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THE ANALYSIS OF GENETICALLY AND PHYSIOLOGICALLY COMPLEX TRAITS USING CERATOPTERIS: A CASE STUDY OF NaCl-TOLERANT MUTANTS

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Genetic and physiological complexities associated with salt tolerance in plants have limited progress in the analysis of specific factors responsible for the salt-tolerant phenotype. We have used the homosporous fern *Ceratopteris richardii* as a model plant to investigate the physiological basis of salinity tolerance by selecting single gene mutants that confer tolerance in the gametophyte generation. The unique genetic system of homosporous ferns permits the generation of mutants in a genetic background nearly isogenic to the wildtype, such that comparative studies with the wildtype can identify specific physiological responses associated with salt tolerance. One of these mutations, *stl2*, confers a high level of tolerance to Na^+ ($I_{50} \approx 175$ mM NaCl) and generates a complex suite of related phenotypes. For example, in addition to Na^+ tolerance, *stl2* exhibits tolerance to Mg^{2+} salts, sensitivity to supplemented K^+, higher K^+-dependent efflux of K^+, altered responses to Ca^{2+} supplementation and moderate tolerance to osmotic stresses. Based upon its physiological attributes, we have proposed that the mechanism of action for this mutation involves an enhanced influx of K^+ and higher selectivity for K^+ over Na^+ in a K^+ channel. The direct and indirect consequences of this alteration can account for NaCl tolerance and the other phenotypes evident in *stl2*. The complex set of phenotypic responses from such a single gene mutation illustrates the potential for even more extreme pleiotropy in multigenic salt-tolerant strains.

Introduction

Model systems can be especially useful when employed to examine traits that are difficult to study in other organisms. Some traits, such as salinity tolerance, have evolved independently in a number of plant taxa and exhibit extremes of genetical and physiological complexity. Consequently, progress has been limited in the identification of specific genetic factors associated with tolerance, as well as the net contributions of these factors and their interactions. To further our understanding of the genetic basis of salinity tolerance, we have successfully used *Ceratopteris* to select single gene mutations that confer tolerance. A large number of stable salt-tolerant mutant lines are now available, and, as they are characterized genetically and physiologically, we are examining their expression both singly and in combination. By this approach, we are attempting to identify synergistic and additive combinations of genetic modifications that result in significant levels of tolerance.

Physiological complexity of salt tolerance

The study of the mechanisms of salt tolerance in plants not only addresses the important agricultural problem of crop response to soil salinization but also may provide insight into the fundamental processes of ion transport, accumulation, and regulation across the plasma membrane as well as information on the biophysical events that affect a plant exposed to salt stress. Although several other ions, e.g., SO_4^{2-} and Mg^{2+}, may contribute to soil salinity stress, the toxic effects of Na^+ and Cl^- are considered important (Flowers and Yeo 1986) and serve predominantly as model ions in experimental studies. Despite numerous studies of plant responses to salt stress, little is known about the specific genetic basis and associated physiological mechanisms that can confer tolerance to salt (Cheeseman 1988; Dracup 1991). Many basic physiological attributes have been suggested as important components of a salt-tolerant phenotype, including direct modification of the influx and/or efflux of ions such as K^+ and Na^+ across the plasma membrane and tonoplast (Jeschke 1984; Flowers and Yeo 1986; Yang et al. 1990*b*); synthesis of compatible osmotica such as proline, other amino acids, soluble carbohydrates, and glycine betaines (Jones and Storey 1981; Sumaryati et al. 1992); and modifications of membrane composition (Kuiper 1984, 1985; Hirayama and Mihara 1987; Peeler et al. 1989; Blits and Gallagher 1990). These physiological mechanisms and numerous others that are presumed to confer some level of salinity tolerance have been reviewed extensively (Rains 1972, 1987; Flowers et al. 1977; Epstein et al. 1980; Greenway and Munns 1980; Yeo 1983; Läuchli and Epstein 1984; Flowers and Yeo 1986; Rhodes et al. 1986; Epstein and Rains 1987; Cheeseman 1988; Munns 1993). In general, halophytes compartmentalize both Na^+ and Cl^- into the vacuole and regulate ion transport at both the plasma membrane and tonoplast (Flowers et al. 1977, 1990; Flowers 1985; Flowers and Yeo 1986). The osmotic imbalance of the cytoplasm relative to the vacuole and the external environment may be adjusted by the accumulation of compatible

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organic solutes, such as proline, betaine, or sugars in the cytoplasm (Flowers et al. 1977; Greenway and Munns 1980; Motah et al. 1987; Binzel et al. 1988). Some halophytes have differential responses to salinity levels, e.g., *Spergularia marina* "includes" Na^+ at low levels and "excludes" it at high levels (Cheeseman and Wilkens 1986). Other adaptations associated with some halophytes include transport of Na^+ to specialized glands or bladders and succulence (Flowers et al. 1977). Some nonhalophytes, i.e., glycophytes, respond to high external salinity by the accumulation of Na^+ and Cl^- (Rush and Epstein 1976; Marschner et al. 1981; Binzel et al. 1988). In other glycophytes, tolerance is apparently associated with enhanced K^+/Na^+ selectivity, increased Na^+ efflux at the plasma membrane, and/or restricted transport of Na^+ and Cl^-, especially to the shoot (Greenway and Munns 1980; Jeschke and Nassery 1981; Jeschke 1984; Walker 1986; Gorham et al. 1987; Yeo and Flowers 1989; Chow et al. 1990; Yang et al. 1990*a*). The maintenance of relatively lower cytoplasmic concentrations of incompatible ions, e.g., Na^+, and adequate concentrations of compatible ions, e.g., K^+, emerges as an apparent fundamental property of salt tolerance at the cellular level in some taxa (Leigh and Jones 1984; Ben-Hayyim et al. 1987; Chow et al. 1990). Critical concentration of K^+ contributes to several important cellular functions, including cytoplasmic and organellar osmotic potential, activation of enzymes in glycolysis, starch synthesis and protein synthesis, maximum photosynthetic activity, control of cytoplasmic pH and membrane potential, and a correct thermodynamic environment for active form protein folding (Leigh and Jones 1984). Such modifications of K^+ and Na^+ contents must result from appropriate K^+ influx and efflux relative to Na^+ fluxes or subcellular partitioning of these ions principally between the cytoplasm and vacuole. However, little is known about the specific physiological mechanisms that can yield these responses.

It is apparent even from this brief summary that ion transport and its regulation play key roles in the salt-tolerant phenotype (Kramer 1984; Flowers and Yeo 1986) and that the tolerance responses of plants to excess salinity involve potentially numerous, extremely complex, and highly integrated responses at the subcellular, cellular, and interorgan and organismal level (Epstein and Rains 1987; Cheeseman 1988; Yeo and Flowers 1989; Claes et al. 1990; Adams et al. 1992). This complexity is not merely a function of the large numbers of potential processes but is also the result of the indirect interactions and complexities associated with such ion transport phenomena themselves (Glass 1989). For instance, alterations in ion transport not only may directly modify intracellular ion contents but also may change the fundamental driving forces for ion transport, i.e., $\Delta\mu_{H+}$ and E_m. Changes in these membrane gradients, in turn, could immediately influence any number of transport processes driven by or coupled to an electrochemical gradient independent of their involvement with salt tolerance. These alterations, especially in conjunction with any additional genetic differences among strains being compared, could result in an almost indecipherable phenotypic response. In addition, the responses of whole plants reflect the integration of processes operating among the external environment, cell wall, root cells, vascular system, shoot cells, and leaf cells and make the dissection of individual mechanisms exceedingly difficult.

Genetic complexity of salt tolerance

Because of the unavailability of isogenic lines differing only in salt tolerance, nearly all physiological studies have been restricted to comparisons between different taxa or varieties that likely have significant genetic differences in addition to those differences specifically related to responses to salt stress. Naturally evolved salt tolerance is a quantitative and polygenic trait associated with numerous morphological and biochemical adaptations (Shannon 1984, 1985; Tal 1984, 1985; Epstein and Rains 1987; Cushman et al. 1989). The level of genetic complexity is evidenced by the multigenic inheritance, e.g., various heritabilities, of salt tolerance traits in such taxa as rice (Yeo et al. 1988), barley (Forster et al. 1990), tomato (Saranga et al. 1992), various grass species (Ashraf et al. 1986), pigeonpea (Subbarao et al. 1990), and wheat (Schachtman and Munns 1992). In the Triticaceae, responses to salt of chromosome addition lines from *Thinopyrum elongatum* into tetraploid (*Triticum durum* cv Stewart) or hexaploid wheat (*Triticum aestivum* Chinese Spring) show that several chromosomes may contribute to Na^+ exclusion, distinct from the K^+/Na^+ discrimination trait controlled by *Knal* in the D genome of wheat (Gorham 1994).

The potential for genetic and physiological complexity is also evident in changes in gene expression analyzed by 2-D gel electrophoresis of in vivo labeled proteins and in vitro translation products from mRNA (Bohnert et al. 1989; Cushman et al. 1989; Claes et al. 1990; Hurkman et al. 1991). For instance, salt stress results in quantitative changes in the synthesis of some proteins and may or may not induce unique proteins (Hurkman et al. 1988, 1989; Hurkman 1990). Salt-induced changes in polypeptide and mRNA levels in the roots of sensitive (cv Prato) and tolerant (cv CM72) cultivars of barley (*Hordeum vulgare* L.) were small and similar (Hurkman et

al. 1989). Various techniques have identified several proteins such as osmotin (Singh et al. 1985), the rice *salT* (Claes et al. 1990), and the barley germin-like polypeptide (Hurkman et al. 1991) that are induced or modulated by salt stress. Although these molecular approaches are useful for descriptive analyses of salinity responses, they cannot distinguish between the general effects of salt stress, a salt-induced response that confers tolerance, and/or whether the changes are genetically related to a salt-tolerance trait (Cheeseman 1988; Hurkman 1990).

An alternative approach is needed to identify and effectively resolve the contributing individual physiological mechanisms associated with a salt-tolerant phenotype. One such approach, recognized for a number of years, is the use of strains bearing single gene mutations that confer some level of salinity tolerance. However, even given more than 30 yr of exploration, few well-defined mutants are available. The few mutants that have been studied typically exhibit either greater sensitivity to salt stress or tolerance that is restricted developmentally. For instance, the wilty mutant of pepper (*Capsicum annuum, scabrous diminutive*) accumulates more Na^+ and less K^+ and effluxes less Na^+ and more K^+ in 100 mM NaCl-supplemented medium than the wildtype genotype (Tal and Benzioni 1977; Benzioni and Tal 1978). This wilty mutant is also associated with excessive stomatal opening and possible cuticular changes that result in lower leaf resistance to water loss and alterations in leaf anatomy (Tal et al. 1974). Tal (1985) outlined a possible series of epigenetic events that could account for the pleiotropic effects associated with this wilty mutant and has proposed an unidentified change in "membranal components" as the likely site of the lesion that subsequently influences several transport processes and morphology. Likewise, the Cl^- transport mutant of soybean shows enhanced sensitivity to salt stress (Abel 1969). The recently reported salt-tolerant mutants of *Arabidopsis thaliana* express tolerance only in germination responses and are reportedly based on changes of osmotic relations and an enhanced capacity for seed imbibition under water stress conditions (Saleki et al. 1993).

In addition to traditional breeding approaches and selections based on whole plants, cell and tissue culture selections have been used to isolate salt-tolerant lines. This approach has resulted in the successful establishment of salt-tolerant cell or callus variants for a large number of taxa (Nabors et al. 1975; Nabors et al. 1980; Croughan et al. 1981; Tyagi et al. 1981; Kochba et al. 1982; Rangan and Vasil 1983; Smith and McComb 1983; Watad et al. 1983; Chandler and Vasil 1984; Pandey and Ganapathy 1984; Hassan 1988; Winicov 1991; Dix 1993). Although cell and tissue culture approaches provide some advantages over conventional breeding, many difficulties continue to plague this approach (Stavarek et al. 1980; Epstein and Rains 1987; Dracup 1991). Only a few of the salt-tolerant variants have been successfully regenerated as whole plants, which has limited their usefulness in associating specific genetic changes with a salt-tolerant phenotype; and often regenerated plants lack expression of the salt-tolerant trait (Dix et al. 1984; Epstein and Rains 1987).

Currently, substantial information on the genetics of salt-tolerant variants is limited to *Nicotiana tabacum* (Nabors et al. 1980; Bressan et al. 1987), *Nicotiana plumbaginifolia* (Sumaryati et al. 1992), and alfalfa (Winicov 1991). In alfalfa, salt tolerance was maintained in callus cultures derived from regenerated salt-tolerant plants. The segregation of tolerant and sensitive phenotypes in progeny of selfed regenerated plants indicated a dominant-type inheritance. However, the autopolyploid nature of alfalfa and non-Mendelian segregation ratios precluded a formal genetic interpretation. Sumaryati (Sumaryati et al. 1992) identified individual mutants resistant to NaCl, KCl, and PEG from protoplast-derived colonies of haploid *N. plumbaginifolia* (Vivani). Following regeneration of diploid plants, presumably by spontaneous chromosome doubling, the tolerance trait was inherited in each mutant as a single dominant gene. However, the NaCl- and KCl-tolerant mutants were male sterile even after two successive backcrosses with the wildtype. In the most comprehensively studied system, *N. tabacum* var. Wisconsin (W38) cells, stable expression of tolerance in the absence of salt depends on the level of salt used for adaptation (Bressan et al. 1985). In cells adapted to 428 mM NaCl (S25) the non-Mendelian inheritance of NaCl survivability and of reduced growth rate, a trait associated with the salt-tolerant phenotype, indicates polygenic and/or cytoplasmic inheritance (Bressan et al. 1987). Inheritance and expression of tolerance and associated traits may be further complicated by polyploid chromosome number changes in the S25 cell line (hexaploid) (Kononowicz et al. 1990*a*, 1990*b*).

In addition to these noted genetic complexities, extensive comparative physiological analyses of salt-tolerant *N. tabacum* S25 cells (Binzel et al. 1985) have identified several independent physiological mechanisms that may contribute to the salt-tolerance trait in this cell line, e.g., modified plasma membrane H^+-ATPase activity (Reuveni et al. 1993); accumulation of osmotica, including Na^+, Cl^-, and organic solutes (Binzel et al. 1987); cytoplasmic volume changes and differential compartmentation of Na^+ and Cl^- to the vacuole (Binzel et al. 1988); more effective Na^+ exclusion (Binzel et al. 1989); and accumulation of osmotin

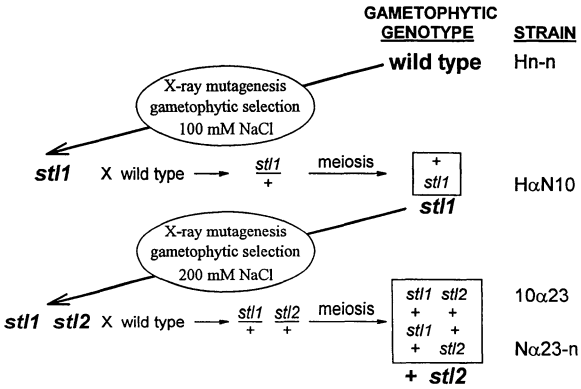

Fig. 1 Selection strategy for modified serial selection procedure and identification and isolation of single gene salt-tolerant mutants in the homosporous fern *Ceratopteris.*

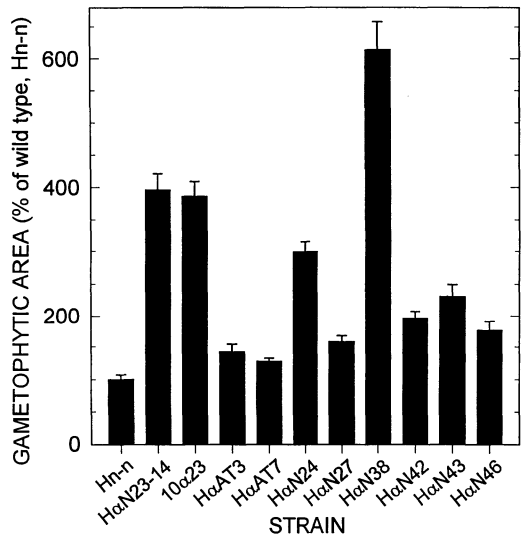

Fig. 2 Gametophytic areas of *Ceratopteris* strains carrying known salt-tolerant mutations, i.e., *stl2* (HαN23-14) and *stl1 stl2* (10α23), various putative mutants that express salt tolerance in M2 generation gametophytes and the wildtype (Hn-n). HαNn represents sister selections of HαN10 from a screen on 100 mM NaCl (fig. 1). HαATn represents selections from aluminum-EDTA screen that are also tolerant to low pH (Wright et al. 1990). Precordate gametophytes were transferred to medium supplemented with 175 mM NaCl and cultured for 17 d. Areas of gametophytes stained and mounted in a Hoyer's medium–acetocarmine mixture were measured using a computer-interfaced image analysis system (Bioquant IV). Values represent the mean ± SE of $n = 10$.

I and reduced growth rate (Bressan et al. 1987). The wide diversity of these traits indicates extensive pleiotropy or epigenetic interactions that limit the potential to associate physiological mechanisms with specific genetic changes.

Ceratopteris and salt-tolerant mutants

Ideally, the physiology of single gene mutations should be studied in a genetic background isogenic to the wildtype to allow for identification of specific physiological processes associated with the salt-tolerance trait. To obtain such single gene mutations requires a system that is capable of rapidly screening very large numbers ($>10^6$) of individuals, is highly sensitive, and can easily discriminate minor differences in tolerance, is morphologically simple, and, most important, is genetically well defined such that mutants arise in a nearly isogenic background. We have successfully used *Ceratopteris* to select for single gene mutations that confer salt tolerance to gametophytes. The unique life cycle and genetics of homosporous ferns allow an unlimited population of genetically identical spores to be generated after a single generation of intragametophytic selfing (Hickok 1987; Hickok et al. 1987; Hickok et al. 1995). Such a completely isogenic population of spores is then mutagenized, either by X-irradiation or chemical (EMS) mutagenesis, and cultured on medium supplemented with a selection agent, e.g., 100 mM NaCl (Warne and Hickok 1987) (fig. 1). Tolerant individuals can be isolated and self-fertilized to generate a completely homozygous M1 sporophyte carrying the putative mutation of interest in a homozygous state. Both dominant and recessive mutants can be recovered in a single generation. Gametophytes derived from M1 spores can be tested to confirm tolerance and crossed to carry out genetic studies. Since selection is carried out in the nonvascular haploid gametophyte, complications associated with the vascular system are avoided. Gametophytes are exposed to NaCl for usually less than 21 d. Individuals that exhibit tolerance are removed from salt, isolated onto salt-free medium, and watered to effect intragametophytic selfing. Only those gametophytes that develop functional gametangia and produce sporophytes are used to generate material for further analyses.

Genetic characterization of salt-tolerant mutants: *stl1, stl2,* and *stl3*

An original screen of 1.25×10^6 spores on 100 mM NaCl-supplemented medium resulted in a selection of over 40 individuals that showed wide-ranging tolerance to NaCl (Warne and Hickok 1987). A retest of tolerance for several of these selections show that original M1 spores have retained tolerance for over 8 yr in storage (fig. 2). Two of these original selections were characterized genetically as unlinked single nuclear gene mutations; strain HαN16, carrying the *stl3* mutation, and HαN10, which carries the *stl1* mutation (Warne and Hickok 1987). We commonly designate individual selections as separate strains even though they are selected directly from identical populations of mutagenized wildtype spores and can be considered nearly isogenic to each other and the wildtype with the exception of any induced mutation. In addition, once they are defined genetically, we refer to such strains merely

Table 1

RESPONSE OF THE WILDTYPE, *stl1*, AND *stl2* TO VARIOUS IONIC AND OSMOTIC STRESS AGENTS

Treatment	I_{50} (mM)		
	Wildtype	*stl1*	*stl2*
NaCl	100	110	175
Na_2SO_4	25	35	120
$MgCl_2$	25	25	80
$MgSO_4$	25	25	100
KCl	50	50	50
K_2SO_4	25	25	25
Mannitol	225	260	260
Melibiose	190	225	280

I_{50} is the approximate concentration (mM) that gives 50% inhibition of gametophytic growth. Values were obtained from dose response curves in Hickok et al. (1991), Vogelien (1993), and Vogelien et al. (1995).

by the salt-tolerance mutation they carry, e.g., *stl1*.

Because spore germination in *stl1* under salt stress was substantially higher than the wildtype, we used *stl1* in a subsequent selection at a higher NaCl concentration (200 mM). From this, we obtained the highly tolerant mutant strain 10α23. Segregation tests subsequently showed that 10α23 carried two unlinked mutations, *stl1* and *stl2* (fig. 1; Hickok et al. 1991). The new mutation, *stl2*, exhibited partial additivity with *stl1* and independently conferred tolerance up to 275 mM NaCl. *stl2* was segregated from *stl1* by backcrossing to the wildtype and was established as a single mutant homozygous Nα23-14 strain. It is highly likely that such strains, which originate from a single haploid gametophyte, bear only a single mutation for the trait in question. The induced mutation rate, based on estimates from selections with lethal concentrations of paraquat and FdUR, is ca. 10^{-5}. Given this as an estimate of the mutation rate for any given gene, the probability of any spore bearing two mutations that affect the same physiological process would be very small.

These single gene mutations provide the means to identify genetically regulated individual physiological processes that confer NaCl tolerance. The ability to use this type of discrete serial selection approach coupled with the subsequent isolation of individual mutations can also permit identification of genetic changes that are in themselves phenotypically cryptic but that act in concert to enhance tolerance, as has been done with paraquat tolerance (Hickok and Schwarz 1989). Since haploid gametophytes are essentially a single cell layer thick, only a few square millimeters at maturity, and have relatively limited cellular differentiation, it is likely that mutations conferring tolerance are associated with a cellular-based response. Mutations that confer known tolerance at a cellular level can subsequently be independently tested and characterized for tolerance in the sporophyte phase of the life cycle (Augé et al. 1989). With this system, given a single gene mutation in a genetic background essentially isogenic to the wildtype, differential responses of the mutant strain compared with its nearly isogenic wildtype will indicate those physiological mechanisms responsible for the salt-tolerant phenotype. This approach is based on the unique genetics of homosporous ferns and serves as a powerful means to examine the individual contributing mechanisms of physiologically complex traits.

Physiological characterization of the salt-tolerant mutants: *stl1* and *stl2*

OSMOTIC AND IONIC RESPONSES

To date, we have undertaken a comparative evaluation of *stl1*, *stl2*, and the wildtype with regard to a large number of physiological traits commonly associated with salt tolerance, including water relations, accumulation of inorganic (ions) and organic (amino acids, betaine, soluble carbohydrates, malate) solutes, responses to various osmotic and ionic stresses, K^+, Mg^{2+}, and Ca^{2+}, as well as K^+ influx and efflux and electrophysiological responses, in order to identify physiological differences associated with these mutations.

stl1 confers a low level of tolerance to NaCl and to Na_2SO_4, as well as to mannitol and melibiose, yet can be clearly identified both quantitatively and qualitatively in dose response and segregation tests (table 1; Warne and Hickok 1987; Vogelien 1993; Vogelien et al. 1995). *stl1*, like the wildtype, is sensitive to Mg^{2+} and K^+ salts (table 1). In response to 60 mM NaCl, both the wildtype and *stl1* exhibit substantial changes in contents of total amino acids, soluble carbohydrates, malate, and major ions (Vogelien et al. 1993). However, both *stl1* and the wildtype are essentially similar in their content of most major ions (K^+, Ca^{2+}, Mg^{2+}, Cl^-) and organic solutes (amino acids, betaines, soluble carbohydrates, and malate), both constitutively and in response to long-term exposure (21 d) to 60 mM NaCl. In addition, there were no substantial differences between *stl1* and the wildtype in osmotic responses (turgor, water, and osmotic potentials) (Vogelien et al. 1993). Supplemented Ca^{2+} (1 mM vs. 0.2 mM) alleviated growth inhibition to a similar extent in both the wildtype and *stl1* during NaCl or $MgCl_2$ stress (Vogelien 1993; Vogelien et al. 1995). The enhancement of tolerance to NaCl by Ca^{2+} was accompanied by an increased K^+ and lower Na^+ content. The only difference of note between the wildtype and *stl1* was the reduced accumulation of Na^+ in *stl1* (33 mM) compared

with that of the wildtype (47 mM) under long-term exposure to 60 mM NaCl and the associated increased K^+/Na^+ ratio. With shorter-term exposures (3 d), *stl1* had reduced Na^+ content compared with the wildtype only at external concentrations greater than 120 mM. The absence of any clearly defined physiological response indicates that tolerance may be associated with a more subtle process, such as a minor increase in selectivity against Na^+.

In contrast to *stl1*, the phenotypic suite of characters exhibited by *stl2* indicates a mutation with a complex mechanism of action. *stl2* confers a high level of tolerance not only to Na^+ (NaCl, Na_2SO_4) but also to Mg^{2+} salts ($MgCl_2$, $MgSO_4$) (table 1; Hickok et al. 1991; Vogelien 1993; Vogelien et al. 1995). Tolerance to mannitol was equivalent to *stl1*, and higher tolerance than either wildtype or *stl1* was evident with melibiose (table 1). *stl2* was not different from *stl1* and the wildtype in osmotic responses and in the contents of organic solutes, Ca^{2+}, Mg^{2+}, and Cl^-, both constitutively and in response to long-term exposure (21 d) to 60 mM NaCl (Vogelien et al. 1993).

However, *stl2* exhibited substantial differences from *stl1* and the wildtype in the accumulation of both K^+ and Na^+ during long-term exposure to NaCl (21 d) (Vogelien et al. 1993). In the presence of 60 mM NaCl, the K^+ content in *stl2* was nearly twice that of the wildtype (99 vs. 55 mM) and the Na^+ content was nearly one-half of the wildtype (24 vs. 47 mM). These differences result in a substantially high K^+/Na^+ ratio in *stl2*, i.e., 4.0 compared with 1.1 in the wildtype. *stl2* maintains a higher K^+, lower Na^+ content, and subsequently higher K^+/Na^+ ratio for concentrations up to at least 175 mM NaCl during short-term exposure (3 d).

Even though supplemental Ca^{2+} (0.2 vs. 1 mM $CaSO_4$) did not alleviate growth inhibition by NaCl (up to 200 mM) or $MgCl_2$ (up to 100 mM), it did result in a reduced accumulation of Na^+ (30 vs. 16 mM) and an increased accumulation of K^+ (72 vs. 116 mM) in gametophytes grown at 175 mM NaCl (Vogelien et al. 1995). This response to Ca^{2+}, which was substantially different from that of the wildtype, indicates the possible involvement of this ion in a salt-tolerance response.

stl2 also differs substantially from both *stl1* and the wildtype in its response to external concentrations of K^+ (Vogelien 1993; Vogelien et al. 1995). Under standard conditions (3.7 mM K^+), growth of *stl2* is only 60% of the wildtype. Reduction of extracellular K^+ to trace levels or that present only as a contaminant in the agar resulted in a ca. 40% increase in growth to a size equivalent with the wildtype under the same conditions. In addition to K^+ sensitivity, *stl2* maintained substantially greater tolerance to low NaCl levels (75 mM) even when K^+ was at trace levels. Tissue ion content of K^+ and Na^+ were similar for *stl2* and the wildtype with different levels of K^+ supplementation (0.37 vs. 3.7 mM) in the absence of NaCl. In the presence of 175 mM NaCl, 3.7 mM K^+ resulted in a substantially greater K^+ and lower Na^+ content in *stl2* compared with the wildtype.

K^+ INFLUX, EFFLUX, AND ELECTROPHYSIOLOGICAL RESPONSES

Recent examination of very short term Rb^+ influx (10 min) and efflux (2 h) and electrical responses have shown substantial differences between *stl2* and the wildtype that are consistent with K^+ and Na^+ contents in *stl2* both in the presence and absence of NaCl (long and short term) and the observed growth inhibition of *stl2* by ≥ 1 mM K^+ (Warne et al., unpublished). In these influx experiments, RbCl completely replaced KCl so that the direct effects of any putative difference in K^+ transport between the wildtype and *stl2* could be observed independently of any differential selective effects of K^+ over Rb^+. Rb^+ has been classically and extensively used as a tracer for K^+ and has been shown to move through K^+ channels (Bentrup 1990; Tester 1990). Though uptake rates are identical to the wildtype at concentrations less than 0.5 mM and greater than 50 mM, *stl2* has substantially higher uptake (ca. 30% greater) at 0.5–10 mM Rb^+. In addition, RbCl (5 mM) induced about a twofold times greater membrane depolarization in *stl2* than the wildtype. However, the experimental use of Rb^+ may underestimate the extent of the difference between *stl2* and the wildtype, since 5 mM KCl resulted in a 2.6-fold greater depolarization in *stl2*. The greater depolarization by Rb^+ or K^+ and enhanced uptake in *stl2* was not the result of different driving forces available for cation uptake since membrane potentials in both the wildtype and *stl2* were ca. −150 mV ($n = >30$). In addition to these differences in uptake and electrical responses to Rb^+ and/or K^+, *stl2* also exhibits consistently higher K^+-stimulated secretion of H^+ (fig. 3).

In the presence of NaCl, differences in K^+ uptake and electrical responses between *stl2* and the wildtype are maintained. Na^+ inhibits Rb^+ uptake in both the wildtype and *stl2;* however, *stl2* retains both higher absolute and normalized uptake of Rb^+ up to ratios of 0.5 mM Rb^+ : 180 mM Na^+. In addition, following treatment with NaCl (60 and 120 mM), subsequent depolarization induced by 5 mM K^+ was greater in *stl2*.

Unlike Na^+, short-term exposure to Mg^{2+} is not associated with significant inhibition of Rb^+ uptake even though chronic exposure to the same concentrations is strongly inhibitory to growth.

Though 5 mM Mg^{2+} ($MgCl_2$) inhibited Rb^+ uptake in *stl2* and the wildtype by about 20%, additional Mg^{2+}, up to 40 mM, had little further effect. Since the mechanism(s) of Mg^{2+} toxicity are unknown—for example, whether it is associated with excessive cytoplasmic accumulation or interaction with Ca^{2+}—the exceptional tolerance by *stl2* to this ion remains enigmatic. Toxicity, as reflected in an alteration of K^+ influx, may only occur with longer-term exposure to Mg^{2+}. For example, a longer exposure may be necessary for sufficient Mg^{2+} to accumulate to affect gating properties in K^+ channels (Matsuda 1988).

Though Ca^{2+} had a substantial effect on K^+ and Na^+ content of wildtype and *stl2* gametophytes grown in NaCl-supplemented medium, in the absence of NaCl, Ca^{2+} had no effect on the content of these ions. Likewise, Ca^{2+} supplementation up to 1.5 mM had no effect on Rb^+ uptake in either genotype. These responses indicate that Ca^{2+} may act independently of K^+ uptake to modulate levels of Na^+ and K^+. Ca^{2+}-induced alterations in K^+ and Na^+ contents are sufficient to ameliorate salt stress in the wildtype, but not in *stl2*.

Rb^+ influx was also measured in the presence of the quaternary ammonium salt, tetraethylammonium chloride (TEA; 10 mM), and CsCl (10 mM). Both of these compounds have been shown to block potassium channels or uptake in the excitable membranes of animal cells, *Chara,* and corn roots presumably by reversibly occluding the mouth of the pore (Hille 1967; Tester 1988; Bentrup 1990; Jan and Jan 1992). Both the wildtype and *stl2* showed similar inhibition of Rb^+ influx for TEA (20%–30%) and Cs^+ (70%–80%). The reduced effectiveness of TEA as an inhibitor of K^+ transport has been observed in low-salt roots of corn and other tissue in which TEA fails to accumulate readily (Kochian and Lucas 1988). Since gametophytic tissue was pretreated in a "low-salt"-type medium for up to 6 h prior to experimental use, it is possible that it responds in a similar TEA-insensitive manner. A dramatic differential response to inhibition of Rb^+ uptake occurred with exposure to NH_4Cl. NH_4^+ has been shown to inhibit K^+ influx (Bloom and Finazzo 1986; Vale et al. 1987; Topa and Jackson 1988), cause significant depolarization of membrane potential (Ayling 1993), and pass through K^+ influx channels (Tester 1990). Inhibition of Rb^+ influx by NH_4^+ was substantially greater in the wildtype (80%) compared with *stl2* (20%). Similarly, NH_4^+ gave a lower depolarization in *stl2* compared with the wildtype, and the capacity for subsequent depolarization by K^+ was likewise greater in *stl2*. The current evidence from influx studies using Rb^+ and the responses to various inhibitors of transport (Na^+, TEA, Cs^+, NH_4^+) as well as electrical data are consistent with a modification in velocity and selectivity in a K^+ influx transport system in *stl2*. Differences in influx occur only in the concentration range associated with low-affinity K^+ transport (ca. >1 mM) and are absent at those concentrations ascribed with high-affinity uptake (ca. <1 mM). Low-affinity K^+ influx is generally attributed to inward K^+ channels (Schroeder and Fang 1991; Gassmann et al. 1993; Kochian and Lucas 1993).

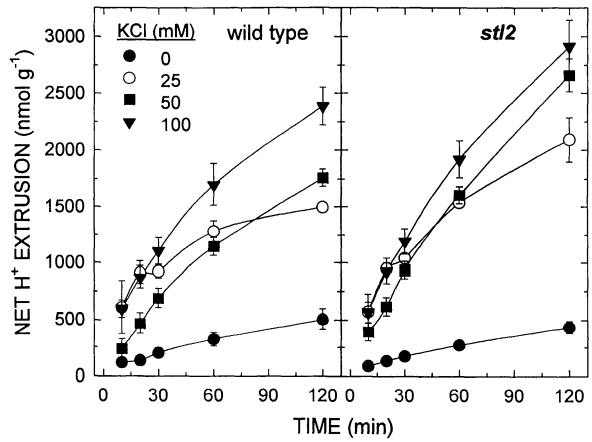

Fig. 3 K^+-induced H^+ secretion in wildtype and *stl2* gametophytes. Fourteen-day-old gametophytes (liquid culture) were rinsed and preincubated in CM buffer (0.5 mM Ca_2SO_4, 250 μM MES, pH 5.5) for 3–4 h and then resuspended in fresh buffer and treated with KCl. Aliquots of buffer were removed and pH was measured using a microelectrode in a flow-through chamber. Standard curves, generated from additions of known amounts of HCl (5.00 N) to CM buffer, allowed conversion of measured pH to quantity of secreted H^+. Net H^+ secreted was calculated by subtracting appropriate controls, i.e., no gametophytes.

Since constitutive levels of K^+ in the absence of NaCl stress are similar in both the wildtype and mutant, i.e., ca. 50 mmol kg^{-1} plant water, the higher uptake that occurs in *stl2* must be associated with a mechanism that can regulate cytoplasmic K^+ content. For example, such high uptake may be a transient phenomenon that is feedback regulated by increasing cytoplasmic content or coupled with an effective efflux mechanism (Pettersson 1986; Lew 1991). We measured loss of radiolabeled Rb^+ from gametophytes and into unlabeled rinse medium following a 2-h preloading with Rb^+ alone. Loss from both genotypes was similar and low when the rinse solution did not contain K^+; however, when the rinse solution contained K^+ at concentrations identical to the Rb^+ preloading treatment, loss was substantially higher in *stl2*. Ca^{2+} has no effect on this K^+-dependent Rb^+ efflux.

Co-segregation analysis is consistent with single gene basis

Given the complex physiological responses exhibited by *stl2,* it is important to establish wheth-

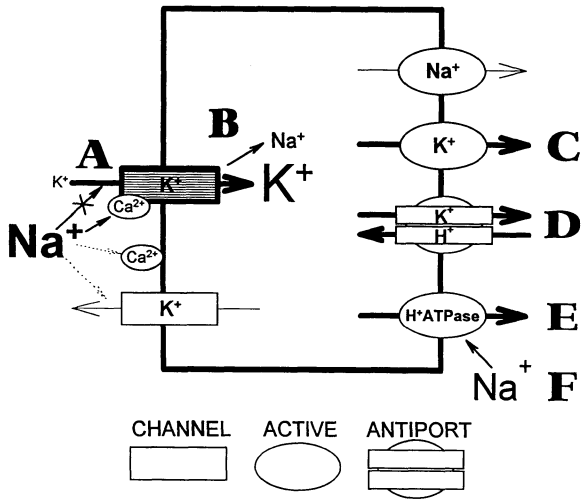

Fig. 4 Proposed model for the mechanism of action of *stl2*. An altered K^+ influx channel (*A*) possesses a higher selectivity and velocity for K^+ over Na^+ such that a greater intracellular K^+/Na^+ ratio (*B*) is maintained in the face of Na^+ stress. In the absence of Na^+ stress, a constitutive intracellular K^+ level, similar to that of the wildtype, is maintained by K^+ efflux mechanisms, e.g., K^+ pump (*C*) or K^+/H^+ antiport (*D*). Both of these mechanisms directly (*C*) or indirectly (*D, E*) use ATP and create an energy debt reflected in growth inhibition that can be alleviated by reduction of extracellular K^+ or by the interaction of other cations, e.g., Na^+, that reduce K^+ uptake. In the face of Na^+ stress, the intracellular content of $K^+ + Na^+$ increases as a consequence of direct or indirect (osmotic) stimulation of H^+-ATPase (*F*) and subsequent decrease in the membrane potential (E_m).

er all these phenotypes, in fact, are a consequence of a single mechanism. We tested for co-segregation of K^+ sensitivity and salt tolerance in gametophytes from *stl2*-type, F2 individuals that segregated from a hybrid between the wildtype and strain 10α23, which carries *stl1* and *stl2* (fig. 1). These individuals represent sister strains to Nα23-14, which is the *stl2*-carrying strain used in all the above studies, and are designated Nα23-n, where n = 1, 3, 4, 9, 10, and 16. Precordate gametophytes from each strain were transferred to control medium (3.7 mM K^+), to medium supplemented with 200 mM NaCl, and to medium without added K^+, i.e., trace K^+. All strains examined exhibit co-segregation of *stl2*-type salt tolerance (Hickok et al. 1991) and K^+ sensitivity (Vogelien et al. 1995). These data along with the genetic origin of the mutation in a completely homozygous line and subsequent genetic analyses lend further support for a single gene basis for the salt-tolerant phenotype and corresponding ion transport phenomena.

Model for mechanism of action of *stl2*

Given a discrete and single gene basis for the salt-tolerant phenotype of *stl2,* the above data derived from several different approaches provides compelling evidence for a physiological mechanism of action based upon the operation of an altered K^+ influx channel at the plasma membrane (fig. 4). In this model, all phenotypic responses are regarded as a direct or indirect consequence of the activity of a modified K^+ influx channel that has a higher effective velocity of transport and exhibits increased selectivity of K^+ relative to Na^+ and other competing cations, such as NH_4^+. In the absence of NaCl stress, the higher K^+ uptake does not result in a substantial net increase in cytoplasmic K^+ content, since local increases in K^+ could promote efflux by a K^+/H^+ antiport or by an outward K^+ pump. These efflux mechanisms ultimately utilize ATP either directly or indirectly by the action of a H^+-ATPase and result in ATP consumption that is reflected in growth sensitivity to K^+. Alleviation of this sensitivity occurs when the external K^+ concentration is reduced to near trace levels or by the addition of small amounts of other cations, such as Na^+ (Hickok et al. 1991). In this case, Na^+ may interfere with high-velocity K^+ influx and reduce it to levels more comparable with the wildtype. In the presence of salt stress, the altered selectivity of this channel promotes K^+ uptake relative to Na^+. Since Na^+ uptake is relatively reduced, those mechanisms responsible for efflux of Na^+ in the cytoplasm are not swamped and remain relatively more effective in reducing cytoplasmic Na^+ (Jeschke 1984; Braun et al. 1988). Thus, *stl2* can maintain a higher K^+/Na^+ ratio. As external salt stress increases, the total intracellular cation content, dominated by K^+ and Na^+, in both wildtype and *stl2* increases similarly (Vogelien 1993; Vogelien et al. 1995). Such an increase may result from a stimulation of H^+-ATPase activity directly by external Na^+ or in response to a general decrease in water potential (Reinhold et al. 1984; Sze 1985; Reuveni et al. 1987). Over a longer-term exposure to Na^+, promotion of higher H^+-ATPase activity would result in a lower membrane potential and a greater capacity for cation accumulation. In the face of these changes, *stl2* continues to maintain its higher K^+/Na^+ ratio, whereas the wildtype accumulates these cations in a lower ratio that is presumably more toxic.

Unique single gene mutations, such as *stl2,* provide an unparalleled opportunity to study salt tolerance and to explore in further detail the metabolism and physiology of K^+, Na^+, and other ions. The complex phenotypic response observed in *stl2* has important implications for studies of salt tolerance, especially those associated with ion transport. If such richly complex phenotypes are generated in single gene mutations arising in a nearly isogenic background, interpretations of tolerance mechanisms based upon comparative

studies of genetically different salt-tolerant and sensitive taxa must necessarily be highly compromised. Analyses of such mutants, first singly and then in combination, can further our understanding of individual physiological traits that contribute to salt tolerance and ion metabolism as well as afford the means to examine their complex interactions.

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