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Selection of a mutation conferring high NaCl tolerance to gametophytes of *Ceratopteris*

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Summary. Spores from a weakly salt tolerant strain of *Ceratopteris richardii* containing the mutation *stl1* were irradiated and sown on nutrient medium supplemented with 200 m*M* NaCl. A single highly salt tolerant gametophyte was recovered and selfed to generate a homozygous sporophyte. Spores from this strain, 10α23, were used to document the sexual transmission of the trait and to monitor the inheritance of tolerance in crosses to both the wild type and to the parental salt tolerant strain. Genetic analysis showed the 10α23 strain to possess both the original *stl1* mutation and an additional semi-dominant nuclear mutation, *stl2*, that individually conferred a high level of tolerance to gametophytes. In combination, both mutations had additive effects. Tolerance was also evident in sporophytes, but at a lower level than in gametophytes.

Key words: *Ceratopteris richardii* – Fern – Salt tolerant

Introduction

The responses of plants to excess salinity involve complex and highly integrated processes at both the cellular and organismal levels (Cheeseman 1988; Claes et al. 1990; Epstein and Rains 1987). In naturally evolved salt tolerant taxa, tolerance is a quantitative and polygenic trait associated with a variety of possible morphological and biochemical adaptations (Greenway and Munns 1980; Shannon 1984; Tal 1984). Although substantial information is available concerning physiological characteristics of glycophytes and halophytes under salt stress (Greenway and Munns 1980; Flowers et al. 1977; Yeo 1983), most studies are restricted to comparisons of different taxa or varieties. This limits the ability to associate particular responses with specific genetic differences. Because of the complexity at both the genetic and physiological levels, success in the development of salt tolerant crops through traditional breeding approaches has been limited (Shannon 1984; Epstein and Rains 1987).

Alternative approaches for the development of salt tolerant varieties, and as a means of furthering our understanding of the basic mechanisms involved in response to salt stress, include the use of in vitro cell or tissue culture selection systems. This approach can effectively increase the number of variants available for study, and stable salt tolerant cell or callus lines have been developed in a number of genera (for citations see: Bressan et al. 1987; Hassan and Wilkins 1988; McHughen 1987). Only a few of the available lines have been successfully regenerated as whole plants (see McHughen 1987), and of these, information about the inheritance is limited to two tobacco examples (Bressan et al. 1987; Nabors et al. 1980). In these two cases inheritance patterns were complex and not strictly Mendelian, suggesting multiple mutational events and/or epigenetic changes. This probably reflects the fact that recurrent selections were performed over an extended period with the use of gradually increased levels of salt (serial selection). Other recent studies have involved molecular approaches to the study of adaptation responses of individual genotypes of salt stress (Cushman et al. 1989; Claes et al. 1990). These studies provide valuable information concerning basic responses and regulation at the molecular level, but do not provide a direct means of identifying discrete genetic differences between salt sensitive and salt tolerant genotypes.

Because salt tolerance in naturally evolved taxa is undoubtedly polygenic, it is predictable that most suc-

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cessful in vitro selections would result in genetically complex lines. Nonetheless, it is likely that even with polygenic traits some of the contributing alleles have greater effects than others and may be themselves contribute a measurable amount of tolerance (Tal 1984). A few documented examples of single gene traits associated with salt tolerance are available. Abel (1969) described a chloride exclusion gene in soybean that was associated with varietal differences in salt sensitivity. Kueh and Bright (1982) selected for proline-accumulation mutants of barley and showed that they were somewhat tolerant to low (<100 mM) concentrations of NaCl. More recently, Warne and Hickok (1987) described two single gene nuclear mutations associated with salt tolerance in gametophytes of the fern *Ceratopteris*. The general lack of available mutants for study probably reflects the low level of tolerance generally associated with any single mutation and the consequent inability to readily detect the slight difference in phenotype. In the *Ceratopteris* gametophyte system, the ability to distinguish mutants with only subtle differences in response (e.g., Hickok and Schwarz 1989) has allowed the isolation of a number of putative salt tolerant mutants that are yet to be characterized (Hickok et al. 1987). The availability of single gene mutants provides opportunities for an expanded approach to selection for salt tolerance. For instance, mutant strains that exhibit some level of enhanced germination or growth under salt stress can be used as the parental source in continued selection experiments. In this way, it is possible to select at higher salt concentrations for additional mutations that may exhibit additive or synergistic effects in combination with the first mutation. If this is carried on for two or more rounds of selection it constitutes, in effect, a serial selection technique, but one that is carried out in discrete steps so that the individual components of the derived polygenic system are available and defined for individual study and characterization. Knowledge of individual components of tolerance can be of significant value in the identification of markers for salt tolerance, which could aid in the development of salt tolerant crops (Epstein and Rains 1987). This paper documents the use of a previously characterized single gene mutant strain of *Ceratopteris* in the selection and isolation of a mutation conferring a high level of salt tolerance to gametophytes.

Materials and methods

The homozygous diploid ($2n=78$) strain of *Ceratopteris richardii* Brongn., Hn, was used as the wild-type spore source (Hickok et al. 1987). Strain N10, containing the nuclear gene mutation designated *stl1*, was derived previously from the Hn strain by mutagenesis and selection for tolerance to NaCl (Warne and Hickok 1987). To establish cultures, spores were disinfected and gametophytes axenically cultured on agar-solidified mineral nutrient medium according to standard procedures as noted by Warne and Hickok (1987), except that the pH of the medium was adjusted to 6.0. In addition, all experiments involving comparisons between different strains were initiated by sowing disinfected spores onto medium lacking NaCl. After germination (3–4 days following soaking of the spores [DFS]) and growth to a stage just prior to meristem development (about 6 DFS) (Hickok et al. 1987), gametophytes were transferred to the various treatments. Each treatment consisted of 15 gametophytes per 60×15 mm petri plate, that were cultured for 9, 12, 14, or 26 days following transfer (DFT). Four or more replicates of each experiment were run simultaneously and data were combined for analysis. Cultures were maintained under constant conditions at $28° \pm 2$ °C and about 85 μmoles m^{-2} s^{-1} photosynthetic active radiation (PAR) from 40 W cool white fluorescent tubes.

Selection of gametophytes showing high tolerance to NaCl was accomplished by irradiating with X-rays (400 R min^{-1}, 28 min) 1.0 g of spores (about 1.25×10^6 spores) from the N10 strain (Hickok et al. 1987) and sowing on medium supplemented with 200 mM NaCl. After several weeks of culture, putative M1 mutant tolerant individuals were identified visually, transferred to unsupplemented medium, self fertilized, and cultured to the sporophyte stage for the production of M2 generation spores. M2 spores were used in all subsequent tests of the selected mutants. Reciprocal F1 hybrid combinations were made between the strains using previously described methods (Hickok 1985). Segregation analyses were performed by comparing the growth on NaCl of individual gametophytes generated from spores of the F1 hybrids with growth of the parental mutant or wild-type strains.

Comparative studies of the growth of gametophytes of the various strains in response to different NaCl treatments were performed by measuring areas of the essentially two-dimensional gametophytes. Gametophytes were removed from the treatment plates at 9, 12, or 14 DFT 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™ Image Analysis System. Data from replicate experiments were combined.

Comparative studies of homozygous and heterozygous sporophytes were performed by measuring the area of the first leaf of 10-day-old sporophytes generated on agar-solidified culture medium containing 60 mM NaCl. Homozygous sporophytes were generated by flooding, at 13 DFS, multispore cultures (about 10 spores cm^{-2}) of each homozygous line with sterile distilled water to effect fertilizations. This resulted in a uniform population of genetically identical sporophytes for each line. Heterozygous sporophytes were generated in the same manner except that cultures of the intended female parent (either wild type or N10) were flooded with a sperm suspension of the intended male parent. The male parent always showed greater tolerance to NaCl than the intended female. Although this resulted in both selfs and crosses, heterozygotes that exhibited tolerance greater than that shown by the female parent could be visually identified by their superior growth.

Results

Selection

After 4 weeks of culture on 200 mM NaCl, about 22% of the irradiated spores had germinated and 23 viable gametophytes were evident, but only 1 exhibited vigor-

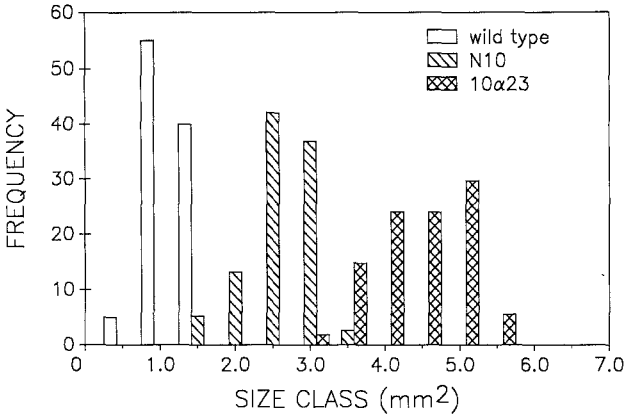

Fig. 1. Comparison of gametophyte growth after 12 days on 100 m*M* NaCl of the wild type and strains N10 and 10α23 ($N=54$ for each strain)

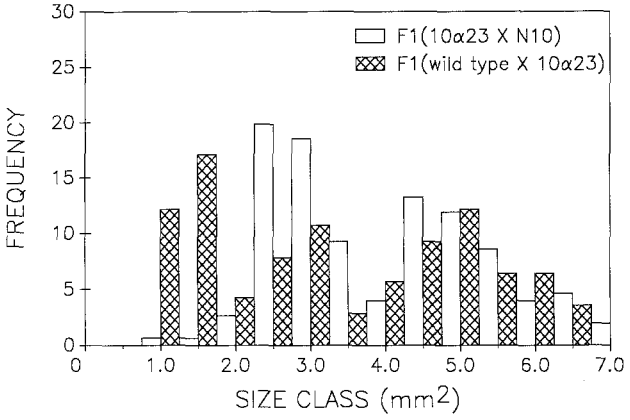

Fig. 2. Comparison of gametophyte growth after 12 days on 100 m*M* NaCl of segregates from crosses of strain 10α23 with the wild type ($N=140$) and strain N10 ($N=151$)

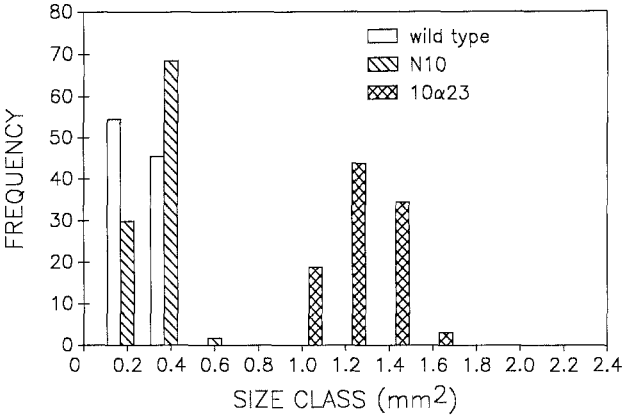

Fig. 3. Comparison of gametophyte growth after 14 days on 200 m*M* NaCl of the wild type ($N=55$) and strains N10 ($N=57$) and 10α23 ($N=64$)

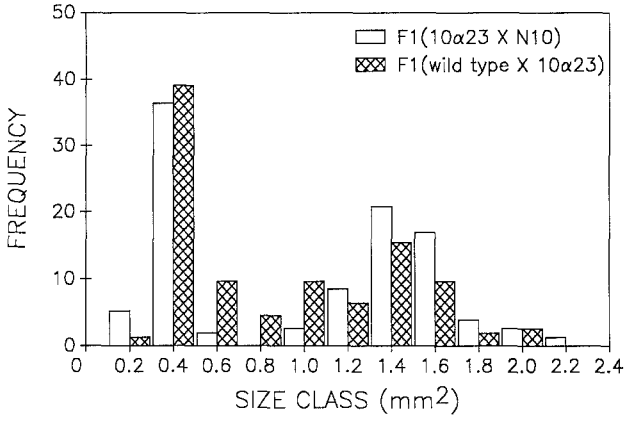

Fig. 4. Comparison of gametophyte growth after 14 days on 200 m*M* NaCl of segregates from crosses of strain 10α23 with the wild type ($N=156$) and strain N10 ($N=154$)

ous growth. This putative M1 mutant individual was transferred to medium without NaCl for 4 weeks and then watered to allow self fertilization (Hickok et al. 1987). The resulting homozygous M1 sporophyte (strain 10α23) was cultured to maturity in the greenhouse, and M2 spores were collected. M2 spores from strain 10α23 were used in all of the experiments described below.

Segregation from hybrids

Two hybrid combinations were tested for segregation of gametophyte types: F_1 (wild type × 10α23) and F_1 (10α23 × N10), where the female parent is listed first. Because preliminary tests of reciprocal crosses showed similar results, only data from these combinations are reported here. Segregating gametophyte types were compared to the parental strains on 100 m*M* NaCl at 12 DFT (Figs. 1, 2) and on 200 m*M* NaCl at 14 DFT (Figs. 3, 4). The use of two different concentrations allowed better discrimination of low and high tolerance types.

Figure 1 shows the size differences on 100 m*M* NaCl between the wild type, N10, and 10α23 parental strains. Although some overlap exists between the strains, strain N10 clearly exhibits low tolerance and 10α23 exhibits high tolerance, relative to the wild type. Figure 2 shows the segregation pattern from the two hybrid combinations. The F1 (wild type × 10α23) size distribution is trimodal, generally corresponding to the wild type, N10, and 10α23 classes in Fig. 1. A somewhat arbitrary division of the distribution into 1.0–1.5, 2.0–3.5, and 4.0–7.0 mm^2 classes suggests a 1:1:2 ratio of 41, 36 and 63 individuals, respectively. The F1 (10α23 × N10) size distribution is bimodal and generally corresponds to the N10 and 10α23 classes in Fig. 1. Division of the distribution into 1.0–3.5 and 4.0–7.0 mm^2 classes suggests a 1:1 ratio of 78 and 73 individuals, respectively. Both hybrids show segregation of gametophytes that are more highly tolerant than the 10α23 parent (i.e., >5.5 mm^2).

Figure 3 shows the gametophyte size differences on 200 m*M* NaCl between the wild type, N10, and 10α23

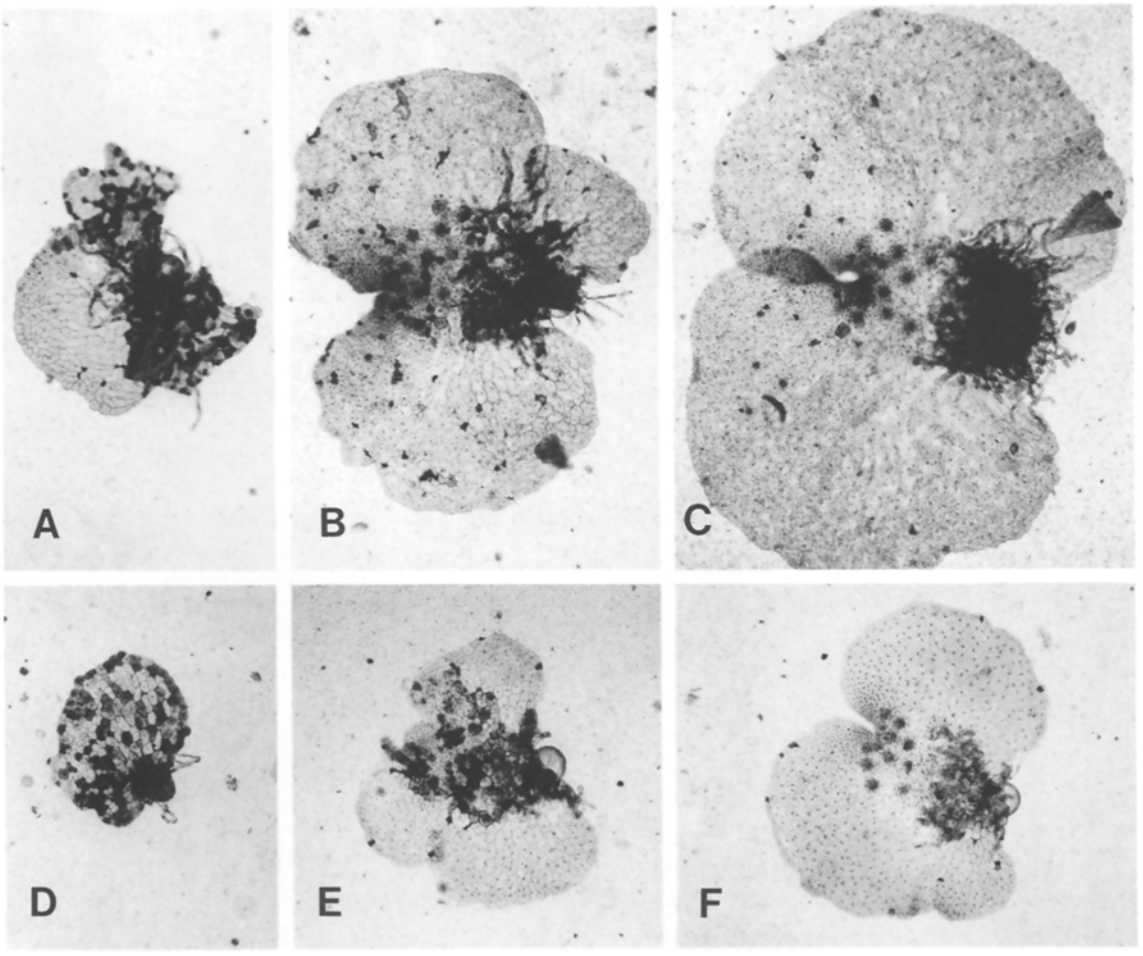

Fig. 5A–F. Gametophyte segregants from the F1 (wild type × 10α23) cross. **A–C** show, respectively, the responses of genotypes + +, *stl1* + and + *stl2* or *stl1 stl2* after 12 days on 100 m*M* NaCl. The gametophyte in **D** shows a response after 14 days on 200 m*M* NaCl similar to both the + + and *stl1* + genotypes. **E** and **F** show, respectively, responses after 14 days on 200 m*M* NaCl of the + *stl2* and *stl1 stl2* genotypes. Area measurements for **A–F** are, respectively, 0.80, 2.41, 4.10, 0.38, 0.94, and 1.36 mm^2

parental strains. The wild type and N10 are indistinguishable on the basis of size, while 10α23 is clearly tolerant. Figure 4 shows the segregation pattern on 200 m*M* NaCl from the two hybrid combinations. Both distributions are bimodal. The F1 (10α23 × N10) hybrid shows two distinct classes with 67 smaller and 87 larger gametophytes. This approximates a 1:1 ratio ($\chi^2 = 2.34$, $P > 0.05$). Segregation from the F1 (wild type × 10α23) hybrid, if divided into 0.0–0.8 and 1.0–2.4 mm^2 size classes, suggests a 1:1 ratio with 84 and 72 gametophytes, respectively. Both hybrids show a segregation of gametophytes that are more highly tolerant than the 10α23 parent (i.e., > 1.8 mm^2).

Based on these segregation data, it was hypothesized that strain 10α23 contained two unlinked nuclear gene mutations, *stl1* and *stl2*. Mutation *stl1* confers low tolerance and was present in the N10 strain, and *stl2* confers high tolerance and was recovered from the second round of selection. Therefore, gametophyte genotypes of the wild type and strains N10 and 10α23 can be tentatively represented as + +, *stl1* + and *stl1 stl2*, respectively. Segregation from the F1 (10α23 × N10) hybrid would yield a 1:1 ratio of *stl1* + (N10 type) and *stl1 stl2* (10α23 type) gametophytes. Segregation from the F1 (wild type × 10α23) hybrid would yield a 1:1:1:1 ratio of + + (wild type), *stl1* + (N10 type), + *stl2* (new type), and *stl1 stl2* (10α23 type) gametophytes. Because of the absence of a fourth size class in Fig. 2, it was assumed that the + *stl2* segregant could not be distinguished quantitatively from *stl1 stl2* on 100 m*M* NaCl. Therefore, the observed ratio was 1:1:2. On 200 m*M* NaCl (Fig. 4), it was assumed that + + and *stl1* + were indistinguishable as were + *stl2* and *stl1 stl2*. Therefore, a 1:1 ratio was evident.

In addition to the quantitative analysis of segregating types, it was also possible to distinguish certain gametophyte types visually. Figure 5A–C shows segregating types from the F1 (wild type × 10α23) hybrid on 100 m*M*

Table 1. Segregation of gametophyte types from the F_2 (wild type × 10α23) hybrid

Treatment	Genotypes[1]			
	++	*stl1*+	+*stl2*	*stl1 stl2*
100 m*M*[2]	133	114	– (227)[3]	–
200 m*M*[2]	– (270)[3]	–	110	102

[1] Visual distinctions of the genotypes (see text and Fig. 5) were made at 26 DFT after culture on NaCl-supplemented medium
[2] Chi-square test for hypothesis of a 2:1:1 ratio: 100 m*M*– $\chi^2=2.36$, $P>0.2$; 200 m*M*– $\chi^2=7.24$, $P>0.01$
[3] Categories combined because of the inability to distinguish individual types (see text)

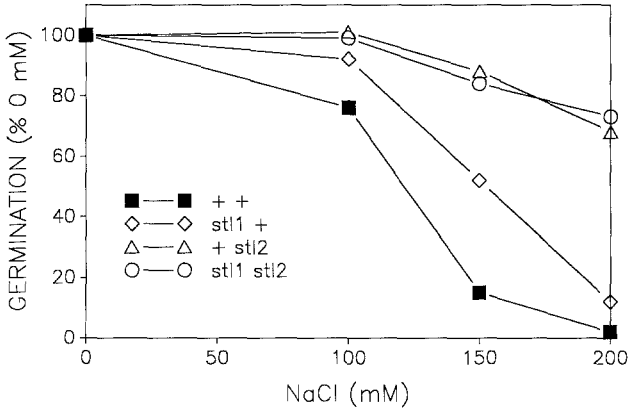

Fig. 6. Germination responses at 21 DFS of the four genotypes. Values represent the average of two counts and are expressed as a percentage of spore germination on unsupplemented medium

at 12 DFT. These closely resemble, respectively, the wild type, N10, and 10α23 strains cultured under identical conditions. In accordance with the above mode, Fig. 5A and 5B were assigned the genotypes ++ and *stl*+. Fig. 5C is assumed to represent both the + *stl2* and *stl1 stl2* genotypes, which are indistinguishable on 100 m*M* NaCl. The wild type is distinguished by irregular growth, significant necrosis, and the lack of an organized meristem. The *stl1* + type shows some necrosis and irregularity, but contains an organized meristem. The + *stl2* and *stl1 stl2* types, with the exception of a few dead cells, are largely unaffected by the treatment. Figure 5D–F shows segregating types from the F1 (wild type × 10α23) hybrid on 200 m*M* NaCl at 14 DFT. The wild type and N10 strains are indistinguishable at this concentration in that they both show little or no growth and die. The segregant in Fig. 5D, therefore, resembles the response of both the wild type and *stl1* + genotypes. Figure 5E and F represents the only viable gametophyte types present at this concentration. Although a size difference between 5E and 5F is apparent, no distinct size class differences were evident (compare Fig. 4). The most conspicuous difference between these two types is the greater irregularity and degree of necrosis in Fig. 5E as opposed to near normal morphology and little cell death in Fig. 5F. The 10α23 strain, under identical conditions, closely resembled the segregant in Fig. 5F, which was accordingly assigned the *stl1 stl2* genotype. No Fig. 5E types were evident in any of the parental strains. Because of this, the segregant represented in Fig. 5E was assumed to represent the +*stl2* genotype. Segregants from the F1 (N10 × 10α23) hybrid could also be distinguished as above, with the exception that, as predicted, no + *stl2* or ++ types were evident.

Because of the ability to visually distinguish the nonparental + *stl2* segregant, an additional experiment was conducted to determine the frequencies of the various segregating types from the F1 (wild type × 10α23) hybrid. This experiment was set up, as above, on 100 m*M* and 200 m*M* NaCl, with the exception that cultures were not visually scored until 26 DFT. Preliminary tests indicated that this longer time allowed a clearer visual distinction of the various segregating types. The results are summarized in Table 1.

Comparison of homozygous strains

In association with the above experiment, individual +*stl2* gametophyte segregants from the F1 (wild type × 10α23) hybrid were identified on 200 m*M* NaCl, isolated onto plain medium, and self fertilized to obtain homozygous sporophytes. Spores from one of these sporophytes were used in comparative studies of the +*stl2* genotype along with the wild tpye, *stl1*+, and *stl1 stl2*.

Germination responses of the four genotypes at 21 DFS are shown in Fig. 6. Germination of the wild type is reduced substantiallly above 100 m*M* NaCl while *stl1*+ shows enhanced germination over the range tested. Both +*stl2* and *stl1 stl2* show relatively high germination at all concentrations.

Growth comparisons were made by culturing gametophytes on a range of NaCl concentrations and measuring sizes at 9 DFT. This treatment time was chosen because a longer period would make measurement diffficult at lower concentrations (i.e., larger gametophytes become multiple winged and three dimensional). Figure 7 shows the response of the four genotypes to NaCl. At 0 m*M* NaCl *stl1* + grows slightly better, while both +*stl2* and *stl1 stl2* show restricted growth relative to the wild type. Figure 8 illustrates these data normalized to the 0 m*M* control for each strain. The concentration at which a 50% reduction in growth is evident is approximately 80, 95, 185 and 190 m*M* NaCl for the wild type, *stl1* +, + *stl2* and *stl1 stl2* genotypes, respectively.

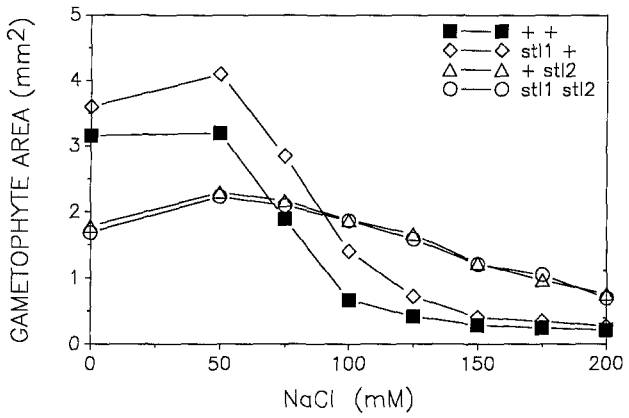

Fig. 7. Growth responses at 9 DFT of gametophytes of the four genotypes. Values represent the average area of from 38–53 gametophytes for each genotype at each treatment. Standard errors were smaller than the symbols

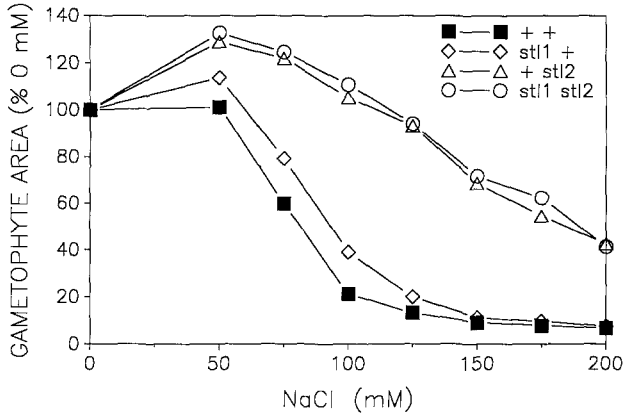

Fig. 8. Data from Fig. 7 represented as a percentage of the 0 m*M* NaCl control for each genotype

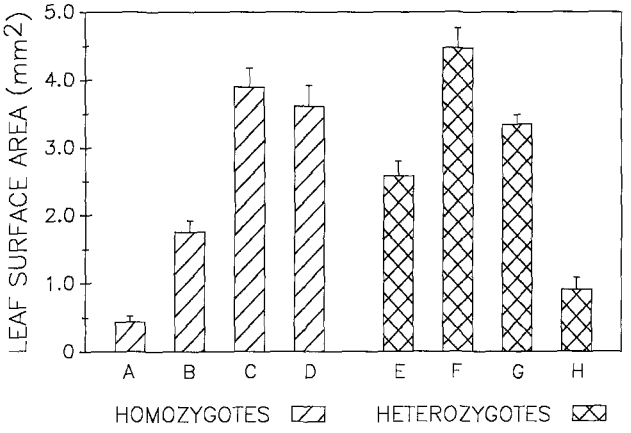

Fig. 9. Areas of the first leaf of 10-day-old homozygous and heterozygous sporophytes cultured on 60 m*M* NaCl. Values represent the average and SE of ten measurements. *A* = +/+ +/+, *B*=*stl1*/*stl1* +/+, *C*=*stl1*/*stl1* *stl2*/*stl2*, *D*= +/+ *stl2*/*stl2*, *E*= +/*stl1* +/*stl2*, *F*=*stl1*/+ +/*stl2*, *G*= +/+ +/*stl2*, *H*= +/*stl1* +/+. (For each allelic pair the genotype of the female parent is listed first)

Sporophyte characterization

Figure 9 illustrates the different tolerances of homozygous and heterozygous sporophytes to 60 m*M* NaCl, as assessed by the area of the first leaf in 10-day-old sporophytes. All mutant homozygotes and heterozygote combinations showed tolerance to NaCl relative to the wild type. In addition to size differences, the first leaf of the wild type and *stl1*/*stl1* +/+ homozygotes and of their hybrid was very irregular in development, being club shaped as opposed to the normal laminate appearance. On 60 m*M* NaCl, leaf size for the wild type, *stl1*/*stl1* +/+, +/+ *stl2*/*stl2*, and *stl1*/*stl1* *stl2*/*stl2* genotypes was 7%, 36%, 74%, and 87% of the size on 0 m*M* NaCl, respectively. Comparable data are not available for heterozygote combinations because the response to NaCl was used as the means of identifying heterozygotes (see Methods) and there was no way to distinguish heterozygotes from homozygotes on medium lacking NaCl.

Discussion

Genetic analyses of the two hybrid combinations, based on both quantitative and qualitative comparisons of segregation patterns, support the model of two unlinked nuclear gene mutations in strain 10α23. The only significant deviation from expected segregations involved the visual analysis of segregant types from the F1 (wild type × 10α23) hybrid on 200 m*M* NaCl (Table 1). However, although in this case the number of wild type and *stl1* + types combined was somewhat larger than expected, the number of + *stl2* and *stl1* *stl2* types closely approximated a 1:1 ratio, as expected.

Some segregates from both hybrids showed tolerance to 100 m*M* and 200 m*M* NaCl at levels higher than the 10α23 parent (Figs. 2, 4). This may be associated with the loss, through segregation, of some deleterious mutation(s) present within the original 10α23 strain. We are currently resynthesizing the *stl1* *stl2* genotype, using selected segregates from hybrids, in order to generate a pure 10α23 line lacking the putative deleterious factor(s).

Comparative dose response tests on NaCl documented low tolerance in the *stl1* + genotype and high tolerance in both the + *stl2* and *stl1* *stl2* genotypes. The + *stl2* and *stl1* *stl2* genotypes grew better on medium containing up to 100 m*M* NaCl than they did on medium lacking NaCl. The high tolerance is associated with the *stl2* mutation, and little quantitative difference was apparent between + *stl2* and *stl1* *stl2* at nine DFT. Longer culture times on high NaCl concentrations result in greater differences. For instance, if the two genotypes are sown directly on 200 m*M* NaCl, substantial differences in growth and cell necrosis are evident at 28 DFS (data not shown). The more normal growth and general lack of necrosis in the *stl1* *stl2* genotype indicates that the *stl1*

mutation positively contributes to tolerance, even at high concentrations. Experiments not reported here have shown that the *stl1 stl2* genotype can be grown on 275 m*M* NaCl without significant cell death or irregularity in development, although growth was severely reduced. In contrast, the wild type begins to show cell necrosis and developmental irregularities at 75 m*M* NaCl.

In the absence of NaCl, growth of the high tolerance types was much poorer than the wild type, indicating that a certain metabolic cost may be associated with the *stl2* mutation. A similar compromise in growth has been documented in comparative studies of wild type and 10α23 sporophytes (Augé et al. 1989). Bressan et al. (1987) also noted a reduction in growth rate in plants derived from NaCl-adapted cell lines of tobacco. Lower growth rates in highly salt tolerant lines may be difficult to avoid in that most halophytes have lower growth rates than glycophytes (Shannon 1984).

Analysis of sporophytes at the first leaf stage clearly showed all mutant combinations to be tolerant to 60 m*M* NaCl, relative to the wild type. In addition, the *+/stl1 +/stl2*, *+/stl1 +/+*, and *+/+ +/stl2* heterozygotes showed intermediate levels of tolerance relative to the wild type and mutant homozygotes. Thus, the *stl1* and *stl2* mutations are semi-dominant. The *stl1/+ +/stl2* combination, which is heterozygous at both loci, showed greater tolerance than the double mutant homozygote. This may be explained on the same basis as the enhanced tolerance in some segregating gametophyte types, as discussed above (i.e., loss of some deleterious mutation(s) via segregation). The difference between the *stl1/+ +/stl2* and *+/+ +/stl2* combinations clearly shows the additional contribution of the *stl1* mutation to tolerance in sporophytes.

Preliminary studies showed that NaCl concentrations above 60 m*M* were restrictive to sporophyte growth in all genotypes. Thus, sporophytes show much lower tolerance than gametophytes. In older sporophytes, differences in tolerance between the genotypes are much less apparent. For instance, although the *stl1/stl1 stl2/stl2* genotype can be distinguished from the wild type (Augé et al. 1989), it is not possible to quantitatively distinguish older heterozygotes or *stl1/stl1 +/+* homozygotes from the wild type (L. G. Hickok and D. L. Vogelien, unpublished).

It is disappointing, but not surprising, that tolerance in sporophytes is so much less than in gametophytes. Selection in gametophytes, which are morphologically simple and avascular, is most likely to yield mutations that are expressed at the cellular level. In contrast, tolerance in sporophytes can be complicated by the fact the Na^+ and/or Cl^- ions may be taken up by the roots and transported to the shoot where they may reach extremely high concentrations.

The ability to culture gametophytes of the mutant strains to sexual maturity on high NaCl concentrations should allow us to directly select for enhanced tolerance in sporophytes at concentrations that would otherwise be restrictive. It is hoped that the use of this step-wise selection scheme involving both gametophyte and sporophyte generations will allow the recovery of strains with substantial tolerance in both generations. Although such strains, as end points, would be somewhat complex genetically, genetic analyses at each step in the selection process will allow a dissection of both genetic and physiological aspects of the mechanisms of tolerance.

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