructed from disposable glass Pasteur pipets (2 ml) packed with glass wool plugs inserted into the bottom of the pipet. Silicic acid (100 - 200 mesh powder) was activated at 100 °C for a minumum of 1 hour. A silicic acid slurry (0.5 g silicic acid suspended in 5 ml chloroform) was used to pack the column. The neutral lipid fraction was eluted with 5 ml chloroform, the GL fraction with 5 ml acetone, and the PL fraction with 5 ml methanol. Each fraction was reduced to dryness under a stream of nitrogen gas and stored under nitrogen at -20 °C for further analysis.

Preparation for GC

Fatty acid methyl esters (FAME) of the individual lipid classes were prepared by mild alkaline methanolic transesterification as reported by Guckert et al. (1985). Dried fractions were redissolved in 1 ml toluene:methanol (1:1, v/v) and 1 ml methanolic KOH (0.2 M KOH in methanol), incubated at 40 °C for 15 minutes, cooled to room temperature, diluted with 2 ml hexane:chloroform (4:1, v/v), and the sample neutralized (pH 6-7) with 0.2 ml 1 N acetic acid. Samples were phase-separated by the addition of 2 ml H_2O. The upper organic phase was recovered and the aqueous lower phase re-extracted with 2 ml hexane:chloroform (4:1, v/v) twice more. FAME samples were evaporated to dryness with nitrogen gas and stored under

nitrogen at -20 °C until separation and quantification.

Trimethyl-silyl (TMSi) derivatives of 3ß-ol sterols were prepared from a total lipid fraction by alkaline saponification as described by Nichols et al. (1983). To dried lipid, 3 ml 5% KOH in methanol:water (80:20, v/v) was added and the samples heated at 60 °C for 2 hours. After cooling to room temperature, 1 ml H_2O and 2 ml hexane:chloroform (4:1, v/v) were added, the sample vortexed for a minimum of 30 sec and the phases separated by centrifugation. The upper organic phase was recovered and the aqueous lower phase re-extracted with 1 ml hexane:chloroform (4:1, v/v) as above twice more. Twenty-four hours before GC analysis, 60 µl N,O-*bis*(Trimethylsilyl)triflouroacetamide (BSTFA) was added, and samples heated at 60 °C for 30 minutes to generate TMSi derivatives.

Analysis

Samples (FAME and TMSi derivatives of 3ß-ol sterols) were separated for quantification using capillary gas chromatography with flame ionization detection. A 60 meter non-polar cross-linked methyl silicone column (Restek RTX-1) was used. The following FAME temperature program was used: 100 °C for 0 min., 10°/min. to 150 °C for 1 min, 3°/min. to 282 °C for 5 min. Injection and detector temperatures were 270° and 290 °C, respectively. Total run time was 55 minutes. Injection volume was 1 µl. Quantification was based on comparison to an internal injection standard (C19:0) added prior to analysis. Species identification was confirmed by comigration with standards and comparison with published Equivalent Chain Lengths

in a library of FAME.

The sterol temperature program was: 200 °C for 0 min., 10°/min. to 280 °C for 0 min., 2 /min. to 310 °C for 5 min. The injector and detector temperatures were 290° and 290 °C, respectively. Total run time was 28 min, injection volume 1 μl. Quantification was based on a comparison to an internal standard (cholestane) added prior to analysis. Identification of sterols involved calculation of relative retention times (RRT) based on cholesterol and sitosterol (Nes, 1989) and comparison of calculated RRT of unknown sterols to a library of RRT for known sterols under given chromatographic conditions. Verification of compound structure was performed by GC/MS as detailed by Nichols et al. (1983).

Statistics

Each salt treatment (0 or 60 mM NaCl) was replicated three times for a given genotype. The data presented in the accompanying tables represent the mean $\pm$ standard deviation of three independent replicates. To test for differences between means, a two-way ANOVA was performed using SAS software for personal computers (PC SAS™), with genotype and salt treatment forming a 2*3 factorial treatment arrangement. When a significant genotype or salt effect was detected, Duncan's Multiple Range Test (α = 0.05, N = 3) was performed to identify the significantly different means.

3. RESULTS

A comparison of the levels of major lipid classes in each genotype in response to 60 mM NaCl stress is shown in Table 1. PL, GL and sterol levels did not differ significantly between the three genotypes. At 60 mM NaCl stress a small increase in the levels of all lipid classes was observed. However, since no genotype x salinity interaction was detected by statistical analysis, 60 mM NaCl stress affected all genotypes equally. The three genotypes also displayed similar sterol/PL ratios in the absence of NaCl stress, and this ratio did not change in response to 60 mM NaCl stress. Similarly, wild type, *stl1* and *stl2* show comparable GL/PL ratios in the absence of salinity stress, and the ratios are essentially unaffected by 60 mM NaCl stress.

Sitosterol, campesterol and stigmasterol were the major sterol species in gametophyte tissues (Table 2). Analysis of variance indicated no overall effect of genotype on sterol composition, however a significant salt effect was detected, namely on stigmasterol and sitosterol levels. Stigmasterol levels decreased in in all genotypes in response to 60 mM NaCl stress (4 - 5 mole%), while sitosterol levels increased (6.5 - 8.6mole%).

The fatty acid compsition of PL and GL was also examined and the results are provided in Tables 3 and 4, respectively. Differences between the fatty acid patterns of wild type, *stl1* and *stl2* were small. Statistical anlayses indicated that there is no significant overall effect of genotype on PL or GL fatty acid composition. The major

fatty acids of the phospholipid class were 16:0, 18:3, 18:2, 18:1 and 20:4, representing approximately 45%, 16%, 11%, 10% and 9.5% of the PL fatty acid pool, respectively. The fatty acid profile for the glycolipid fraction was similar in the fatty acid species present, however the major fatty acids were 18:3, 16:3, 16:0, 18:2 and 18:1, representing approximately 50%, 18%, 11%, 7% and 6% of total GL fatty acid content, respectively. Small changes in the levels of some fatty acids in response to salinity stress were detected. For example, in the phospholipid fraction there was a slight decrease in 18:3 fatty acid during 60 mM NaCl stress, and a slight increase in 18:1 and 20:4 fatty acids. A slight increase in 18:1 fatty acid was detected in the glycolipid fraction, as well as a small decrease in 18:3 fatty acid. In all of these cases, 60 mM NaCl stress was found to affect all genotypes equally.

Table 1. Lipid Composition of Wild Type, *stl1* and *stl2* Gametophyte Tissue Grown in the Absence and Presence of 60 mM NaCl Stress. Each value represents the mean and standard deviation of three individual samples. Statistical analysis (see Statistics in Materials and Methods) indicated no significant difference between genotypes, with 60 mM NaCl stress affecting all genotypes equally.

		nanomoles/ g fresh weight				
strain	[NaCl]	PL	sterol[a]	GL	sterol/PL	GL/PL
wild type	0	756 ± 103	53 ± 1	1164 ± 48	0.070 ± 0.009	1.53 ± 0.14
	60	829 ± 58	56 ± 5	1194 ± 68	0.067 ± 0.002	1.44 ± 0.03
stl1	0	884 ± 110	55 ± 5	1243 ± 248	0.062 ± 0.008	1.40 ± 0.13
	60	902 ± 143	64 ± 15	1314 ± 134	0.073 ± 0.023	1.46 ± 0.08
stl2	0	696 ± 73	47 ± 3	972 ± 35	0.068 ± 0.005	1.40 ± 0.10
	60	786 ± 66	57 ± 3	1281 ± 16	0.073 ± 0.007	1.64 ± 0.14

[a]Sterols were determined from a total lipid sample and include free sterols, sterol esters, steryl glycosides and acylated sterol glycosides.

Table 2. Sterol Composition of Wild Type, *stl1* and *stl2* Gametophyte Tissue Grown in the Absence and Presence of 60 mM NaCl Stress. Each value represents the mean mole % and standard deviation of three individual samples. Statistical analysis (see Statistics in Materials and Methods) indicated no significant difference between genotypes, with 60 mM NaCl stress affecting all genotypes equally.

	wild type		*stl1*		*stl2*	
	0	60	0	60	0	60
sterol[a]	mole %[b]					
cholesterol	4.5±1.5	3.3±0.3	3.8±0.2	7.6±4.3	3.3±0.3	3.7±0.7
campesterol	22.3±1.0	21.5±0.1	21.8±0.8	16.2±9.9	21.2±0.2	19.0±0.4
stigmasterol	25.7±1.9	21.4±2.0	24.2±0.9	20.9±1.7	25.9±0.5	20.8±0.7
sitosterol	45.2±1.4	51.7±2.2	47.7±1.6	56.3±9.9	47.5±0.7	54.0±1.0
fucosterol	2.2±0.2	2.1±0.4	2.8±0.2	2.4±0.5	2.0±0.3	2.4±0.9

[a]Sterols were determined from a total lipid sample and include free sterols, sterol esters, steryl glycosides and acylated sterol glycosides.

Table 3. Fatty Acid Composition of Total Phospholipids in Wild Type, *stl1*, *stl2* Gametophyte Tissue Grown in the Absence and Presence of 60 mM NaCl Stress. Each value represents the mean and standard deviation of three individual samples. Statistical analysis (see Statistics in Materials and Methods) indicated no significant difference between genotypes, with 60 mM NaCl stress affecting all genotypes equally.

	wild type		*stl1*		*stl2*	
	0	60	0	60	0	60
fatty acid			mole %			
16:0	44.4±0.9	45.0±0.5	45.2±0.6	45.5±0.2	44.9±1.3	44.7±0.7
16:1	0.8±0.1	0.8±0.1	1.0±0.2	1.0±0.1	0.9±0.1	0.9±0.05
16:3	1.6±0.2	1.4±0.02	1.4±0.2	1.5±0.2	1.7±0.1	1.8±0.1
18:0	1.5±0.01	1.4±0.05	1.6±0.06	1.4±0.1	1.4±0.06	1.3±0.06
18:1	9.7±0.7	9.9±0.1	9.0±0.4	9.9±0.3	8.9±0.4	10.3±0.3
18:2	11.5±0.3	11.4±0.1	11.7±0.4	11.5±0.3	10.6±0.2	10.9±0.4
18:3	16.8±0.2	15.4±0.3	16.6±1.1	15.5±0.9	17.6±1.1	15.7±0.9
20:0	0.2±0.01	0.2±0.03	0.2±0.02	0.2±0.01	0.2±0.03	0.2±0.03
20:2	1.9±0.1	2.1±0.2	1.8±0.2	1.5±0.9	1.8±0.1	1.9±0.06
20:4	8.9±0.6	9.8±0.2	8.8±0.1	9.5±0.04	9.2±0.4	9.6±0.3
other	2.9±0.2	2.4±0.2	2.8±0.1	2.5±0.1	2.9±0.3	2.6±0.3
total (μmol/gfw)	1.51±0.21	1.65±0.12	1.77±0.22	1.80±0.29	1.40±0.15	1.57±0.13

Table 4. Fatty Acid Composition of Total Glycolipids in Wild Type, *stl1*, *stl2* Gametophyte Tissue Grown in the Absence and Presence of 60 mM NaCl Stress. Each value represents the mean and standard deviation of three individual samples. Statistical analysis (see Statistics in Materials and Methods) indicated no significant difference between genotypes, with 60 mM NaCl stress affecting all genotypes equally.

	wild type		*stl1*		*stl2*	
	0	60	0	60	0	60
fatty acid			mole %			
16:0	11.0±0.5	11.4±1.0	11.6±1.3	11.6±1.2	10.7±0.4	10.2±0.8
16:1	0.6±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.8±0.04	0.7±0.1
16:2	2.6±0.2	2.8±0.1	2.4±0.3	2.8±0.2	2.4±0.3	3.0±0.2
16:3	16.9±1.1	17.1±1.0	17.6±0.9	18.1±0.8	18.3±0.8	19.8±0.7
18:0	1.2±0.1	1.1±0.2	1.1±0.2	1.1±0.3	1.2±0.1	0.9±0.1
18:1	6.1±0.2	8.3±0.6	4.6±0.5	5.9±0.8	4.6±0.3	5.6±1.7
18:2	7.3±0.8	7.7±0.6	6.9±1.4	7.5±0.7	6.5±0.3	7.0±0.7
18:3	51.8±1.2	48.7±0.7	53.1±2.4	50.2±1.3	53.6±0.4	50.0±0.7
20:0	0.8±0.4	0.6±0.3	0.3±0.55	0.5±0.4	0.2±0.2	1.0±0.4
20:4	1.6±0.2	1.6±0.03	1.7±0.2	1.6±0.1	1.7±0.2	1.7±0.1
total (μmol/gfw)	2.33±0.10	2.39±0.14	2.49±0.49	2.63±0.27	1.94±0.07	2.56±0.03

4. DISCUSSION

In a number of studies plants differing in their level of tolerance to salinity stress have been found to also differ in their membrane lipid compositions (Kuiper, 1984 and refs. therein; Douglas and Walker, 1983; Douglas and Walker, 1984; Douglas and Sykes, 1985; Hirayama and Mihara, 1987). The differences, usually in sterol composition, sterol/PL or GL/PL ratios, are often used to explain the observed ion relations for a given plant(s). Other reports document salinity induced changes in lipid composition of salt sensitive and tolerant plants, and these changes have been interpreted as enabling membranes to function more or less effectively during salinity stress (Stuiver, 1981; Kuiper, 1984 and refs. therein; Peeler et al., 1989; Blitts and Gallagher, 1990a and b). Although it is not certain that such differences and changes in the composition of the lipid matrix of membranes holds a physiological significance in salinity tolerance, there is growing evidence to suggest the involvement of membrane lipid composition in the control of both passive (Kuiper, 1984; Kuiper, 1985 and refs. therein; Hirayama and Mihara, 1987), and active transport processes (particularly lipid regulation of the plasma membrane ATPase) in plants (Serrano et al., 1988; Cooke and Burden, 1990 and refs. therein).

In this study lipid content was examined in gametophyte tissue in the absence and presence of 60 mM NaCl stress. Wild type gametophytes grown at this NaCl concentration display a reduction in size (approximately 20%), yet show no signs of tissue necrosis or abnormal development that is typically seen at higher (100 mM)

NaCl concentrations (Warne and Hickok, 1987). Growth and development of *stl2* gametophytes is unaffected by 60 mM NaCl stress (Hickok et al., 1991), while a 10-15% reduction in growth is observed for *stl1* (Warne and Hickok, 1987). Examination of lipid composition under these conditions should limit changes in lipid metabolism associated with stress-induced degradation reactions, thereby reducing the possibility that any detected changes in lipid content and composition would reflect stress-induced deterioration of membranes (Kuiper, 1985).

The present results are based on an examination of total lipid from gametophyte tissue. Many studies examining lipid composition in plants differing in salt sensitivity or in response to salinity stress have used tissue or microsomal samples as the source of lipid (Kuiper, 1984 and refs. therein; Douglas and Sykes, 1985; Hirayama and Mihara, 1987; Blitts and Gallagher, 1990b). Due to the acknowledged importance of the plasma membrane and tonoplast in cellular responses to salinity stress, recent studies have focused on examinations of plasma membrane and endomembrane enriched fractions (Walker and Douglas, 1984; Douglas and Sykes, 1985; Peeler et al., 1989; Brown and DuPont, 1989; Blitts and Gallagher, 1990a). In the present study, lipids from whole tissue samples were examined. If the *stl1* or *stl2* mutations were altering lipid metabolism and influencing the composition of a lipid matrix of one or more membranes, the effect should be detectable from a complete tissue sample. Any differences in lipid composition detected between the wild type and mutant strains would have prompted a more detailed study.

The *stl2* mutation is associated with higher K^+ and reduced Na^+ accumulation

during NaCl stress (Part 3). Higher K^+ levels could result from selective uptake of K^+ or reduced leakage or efflux of K^+ from cells during salinity stress. More efficient exclusion or efflux of Na^+ from cells could account for the lower tissue levels of this ion during salt stress. In any case, the involvement of a membrane transport process(s) is implicated in the tolerant response. A number of factors can account for differences in ion transport, including changes in membrane permeability, selectivity, activity of membrane proteins involved in solute transport (including channels, pumps and carriers), or regulatory/feedback controls. While the composition of the lipid matrix of a membrane can potentially affect each of these, the results of this study indicate that a difference in membrane lipid composition is not a factor in the mechanism conferring enhanced tolerance to NaCl by this mutation. Future studies will begin to examine the involvement of specific plasma membrane proteins in the tolerant response of *stl2*. Likely candidates include K^+ channels and the plasma membrane ATPase.

In conclusion, no significant differences in lipid class composition, sterol/PL and GL/PL ratios, sterol composition or PL and GL fatty acid profiles were detected among the wild type, *stl1* and *stl2* in response to 60 mM NaCl stress. A small increase in PL, GL and sterol levels was observed in all three genotypes in response to 60 mM NaCl stress, but there was no differential effect of salinity on genotypes. Similarly, while salinity did affect the levels of some sterol and fatty acid species, no significant differences were detected between the wild type and mutant strains. These results indicate that a difference in lipid composition is not correlated with the salinity

tolerance imparted by *stl1* and *stl2*.

Acknowledgements

I would like to thank Dr. David White at the Center for Environmental Biotechnology for allowing me to perform these analyses in his laboratory and supplying me with all the necessary materials and equipment. I would also like to thank Steve Nold and Dave Ringelberg for their guidance in every aspect of this study. GC/MS of samples was performed by Dave Ringelberg.

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PART 6

SUMMARY OF PARTS 2 - 5

Our understanding of the physiology of plant-salt interactions has grown considerably over the past several years. However, information concerning the genetics of this trait is comparably modest. For the most part, genetic studies have been limited to demonstrating the polygenic nature of the trait (Norlyn, 1980; Dvořák et al., 1985; Gorham et al., 1987; Forster et al., 1990) and describing gross changes in gene expression at the protein level that result upon exposure to elevated levels of salinity (Hurkman and Tanaka, 1987; Ramagopal, 1987; Bohnert et al., 1989; Hurkman et al., 1989; Cushman et al., 1990). Severely lacking are integrated physiological and genetic studies, which, because of the inherent physiological and genetic complexity of the salt tolerant phenotype, are difficult to undertake. As an approach to this type of study, the fern *Ceratopteris richardii* has been used successfully as a model system to select for single gene nuclear mutations that confer tolerance to NaCl (Warne and Hickok, 1987; Hickok et al., 1991). Two unlinked mutations, *stl1* and *stl2*, have been selected for use in comparative physiological studies with the wild type.

Prior to initiation of this dissertation project, physiological characterizations of *stl1* and *stl2* were largely limited to documentation of the level of NaCl tolerance each mutation confers to gametophytes and sporophytes. The *stl1* mutation confers a low level of NaCl tolerance ($\leq$ 125 mM) (Warne and Hickok, 1987). The *stl2* mutation confers tolerance to NaCl concentrations of 200 mM and above, this being considered a high level of tolerance (Hickok et al., 1991). Both mutations confer tolerance largely to the gametophyte stage of development, for homozygous mutant

sporophytes displayed a much lower level of tolerance to NaCl (60 mM) relative to gametophytes (Hickok et al., 1991). These mutations had also been characterized as to their level of tolerance to the amino acid analogue, hydroxyproline. *stl2* gametophytes showed a higher level of tolerance to increasing concentrations of this compound, as compared to the wild type and *stl1* (Hickok, unpublished data). A number of possible mechanisms can result in tolerance to hydroxyproline, one of which is the overproduction of proline. Thus, prior to initiation of the present studies, circumstantial evidence suggested that the *stl2* mutation may be associated with an enhanced ability to accumulate proline, a compound which has been suggested to play a role in osmotic adjustment of the cytoplasm with the external environment during water and salinity stress (Greenway and Munns, 1980).

The overall objective of this dissertation project was to advance our understanding of the physiological basis of tolerance associated with these mutations. The studies performed employed conventional techniques and, in general, followed the approaches of others making similar inquiries. However, a major difference between these and other studies is in the use of near isogenic lines. Most studies to date have involved the use of plants with considerably different genetic backgrounds, comparing salt-sensitive and salt-tolerant taxa or varieties differing in their level of tolerance to NaCl. The *stl1* and *stl2* mutations are valuable for they provide a means to identify specific physiological characters that are under genetic control.

The following paragraphs summarize the results of these studies and the suggested possible mechanisms contributing to salinity tolerance.

1. *stl1*

The results of cross-tolerance tests comparing the growth responses of *stl1* and the wild type indicated that this mutation confers a similar low level of tolerance to isosmotic levels of osmotic and NaCl stress. While this observation might suggest that *stl1* confers tolerance to osmotic stress imposed by NaCl, additional comparisons involving various salts suggested that tolerance is specific for Na^+. Further physiological comparisons between the wild type and *stl1* revealed no differences between these two genotypes. Specifically examined were (i) the levels of various organic compounds in the presence and absence of moderate NaCl stress (Part 2), (ii) tissue ion content (including major cations and Cl^-) during increasing levels of NaCl stress (Parts 2 and 3), (iii) various aspects of water relations in the absence and presence of moderate NaCl stress (Part 2), (iv) the effects of supplemental Ca^{2+} and K^+ on the salinity response (growth and tissue ion content) (Part 4) and (v) total lipid composition in the absence and presence of a moderate level of NaCl stress (Part 5).

Although differences between *stl1* and the wild type are few in number, the cumulative results do suggest a possible physiological basis of tolerance to NaCl for this mutation. Available results suggest that the *stl1* mutation may be associated with enhanced compartmentation of Na^+ within the cell, perhaps within the vacuole. This hypothesis is primarily based on three observations: (i) no differences in ion profiles, particularly Na^+ and Cl^-, between *stl1* and the wild type gametophyte tissue during NaCl stress, (ii) no significant differences in water relations of these genotypes and

(iii) the suggested specificity of tolerance for Na^+. Uptake and subsequent compartmentation of Na^+ is suggested for many halophytes during osmotic stress (Flowers et al., 1977; Flowers and Yeo, 1986; Greenway and Munns, 1980). Further, it is believed that toxicity in glycophytes may, in part, be due to an inability or inadequacy in compartmentalizing Na^+ and/or Cl^- once these ions have accumulated in the cytoplasm (Greenway and Munns, 1980).

The most definitive way to confirm or refute the above hypothesis involves the use of X-ray microprobe analysis to determine the level and distribution of ions, particularly Na^+, in subcellular compartments. Several groups have been successful in the use of this technique (Sacher and Staples, 1984 and refs therein; Hajibagheri et al., 1987; Binzel et al., 1988). Unfortunately, attempts to use this approach on *Ceratopteris* gametophytes, through collaboration with Dr. John Dunlap of the EM facility at the University of Tennessee, Knoxville, have been unsuccessful due to difficulties in fixation and preparation of tissue for analysis.

2. *stl2*

The *stl2* mutation is particularly interesting because it individually confers a high level of NaCl tolerance to gametophytes. In addition, the mechanism of tolerance appears to involve altered ion transport at the plasma membrane. There are few examples of single gene mutations conferring tolerance to NaCl stress, and even

fewer such mutations associated with altered ion relations.

The results obtained from these studies are presented in Figure 1. These results point out several interesting characteristics of the *stl2* mutation. First, this mutation is not associated with an enhanced ability to accumulate organic solutes, such as proline, that could be used in osmotic adjustment of the cytoplasm. Rather, *stl2* maintains higher K^+ levels and lower Na^+ levels in gametophyte tissue than the wild type in response to NaCl stress. This altered ion accumulation results in more favorable K^+-Na^+ relations, as indicated by the higher K^+/Na^+ ratio observed during NaCl stress. Results from cross-tolerance tests indicate that tolerance to NaCl conferred by *stl2* is largely associated with the ability to respond to ionic, rather than osmotic, stress imposed by NaCl. Further, tolerance to both Na^+ and Mg^{2+} salts suggests a nonspecific tolerance for cations and anions. The differential response of *stl2* to supplemental Ca^{2+} and the ability of supplemental Ca^{2+} to increase K^+ and reduce Na^+ accumulation in the wild type implicates the involvement of this ion in the salinity response of *stl2*. Finally, *stl2* is highly sensitive to K^+, with reduced levels of K^+ in the culture medium resulting in enhanced gametophytic growth. This observation suggests that *stl2* gametophytes suffer from K^+ toxicity under normal culture conditions.

In accordance with these results and information obtained from both the plant and animal literature a model was advanced to explain the possible mechanism of action of *stl2* (Part 4). It was proposed that the *stl2* mutation affects a K^+/cation influx channel located in the plasma membrane. Selectivity of the wild type channel protein and K^+

- *stl2* and the wild type accumulate similar levels of free amino acids, betaine, soluble sugars and organic acids. In addition, these genotypes display similar water relations.(Part 2)

- *stl2* is associated with the selective accumulation of K^+ and reduced accumulation of Na^+ during salinity stress, resulting in higher K^+/Na^+ ratios. (Part 2 and Part 3)

- Tolerance to NaCl conferred by *stl2* is largely associated with the ability to respond to ionic, rather than osmotic, stress imposed by NaCl. (Part 3)

- *stl2* confers a high level of tolerance to Na^+ and Mg^{2+} salts. During low levels of stress from these salts a small stimulation in growth is detected. (Part 3).

- Supplemental Ca^{2+} has very little effect on the growth response of *stl2* gametophytes during NaCl and $MgCl_2$ stress, whereas the toxic effects of these two salts can be alleviated by Ca^{2+} supplementation in the wild type. (Part 4)

- Ca^{2+} supplementation did present changes in K^+ and Na^+ content during NaCl stress in both *stl2* and the wild type, resulting in higher K^+/Na^+ ratios. (Part 4)

- *stl2* maintains tolerance to NaCl even under reduced external levels of K^+ (Part 4)

- Growth of *stl2* gametophytes is sensitive to K^+ concentration in the external medium, in that growth is greatly increased on medium containing reduced levels of K^+. (Part 4)

- Similar K^+ and Na^+ levels are found in *stl2* and wild type tissue under conditions of reduced K^+ and 0 mM NaCl. During 175 mM NaCl stress and reduced K^+ *stl2* looses the ability to maintain high levels of K^+ and reduced levels of Na^+. (Part 4)

- No differences in total lipid composition were detected between *stl2* and the wild type in the absence or presence of moderate NaCl stress. (Part 5)

Figure 1. Summary of Physiological Studies Comparing *stl2* and the Wild Type.

uptake is enhanced by extracellular Ca^{2+} binding to the channel protein, such that when Ca^{2+} is bound to the protein, the channel is selective for K^+ and K^+ uptake is greater. Removal or replacement of Ca^{2+} results in the loss of channel selectivity for K^+ and the subsequent movement of other monovalent cations through the channel. A higher affinity of the *stl2* channel protein for Ca^{2+}, relative to the wild type protein, would result in a greater level of bound of Ca^{2+} at any given time. In the absence of NaCl stress this altered binding results in enhanced K^+ uptake compared to that for the wild type. During salinity stress, the tighter binding of Ca^{2+} to the mutant channel protein would, in effect, result in maintanence of channel selectivity for K^+ and uptake. Alternatively, *stl2* may result in a loss in channel sensitivity to Ca^{2+}.

Tolerance to Na^+, and the resulting higher K^+/Na^+ ratio, is explained by the channel's ability to maintain selectivity for K^+ over Na^+ during NaCl stress. During NaCl stress Na^+ is suggested to displace Ca^{2+} (extracellular) from the wild type K^+/cation channel, consequently altering channel function, which in turn reduces K^+ uptake (and results in Na^+ uptake). In the proposed model the *stl2* mutation reduces the ability of Na^+ to displace externally bound Ca^{2+}.

The growth sensitivity of *stl2* gametophytes to basal levels of K^+ in the absence of NaCl stress is the result of enhanced uptake of K^+. Under these conditions cells would attempt to maintain acceptable cytoplasmic levels of K^+ and electrical homeostasis, and this would likely require an expenditure of energy. The enhancement in *stl2* gametophytic growth that is observed on medium containing reduced levels of K^+ would presumably be due to a reduction in K^+ uptake.

Tolerance to Mg^{2+} stress may be explained in the same manner as Na^+ tolerance, whereby the *stl2* mutation reduces Mg^{2+} induced displacement of Ca^{2+}. Alternatively, Mg^{2+} toxicity may be associated with channel blockage (Matsuda, 1988). In this case, tolerance would be due to the ability of Ca^{2+} to reduce channel blockage by Mg^{2+}.

Cross-tolerance tests indicated that low levels of Na^+ and Mg^{2+} salts resulted in a small enhancement in growth of *stl2* gametophytes (Part 3). In light of the proposed model, low levels of Na^+ and Mg^{2+} would relieve K^+ toxicity by causing a low level of Ca^{2+} displacement, which in turn would result in a slight reduction in K^+ uptake.

Admittedly, the available results do not dismiss the possibility that the *stl2* mutation is associated with a membrane protein other than a K^+ channel, such as a H^+-ATPase or a K^+/H^+ antiporter. Clearly, electrophysiological studies are needed to specifically look at cation channel proteins in the wild type and *stl2*, identifying possible K^+/cation channel types, and subsequently examining channel selectivity to cations and the effect of Ca^{2+} on selectivity and activity. K^+ uptake and efflux studies involving the wild type and *stl2* are also necessary.

Few examples of mutations conferring tolerance to NaCl in higher plants are available for study. Those available include the proline accumulating mutants of barley (Kueh and Bright, 1982) and *Nicotiana plumbaginifolia* (Sumaryati et al., 1992), the Cl^- transport mutant of soybean (Able, 1969), the mutants of *Arabidopsis* associated with K^+ and Na^+ accumulation in germinating seeds (Saleki et al., 1993), the *stl1* and *stl2* mutants of *Ceratopteris* that involve altered ion relations and those of

alfalfa (Winicov, 1991), the physiological basis of tolerance for which is not known. Clearly, the *stl2* mutation provides an extraordinary opportunity to study the contribution of a single gene in the cellular regulation of ion transport during salinity stress and, ultimately, the role of a cellular-based mechanism of tolerance in a whole plant response.

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VITA

Dale Lynn Vogelien was born in Cooperstown, New York on September 4, 1958. She attended Richfield Springs Elementary School and graduated from Schoharie Central High School in June of 1976. The following September she entered the State University of New York (SUNY) at Cobleskill, and in June of 1978 she received an Associate in Applied Science degree in Science Laboratory Technology. She continued her college education at SUNY Plattsburgh and in June of 1981 received a Bachelor of Science degree in Cell Biology/Microbiology. In September 1981 she began working as a research technician at the W. Alton Jones Cell Science Center in Lake Placid, New York for Dr. Donald K. Dougall, whose major research interest involved secondary metabolism in plant tissue cultures. The position with Dr. Dougall was continued at the University of Tennessee in Knoxville, Department of Botany, through 1986. In September of 1983 she began study towards a Master of Science degree in the Plant Physiology and Genetics Program at the University of Tennessee under the guidance of Dr. Dougall. This degree was awarded in December of 1987. She was introduced to the "wonder fern", *Ceratopteris richardii*, in the summer of 1988 when she began working with Dr. Leslie G. Hickok, also in the Botany Department at the University of Tennessee. A year and a half later she began study towards a Doctorate of Philosophy degree in the Botany Department, with Dr. Hickok serving as a mentor. This degree was awarded in December of 1993.