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CHARACTERIZATION OF MUTANTS SELECTED FOR ALUMINUM TOLERANCE IN THE FERN *CERATOPTERIS*

A Thesis

Presented for the

Master of Science

Degree

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Shawn R. Wright

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ABSTRACT

Aluminum toxicity, associated with acid soil conditions, has received much attention in the recent literature because it can limit plant growth. Although much work has been done, more needs to be done to develop an understanding of the mechanisms of aluminum toxicity and aluminum tolerance. One research method is the development of single gene mutants that have altered response compared to the wild type when grown under aluminum-toxic conditions.

This study uses the fern *Ceratopteris richardii* to select and characterize mutants selected for aluminum tolerance. Mutations were induced in wild type spores with x-rays and screened for tolerance on medium containing Al-EDTA at pH 4.5. Thirty putative mutants were isolated and were available for this study. Of the six confirmed mutants, three (HαAT3, HαAT7, and HαAT29) were characterized.

The mutants were characterized morphologically. The mutants HαAT3 and HαAT29 were normal in the gametophyte stage. The mutant HαAT7 was morphologically normal in the gametophyte stage except it produced fewer rhizoids than the wild type at all levels of pH. This is not presumed to be the basis of tolerance, because the rhizoids would have to have aluminum specific uptake.

The relative tolerance levels of the mutants were determined by comparing their growth to the growth of the wild type on media that contained a range of Al-EDTA. Because of the complexity of the selection medium, testing was done to determine if the mutants were tolerant to aluminum and\or the individual components modified in the selection medium. The presence of additional sulfate in the selection

medium was not considered significant in causing the response shown in the original screening. EDTA tolerance testing showed that the mutants HαAT3B and HαAT29 were EDTA tolerant. All three mutants had a lower pH optimum for growth than the wild type and were acid tolerant.

Genetic analyses were performed to determine the genetic basis of tolerance. Segregation analysis showed that tolerance in HαAT3 is due to a single nuclear gene. Tolerance in HαAT7 is probably controlled by a single nuclear gene, but epigenetic factors also play a large role in the extent of tolerance shown by the gametophytes and prevent the absolute determination of how many genes control tolerance. HαAT29 exhibited a complex mode of inheritance, but tolerance appeared to be under nuclear control. The number of genes controlling tolerance could not be determined from the tests performed.

Tests on sporophytes were performed in an attempt to characterize the parental response. A method that produced a response in the wild type was developed, but the mutants' responses were not fully determined.

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I. INTRODUCTION

Aluminum toxicity in acid soils is an important growth limiting factor for plants in many regions of the world. Approximately 40% of the globe's potentially arable soils have severe enough aluminum toxicity to limit the growth of crop plants (Anonymous, 1977). Consequently, aluminum phytotoxicity and aluminum tolerance in acid soils are areas of research that have received much attention. For reviews see Bollard, (1983) Clarkson (1969), Fageria et al. (1988), Foy (1974, 1983a,b), Foy et al. (1978), Haug (1983), Haug and Caldwell (1985), and Roy et al. (1988). Although these reviews are directed towards toxicity of aluminum as the cation, aluminum may also be toxic as the anion, aluminate. Aluminate toxicity occurs in alkaline soils and has been described in several studies, (i.e., Fuller and Richardson, 1986; Jones 1961; and Rees and Sidrak, 1956). Plants that are tolerant to aluminum in one form are not expected to be tolerant to aluminum in the alternate form, because these plants would have to be adapted to a wide range of soil pH. Also, tolerance mechanisms are not expected to be the same for a cation and an anion. This thesis concentrates on studies involving aluminum effects as the cation, and also the effect of low pH.

Mechanisms of Toxicity

One proposed mechanism for the toxicity of aluminum as the cation is an inhibition of cell division in the root, not related to disturbances of the mitotic apparatus or the chromosomes (Clarkson, 1965). For instance, Sampson et al. (1965) reported the absence of mitotic figures

in barley roots, 24 hours after treatment with aluminum sulfate. In contrast, Levan (1945) reported that aluminum caused the formation of sticky chromosomes during mitosis in *Allium cepa*.

The effect of aluminum on the root is not restricted to the region of cell division. Schaedle et al. (1986) reported that a large portion of aluminum uptake is non-metabolic and occurs in the corticular region. Bennet et al. (1987) studied the effects of aluminum on root cap function and found indications that aluminum affects the golgi apparatus in the peripheral cap cells and not in the meristematic region. They also determined that the deleterious effects of aluminum were initiated at aluminum concentrations where the molar ratio of charge between Ca^{2+} and Al^{3+} favored aluminum, (i.e., at aluminum concentrations above 1.25 mg/dm^3, where $1/2\log Ca^{2+} - 1/3\log Al^{3+} \leq 0$), which may indicate an electro-physiological response to aluminum. Wagatsuma et al. (1987) also have demonstrated that cell injury can occur in regions of the root other than the meristematic region. They postulated that the toxic effects may be related to plasmalemma interactions, and the leakage of potassium out of cells.

A common characteristic of aluminum-injured roots is the corralloid appearance of the root system, with thickened and stubby lateral roots (Fleming and Foy, 1968). The occurrence of stubby lateral roots, and the shallow rooting depth caused by aluminum toxic subsoils, may increase the probability of drought injury (Foy, 1974) or decrease the overall vigor of the plant by increasing its susceptibility to other stresses.

Aluminum has also been shown to interact with other elements in the plant. This interaction may be a primary cause of aluminum toxicity, or a reflection of the toxic effect of aluminum on the physiology of the plant. Hoddinott and Richter (1987) showed that aluminum affects carbohydrate translocation in French bean while Rueter et al. (1987) showed that in the green alga, *Scenedesmus*, aluminum toxicity is due to increased cupric ion activity.

Many researchers have demonstrated a negative effect of aluminum on the calcium nutrition of plants (Foy et al., 1978). Schortle and Smith (1988) reported that increased aluminum uptake resulted in decreased calcium uptake in red spruce. This may limit the rate of sapwood production, leaving the trees more vulnerable to other abiotic and biotic stresses. Several recent studies have also demonstrated a negative correlation between aluminum levels and calcium concentration in the root (Thornton et al., 1986; Ohki, 1987; Baligar et al., 1987). Haug and Caldwell (1985), in a review of aluminum toxicity and calcium nutrition associated with the effects of aluminum on calmodulin, concluded that aluminum impairs the ability of calmodulin to interact with the target proteins. Slaski and Aniol (1987) used calmodulin inhibitors to determine if the interaction of aluminum with calmodulin could affect root-tip ATPase activity enough to be the cause of toxicity. Their results indicated that differences in activity were not directly associated with tolerance.

Aluminum phytotoxicity may also be related to phosphate nutrition (Foy et al., 1978). In acid soils, aluminum binds to phosphate making it unavailable to the plant. In addition, aluminum may bind to phosphate

present in the cell (i.e., in DNA, ATP, or in phospho-lipids) and cause its toxic effect by interfering with cellular metabolism. Cumming et al. (1986) showed that although aluminum in solution increased phosphate uptake by red spruce seedlings, it decreased phosphate translocation from the roots. Phosphorous uptake has even been used as a criterion for selecting aluminum tolerant soybean genotypes (Castells et al., 1985).

Aluminum also affects nitrogen nutrition. Jarvis and Hatch (1985, 1987) found that aluminum had a significant effect on nitrogen fixation, due to effects on nodule initiation, nitrogenase activity, and reduced nitrogen uptake. Keltjens and van Ulden (1987) also noticed a reduced nitrate reductase activity in sorghum when grown in solution with aluminum. They interpreted this lower activity as being due to the effect of aluminum on nitrate uptake and not the direct effect of aluminum on the enzyme. Gomes et al. (1985) showed that aluminum has a significant effect on nitrogen accumulation as well as on nitrogen translocation in *Sorghum bicolor* L. Moench.

Hecht-Buchholz et al. (1987) reported that soluble aluminum may inhibit the uptake of magnesium in Norway spruce seedlings, and Taylor and Foy (1985d) reported that treatment of wheat seedlings with aluminum results in reduced concentrations of manganese in both roots and leaves. Lee (1971) associated a greater uptake of potassium and manganese in potato cultivars with aluminum tolerance. In contrast, Thornton et al. (1987b) reported that aluminum had no effect on potassium and magnesium levels in the roots of *Gleditsia triacanthos* L.. Hecht-Buchholz and Schuster (1982) stated that increasing the concentration of calcium and

magnesium in solution culture will protect the root meristems from damage by aluminum. It appears that both nutrient availability, and ionic strength of the solution are important. Thornton et al. (1987a) reported that greater aluminum translocation occurred in more dilute media.

Mechanisms of Tolerance

Different aluminum tolerance mechanisms have been proposed, including: 1) exclusion of aluminum, 2) detoxification of aluminum, or 3) the ability to absorb limiting nutrients to a greater extent than aluminum sensitive varieties. Aluminum tolerance is a complex process and the separation of these different tolerance mechanisms into specific categories is often difficult.

The exclusion of aluminum has been thoroughly reviewed by Taylor (1987) and includes immobilization of metals at the cell wall, the formation of a redox barrier at the plasma membrane, and the formation of a pH barrier at the plasma membrane. Kennedy et al. (1986) found that certain cultivars of *Gossypium barbadense* and *G. hirsutum* have a low cation exchange capacity. This may provide tolerance to aluminum, although total growth differences might also be expected. Kinraide and Parker (1987) noticed that addition of cations to solution culture could ameliorate aluminum toxicity, suggesting that toxicity might be due to competition for external binding sites. A special form of exclusion exists whereby plants exclude aluminum from sensitive growing areas. Matsumoto et al. (1976, in Foy et al., 1978) found that old leaves of a tea plant contained 30,000 ppm Al, while buds contained only 50 ppm. No mechanism of how this might occur was proposed.

Because aluminum solubility decreases significantly above pH 5.0, the uptake of aluminum can be prevented if the acidification of the growth medium is prevented. This occurs in some wheat cultivars (Taylor and Foy, 1985a,b; Foy et al., 1978). A possible mechanism by which the pH of the media, and thus the solubility of aluminum, might be changed is through the preferential uptake of NO_3^- over NH_4^+. Taylor and Foy (1985c) have shown that certain cultivars of wheat have differences in the rate of NH_4^+ uptake, and possibly NO_3^- uptake. Keltjens (1987) found that differences in nitrogen source preference in two sorghum genotypes are induced by aluminum and are not present under non-stress conditions. Therefore, differences in aluminum sensitivity, and not nitrogen nutrition, are responsible for the differences in aluminum tolerance.

The increase in the pH of nutrient solutions has also been seen for Al-tolerant cultivars of barley, rice, peas, and corn (Foy et al., 1978). Wagatsuma and Yamasaku (1985) studied plant-induced medium pH changes in five barley cultivars. They showed that plant-induced pH change is not responsible for aluminum tolerance, and proposed that aluminum tolerant cultivars exclude aluminum actively outside the plasmalemma of the root cells. At the present time it is difficult to state whether the change in the pH of the solution is a primary mechanism of tolerance or simply the result of differential growth.

Detoxification may be related to an ability to chelate aluminum. Aniol (1984) pretreated wheat seedlings in a nutrient solution which contained low doses of aluminum. When root regeneration was observed, an increase in tolerance was observed. It was postulated that the

preincubation with aluminum induces the synthesis of an aluminum binding protein. Chelation with organic acids is also possible. Lee and Foy (1986) found that an aluminum tolerant snapbean cultivar had higher organic acid concentrations than did a sensitive cultivar. Similarly, Suhayda and Haug (1986) found higher organic acid content in the Al-tolerant maize hybrid W64, than in the Al-sensitive maize hybrid A632. Foy et al. (1987b) correlated aluminum tolerance with the ability to maintain a higher concentration of organic acids when grown in the presence of aluminum.

Another proposed mechanism is that aluminum tolerant plants can absorb limiting nutrients to a greater extent than can sensitive plants. For example, aluminum tolerance in certain cultivars of wheat, barley, soybean, and snapbean is associated with the ability to resist aluminum induced calcium deficiency (Foy, 1983b). If aluminum-induced calcium deficiency is a primary cause of phytotoxicity, plants that have a high calcium use efficiency should be aluminum tolerant. Horst (1987) studied the calcium use efficiency of cowpea genotypes and found that the aluminum-tolerant genotypes had a high calcium use efficiency. However, a high calcium use efficiency did not guarantee aluminum tolerance. Also, the ability to absorb and use phosphate in the presence of aluminum has been associated with tolerance (Foy, 1983b; Cumming et al., 1986). Thus, plants with a high phosphate use efficiency may be better adapted to aluminum-toxic conditions. Ota (1968, in Foy, 1974) relates aluminum tolerance in rice varieties to an ability to absorb significant amounts of silicon, which sequesters aluminum in epidermal cells that

have thickened cell walls. Galvez et al. (1987) also report that silicon fertilization of sorghum will alleviate aluminum toxicity.

Larkin (1987) used the aluminum tolerant wheat selection, Waalt, which differed from the cultivar, Warigal, by a single dominant gene for aluminum tolerance, to compare their sensitivity to calmodulin antagonists. No consistent differences in sensitivity were shown by the cultivars. In addition, another experiment showed no apparent differences in the amount of calmodulin present in the roots of an aluminum sensitive and an aluminum tolerant wheat. Larkin (1987) concluded that different aluminum tolerances are not due to different calmodulin levels and proposed that tolerance to aluminum could result from altered calmodulin structure.

Genetic Studies

Much of the current work being done concerning aluminum toxicity involves screening different cultivars and lines of agronomically important plants for aluminum tolerance. Tolerant lines could be used for breeding purposes and for studies of the mechanisms of tolerance and toxicity. Two general screening approaches have been used, whole plant (Bilski and Foy, 1987,1988; Berzonsky and Kimber, 1986; Caradus, 1987; Culvenor et al., 1986a; Ferreira et al., 1986; Flores et al., 1988; Foy et al., 1967, 1987a,c; Furlani et al., 1986; Jarvis and Hatch, 1987; Nyamangombe and Lefebvre, 1985; Reid et al, 1969; Wersuhn et al., 1986) and tissue culture selections (Conner and Merideth, 1985a,b; Merideth, 1978; Ojima and Ohira, 1983; Smith et al., 1983). In addition, Chaudhry et al. (1987) induced mutations in fertilized egg cells in rice and screened for tolerance. Reviews containing lists of differentially

tolerant lines are also available (Epstein and Jefferies, 1964; Reid, 1976; Rhue, 1977).

Gerloff and Gabelman (1983) reviewed the genetic basis for inorganic plant nutrition, including some work concerning the inheritance of aluminum tolerance. Kerridge and Kronstad (1968) first postulated a mechanism for the inheritance of aluminum tolerance in wheat (*Triticum aestivum, Vill.*,Host). They carried out crosses between what they termed susceptible and moderately resistant wheat varieties. In these crosses they demonstrated that the difference in tolerance appeared to be primarily controlled by a single dominant gene, with one or more major genes and several modifying genes also involved. Lafever et al. (1977), from studies of certain wheat populations, concluded that tolerance involves two or three genes plus modifiers. The location of an aluminum tolerance locus on chromosome 5D in wheat was proposed by Prestes et al. (1975). Aniol and Gustafson (1984) located genes for aluminum tolerance in the wheat variety 'Chinese Spring'. These genes were localized in chromosome arms 6AL, 7AS, 4BS, 2DL, 3DL, 4DL, and 4BL, and on chromosome 7D.

In rye, major genes for tolerance appear to be located on 3R and on 6RS, and associated with other genes on 4R (Aniol and Gustafson, 1984). Slootmaker (1974) proposed that the inclusion of a rye chromosome in triticale makes it tolerant to high soil acidity and aluminum. Aniol and Gustafson (1984) showed that in triticale hybrids, the expression of rye genes for aluminum tolerance is highly dependent on which genes from the wheat genome are also present. Also, the genes responsible for aluminum tolerance are usually suppressed to some extent.

In maize, aluminum tolerance is inherited via one locus with multiple alleles (Rhue, 1977). Reid (1971) showed that in certain barley populations aluminum tolerance is controlled by one major dominant gene. Culvenor et al. (1986b) demonstrated that the inheritance of aluminum tolerance in *Phalaris aquatica* L., can be explained by a two-gene hypothesis in which tolerance requires at least one dominant allele at each locus. Modifiers of the genes may also be involved.

Inheritance of aluminum tolerance has also been shown in 40 *Nicotiana plumbaginifolia* seedlings selected from a tissue culture screening. Conner and Meredith (1985b) showed that aluminum resistance was transmitted by a single dominant mutation in all cases.

Summary

Aluminum tolerance is a complex traits that may involve structural, physiological, and nutritional factors. This traits may be controlled by one or several genes. It should be noted that there is little comprehensive data on aluminum tolerant plants. Most studies have involved physiological approaches without genetic characterization, or genetic approaches without physiological characterization. In addition, few studies have been done that examined the effect of soil acidity on plant growth separate from the interaction of aluminum and other elements. However, Ryan et al. (1986a,b) did attempt to separate the effects of aluminum toxicity from the effects of acidity.

Clearly, there is a need for further studies involving physiological and genetic aspects of aluminum tolerance and/or aluminum toxicity. The isolation of single gene mutants with tolerance to aluminum would allow

comparative studies of aluminum tolerance in genetically defined material.

Because the fern *Ceratopteris* has been successfully used to isolate a variety of mutants tolerant to many different factors (i.e., herbicides, NaCl, plant growth regulators, etc.) (Hickok et al., 1987), the isolation and characterization of aluminum tolerant genotypes was attempted with this organism. This study consists of 1) the preliminary analysis of 30 putative mutant stocks to confirm tolerance and the selection of three stocks to be characterized in detail; 2) cross tolerance testing to determine if the three selected mutants were tolerant to aluminum and/or other conditions of the selection media; 3) gametophytic segregation analysis to determine the genetic basis of the traits; and 4) preliminary sporophytic tests to develop a method for determining whether the mutations are dominant or recessive, and possibly for complementation testing.

II. MATERIALS AND METHODS

Spore Source

Spore stock Hn-n (collection, 11/22/85 A) represents spores from the completely homozygous wild type strain of *Ceratopteris richardii* (L.) Brogn. (Hickok et al., 1987). Thirty previously generated putative mutant strains were available for this study (Warne and Hickok, unpublished). They were produced by exposing 1.0 g of Hn-n spores to x-irradiation at a dose of ≈ 400 Roentgens/minute for 28 minutes (total exposure ≈ 11,200 Roentgens). The mutagenized spores were then sown on plates containing medium supplemented with 200 uM $Al_2(SO_4)_3$ chelated with disodium-dihydrate-ethylenediaminetetraacetic acid (EDTA) at pH 4.5. Those M1 generation gametophytes that expressed tolerance in comparison to non-mutagenized wild type spores were isolated and selfed. The resulting M1 sporophytes were potted in the greenhouse. Fertile fronds were collected in sterile petri plates and dried to collect the spores that will produce M2 generation gametophytes.

Spore Ripening

To increase the germination rate, most spore collections from the mutants were ripened by placing spores in glass bottles which were then placed in a large (1 gal.) jar. A bottle containing 3.9 ml of Etherel ^{Tm} to which 7.8 ml of 1N KOH had been added, was placed in the large jar and the jar was sealed (Warne and Hickok, 1987).

Spore Disinfection and Sowing

Spores were sown at a standard density of 450 spores/ 60 x 15 mm petri plate (multispore sowing). The appropriate amount of spores was

weighed (1 mg ≈ 1250 spores) and soaked for 12 - 24 hours in a conical centrifuge tube with enough distilled water to cover. Corrections in this amount were made as needed to account for the low germination percentage of certain stocks. Spores were then surface sterilized and sown as described by Warne et al. (1986). The soaking water was removed axenically by using a sterile pasteur pipet, and the spores were then suspended in freshly prepared 0.875% sodium hypochlorite for three minutes, including the time needed to remove the sodium hypochlorite. Spores were then rinsed twice using sterile distilled water.

To sow spores, a known amount of sterile distilled water was added to the disinfected spores. Five Pastuer-pipet drops, about 0.15 ml, were used per plate of media.

Media Preparation

All cultures were grown in 60 x 15 mm petri plates, or 24-well plates, containing Parker-Thompson's complete inorganic nutrient medium (Tables 1 and 2) solidified with 1 % (w:v) tissue culture grade agar. The pH of this medium was adjusted to pH 4.4 or 5.4, or as noted otherwise, using HCl and KOH, before autoclaving. Medium was then autoclaved for 20 minutes, liquid cooled for 20 minutes, and poured under axenic conditions.

Preparation of Supplemented Medium

The supplemented medium was prepared from 10 mM stock solutions of sodium sulfate, aluminum chloride, aluminum sulfate and/or (EDTA). All stock solutions were made up in Parker-Thompson's nutrient solution. The appropriate amount of the supplemented nutrient solution was added

Table 1. Composition of Stock Nutrient Solutions[a] (Modified from Klekowski 1969).

Code	Stock Solutions	g/l	molar
	Thompson's Macronutrients		
A	NH_4NO_3	25.0	0.312
B	KH_2PO_4	20.0	0.147
C	$MgSO_4$ (anhydrous)	4.8	0.040
D	$CaCl_2.H_2O$	13.0	0.117
E	Parker's Micronutrients		
	$MnSO_4$(anhydrous)	0.022	0.00015
	$CuSO_4.H_2O$	0.037	0.00021
	$ZnSO_4.7H_2O$	0.052	0.00018
	H_3BO_3	0.186	0.00301
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0037	2.99×10^{-6}
F	Iron Solution		
	$FeSO_4.7H_2O$	2.78	0.010
	$Na_2EDTA.2H_2O$	3.73	0.010

[a]Basal medium is prepared by adding 5 ml of stock A, 25 ml of stock B, 12 ml of stock C, 2 ml of stock D, 10 ml of stock E, and 10 ml of stock F to 900 ml of deionized, distilled, twice-polished water and then bringing to 1 liter volume. This solution is then added to a two liter Erlenmyer flask containing 10 grams of tissue culture grade agar, and the pH is adjusted to 4.4 before autoclaving.

Table 2. Elemental Concentration for Basal Parker-Thompson's Nutrient Solution.

Element or Compound	mMolar
K	3.675
P	3.675
N	3.12
S	0.5854
Mg	0.48
Cl	0.46
Ca	0.23
Na	0.20
Fe	0.10
EDTA	0.10
B	0.030
Cu	0.0021
Zn	0.0018
Mn	0.0015
Mo	0.0002093

to unsupplemented nutrient solution to produce the desired concentration. Unless otherwise noted, agar was maintained at 1% (w:v) and the pH adjusted to 4.4 before autoclaving.

General Culture Conditions

Cultures were grown in a growth room at constant thermo-photoperiod of 27 ± 2 °C and 90 ± 7 μmoles m^{-2} s^{-1}. The 60x15 mm petri plates were placed in gray plastic trays (51x24x6 cm) covered with clear plastic domes. The 24-well plates were placed in 16 x 14 cm clear plastic bags and placed in the gray plastic trays without the dome.

Sporophytes were grown in a greenhouse in a 2:1:1 (v:v:v) mixture of peat:vermiculite:perlite, and fertilized regularly.

Germination Determination

The percent germination of spores was determined visually by examining cultures with a Wild dissecting scope at 25x magnification. Fields of view were successively scored until 100 spores had been counted. Germination was determined based on the presence of an observable primary rhizoid, or on rupture of the spore wall. Germination was monitored beginning at three days from soaking (DFS) with 1 DFS being the day spores were sown.

pH Determination

The pH of the medium was determined prior to autoclaving, at the time of spore sowing and at the termination of experiments using a Fisher Accumet R Model 805 MP pH meter.

Determination of Gametophyte Area

Permanent slides of surface-growing bisexual meristematic gametophytes (cordates) were prepared by mounting them in a 4:1 (v:v) mixture of Hoyer's medium and 0.5% Aceto-carmine stain.

Permanent slides were observed using a Wild dissecting scope with drawing tube attached. Area of the gametophytes was determined using this setup in conjunction with the Bioquant ™ software program, an IBM personal computer, a Hipad ™ digitizing pad, and a Houston electronic cursor. Area was determined to five decimal places. Data were analyzed using Lotus 1-2-3 and SAS software.

Experimental Design

All experiments were designed so that duplicate or triplicate experiments were run simultaneously. A given treatment was present in a tray along with all strains, and more than one treatment was present in one tray for certain experiments.

Confirmation of Putative Mutants

Putative mutants were qualitatively confirmed as true mutants by sowing spores on medium supplemented with 200 μM AL-EDTA and observing for the presence of tolerant gametophytes, relative to the wild type response.

Quantitative confirmation was performed by sowing spores on medium supplemented with 0 - 300 μM Al-EDTA in 50 μM increments and comparing absolute growth of the mutants at 14 DFS to the growth of the wild type.

Component Tolerance Testing

Sulfate tolerance testing of the wild type strain was performed using sodium sulfate in a range from 0 to 300 μM in 100 μM increments. The mutants were not tested.

Testing for tolerance to low pH was performed by sowing spores at standard density on unsupplemented medium. The pH was adjusted prior to autoclaving. The pH range used was from 6.4 to 4.0, below which the media would not solidify well and few bisexual gametophytes were produced.

EDTA tolerance testing was performed by adding EDTA to Parker-Thompson's complete medium. Wild type spores were sown on plates which contained unsupplemented Parker-Thompson's medium adjusted to pH 5.4 prior to autoclaving. Upon germination, 20 individuals were transferred to the plates containing the supplemented media. A suitable concentration range was determined and the experiment was repeated with mutants. Upon germination, 60 spores per treatment (20 spores per plate), were isolated over a range of 0 - 300 μM EDTA.

Aluminum tolerance testing was attempted with aluminum chloride but not used as a means to characterize the mutants. It was not possible to add aluminum chloride to the medium prior to autoclaving because the media did not solidify. Doubling the agar concentration to facilitate media solidification was not acceptable because the growth of the control gametophytes was inadequate. Alternatively, sowing spores on filter paper supports was attempted, but the filter paper sunk into the media and gametophytes appeared stressed even in the control. Transferring germinated spores to Nucleopore ™ membrane filters was

also attempted, but the membrane filters did not appear to allow enough nutrient transfer to the gametophytes. The addition of an aluminum chloride solution to solidified plates was also attempted. If the pH of the aluminum chloride solution was not adjusted prior to adding it to solidified medium, the solution appeared to diffuse into the medium, but the great majority of the gametophytes were males due to the low pH of the media. When the pH of the aluminum chloride solution was adjusted to 4.4 to increase the number of bisexual gametophytes, no diffusion took place and it appeared as though the aluminum had complexed with some substance on the surface of the media.

Segregation Analysis

Prior to segregation analysis, hybrids were confirmed by screening for segregation of tolerant and sensitive gametophytes on 60 x 15 mm plates containing medium supplemented with 200 μM Al-EDTA. Segregation analysis was done on gametophytes from F1 individuals generated from reciprocal crosses between mutant stocks and the wild type stock. M2 gametophytes from parental stocks were also analyzed for comparative purposes. Spores were sown at a lower than normal density (ca. 312 spores/plate) on unsupplemented medium at pH 5.4. Individual gametophytes were isolated when they had developed a meristematic region, but before they had developed a cordate morphology (precordates), to 24-well plates containing ca. 2.0 ml of supplemented media per well. Alternatively, isolation and immersion of 10 precordates to 60 x 15 mm plates containing supplemented medium was used with strain HαAT7 in one experiment. After a period of growth on the supplemented medium, permanent slides were made and a frequency

distribution of gametophyte size was generated. The first day after transfer of precordates was recorded as 1 DFT (days from transfer).

Sporophyte Characterization

Sporophyte characterization was not completed, but a possible method for future studies was developed. Sporophytes of similar development (size, number of roots, and number of leaves) were allowed to acclimate to culture in liquid Parker-Thompson's media for one day. After this period of acclimation, all the roots were trimmed from the sporophyte and plants were placed in liquid culture containing Al-EDTA at pH 4.4. The media was replaced after two days growth in a growth chamber under constant thermo-photoperiod (27 ± 2 °C and 90 ± 7 μmoles m^{-2} s^{-1}). Tolerance levels were evaluated by measuring new root regeneration.

III. RESULTS

Selection of Stocks

Ripened spores from thirty putative mutants were sown on media containing 200 μM Al-EDTA to confirm tolerance after passing through a sexual cycle. Six stocks were qualitatively confirmed as expressing tolerance. One of these did not grow well in the M1 sporophyte generation and was eliminated from further study.

The five remaining mutants (HαAT3, HαAT7, HαAT29, HαAT32, HαAT49) were then quantitatively characterized by sowing cultures on media supplemented with a range of Al-EDTA concentrations (0-300 μM) at pH 4.4. Both germination and growth of the gametophytes were monitored. Percent germination at 12 DFS is shown in Figure 1. The treatments had little effect on the germination of the wild type. Mutants exhibited similar behavior, but the mutants HαAT29 and HαAT32 showed a slight decline at higher treatment concentrations. Also, the mutant HαAT32 showed lower germination relative to the others at all treatment levels. Figure 2 presents gametophyte area expressed as size versus treatment concentration at 14 DFS. At all treatment-stock combinations, except HαAT49 at 0 and 50 μM, the mutants are larger than the wild type. The wild type had no cordates present at the 300 μM treatment. In Figure 3 the data are presented as a percent of the 0 μM control to eliminate absolute growth differences. A large range of responses is evident, but all mutants show tolerance relative to the wild type at concentrations above 100 μM. Three mutants, HαAT3, HαAT7, HαAT29, were chosen for further characterization and genetic analysis.

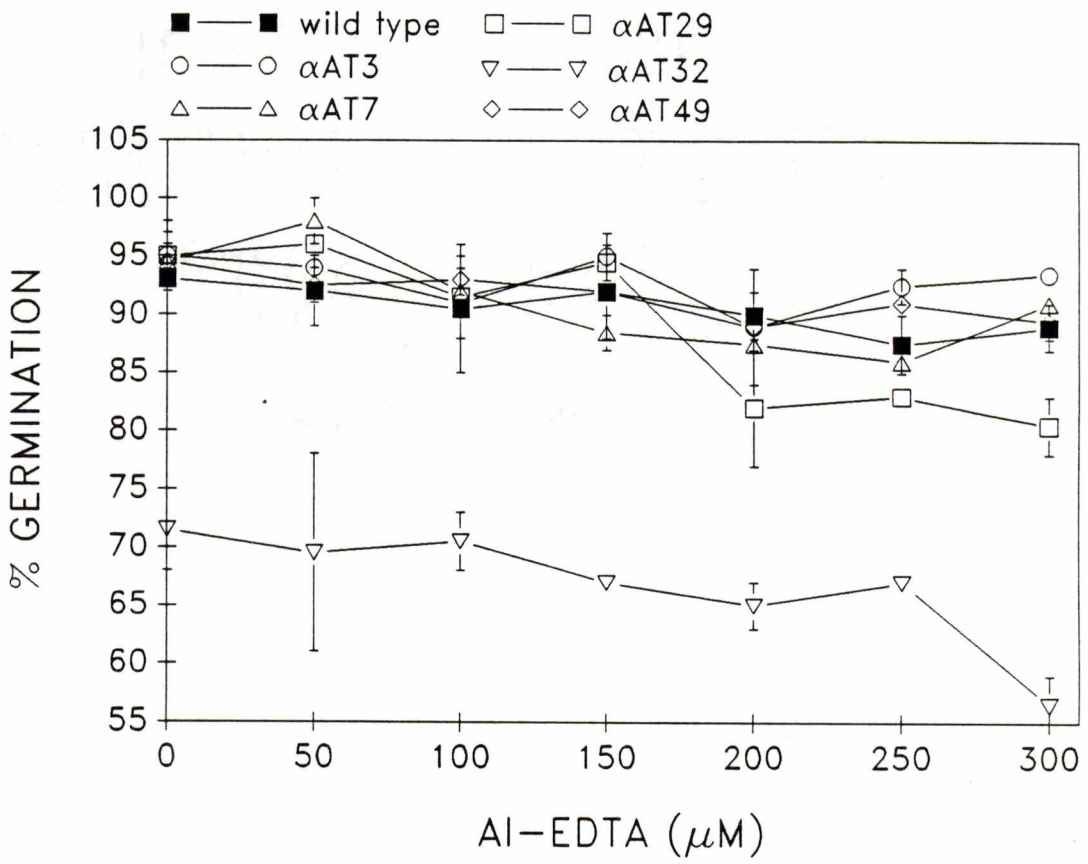


Figure 1. Germination response, in multispore sowings, of the wild type and five mutant strains at 12 DFS over a range of Al-EDTA concentrations at pH 4.4. (Symbols represent means ± S.E.M. of two samples).

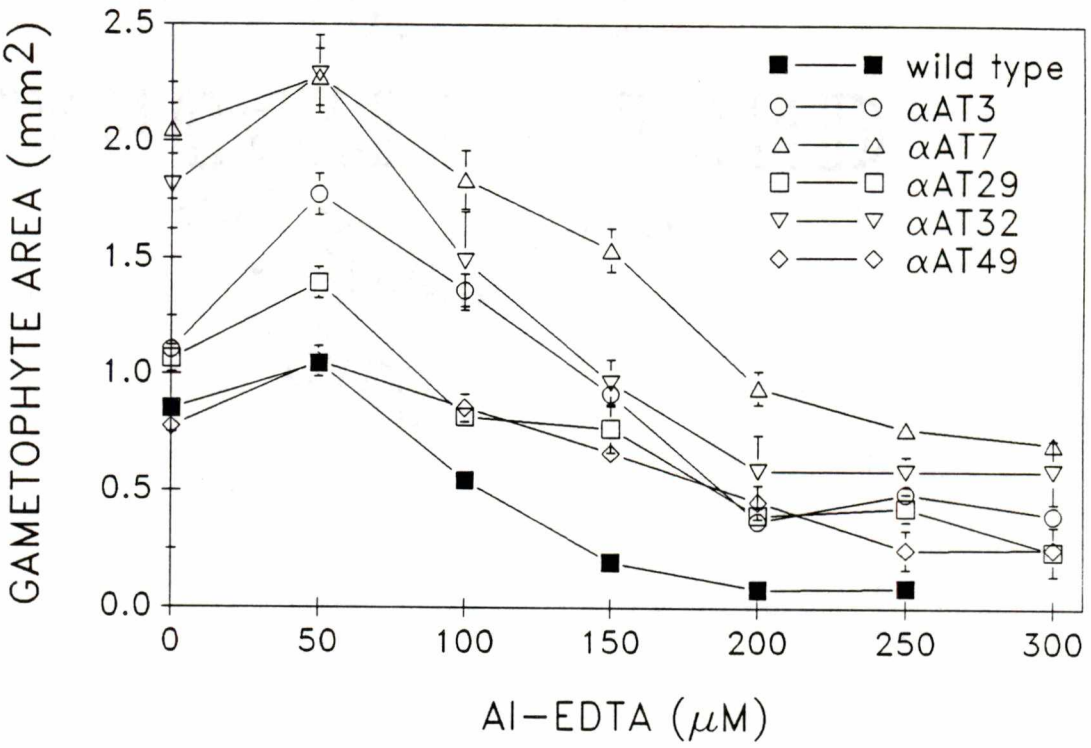


Figure 2. Gametophyte area, in multispore sowings, of surface growing cordates of the wild type and five mutants over a range of Al-EDTA concentrations at 14 DFS, at pH 4.4 (Symbols represent means ± S.E.M., n > 20 from 0 - 150 μM, above which n ranged from 2 - 60). Data from duplicate experiments were combined.

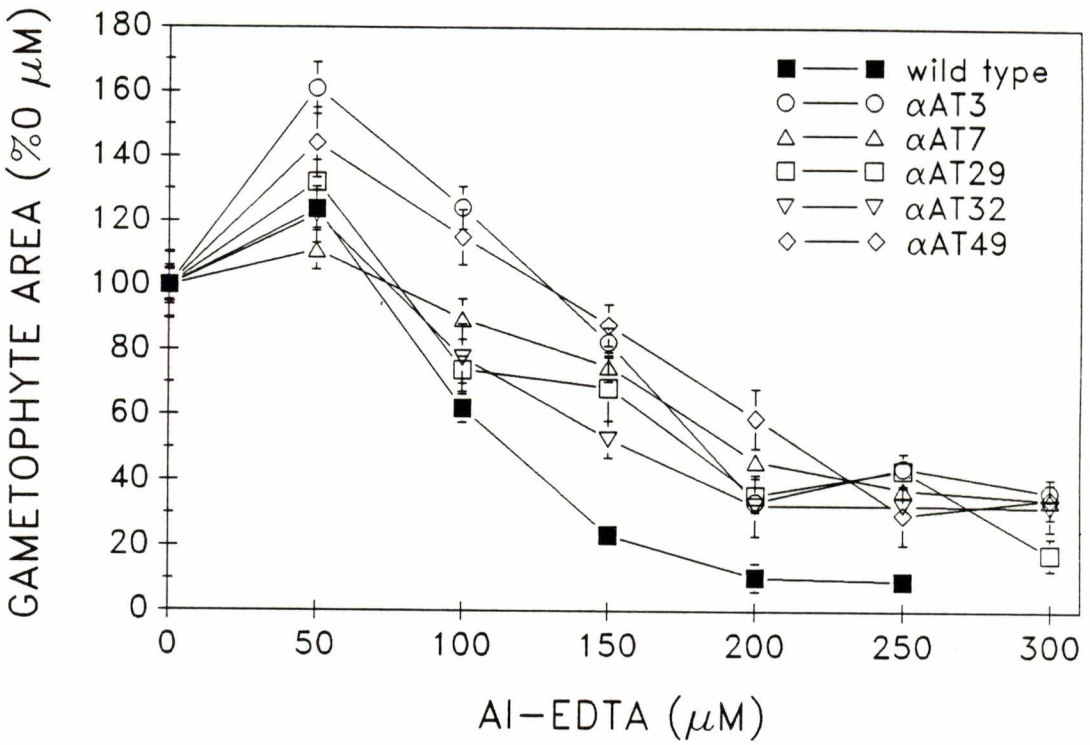


Figure 3. Gametophyte area, in multispore sowings, of surface growing cordates of the wild type and five mutants over a range of Al-EDTA concentrations presented as a percent of the 0 μM control at 14 DFS, at pH 4.4 (Symbols represent means ± S.E.M., n > 20 from 0 - 150 μM, above which n ranged from 2 - 60). Data from duplicate experiments were combined.

Morphological Characterization

At germination, the wild type produces a primary rhizoid. The mutants HαAT3 and HαAT29 were normal in this respect. In contrast, HαAT7 did not produce a primary rhizoid. This trait was examined further by counting the rhizoids present on cordate gametophytes that had been grown on unsupplemented media over a range of pH. Figure 4 shows the mean rhizoid number per gametophyte as a function of pH at 12 DFS. The wild type produces more rhizoids per gametophyte at all pH values, and produces a maximum number at pH 6.0. At pH 6.0 the gametophytes also were at their maximum size. Below pH 5.2 the wild type is dramatically effected by pH, with the mean number of rhizoids decreasing roughly 33% between pH 5.2 and pH 4.8. At pH 4.0 the wild type produces only 11% of the maximum number of rhizoids produced. The mutant produces its maximum number of rhizoids per gametophyte at pH 5.6 which is also the pH that allowed maximum growth. The mutant also produces fewer rhizoids at lower pH and at pH 4.0 produces ca. 9% of the maximum number of rhizoids.

Component Tolerance Testing

Because of the complexity of the selection medium, experiments were performed to determine if the mutants were tolerant to individual components of the selection medium, ie., aluminum, sulfate, EDTA, and acidity. Unless otherwise noted, all experiments were performed on media that had been adjusted to pH 4.4 prior to autoclaving.

The response of the wild type individuals to the presence of additional sulfate in the media was tested by adding sulfate in the same concentration range as was added by the aluminum sulfate in the original

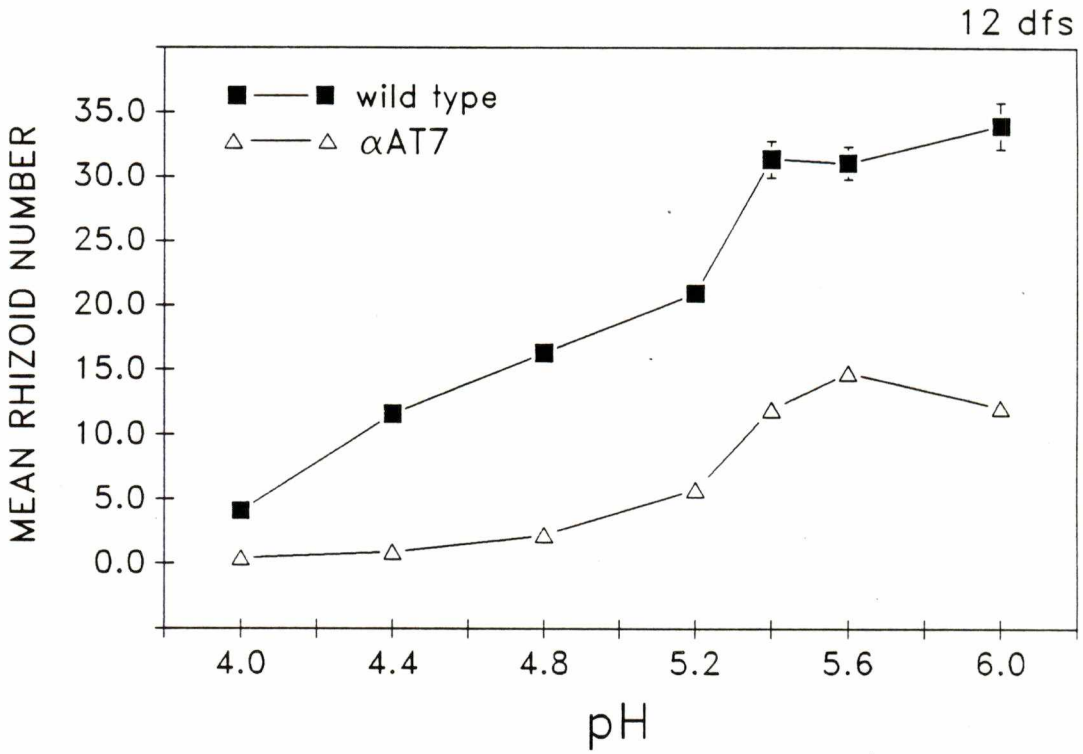


Figure 4. Mean rhizoid number, in multispore sowings, per gametophyte as a function of pH at 12 DFS. (Symbols represent means ± S.E.M., n ranged from 23 - 30 for the wild type, n ranged from 23 - 30 for HαAT7). Data from triplicate experiments were combined.

selection medium (200 μM Al-EDTA contains 300 μM sulfate). Figure 5 shows the area of the gametophytes, as a percent of the 0 μM control, as a function of treatment at 14 DFS. A variable response is observed, but the growth at 200 μM, the lowest point relative to the control, was 71.6% (± 16.1% S.D.) of the growth of the 0 μM control. This decrease is not enough to explain the growth response of the wild type in the original screening. Therefore, the mutants' response to sulfate was not tested. Germination was not effected by treatment. (Data not shown).

The effect of acidity on gametophyte area is seen in Figure 6. The wild type, Hn-n, has a pH optimum of 6.0, and shows a sharp decline in growth below pH 5.6. The mutants, HαAT3 and HαAT29, have a pH optimum for growth near 5.6. The mutant HαAT7 has a pH optimum between 5.2 and 5.6, and an earlier experiment showed a more evident pH optimum of 5.6. The mutants also appear to be less effected by an increase in acidity of the growth medium than the wild type. Germination was not effected by acidity. (Data not shown).

An EDTA tolerance test was performed because additional EDTA was present in the original selection medium. Figure 7 shows gametophyte area as a function of EDTA treatment at 12 DFT of germinated individuals. All mutants are larger than the wild type, but show a decline in growth due to EDTA treatment. The acid tolerance of the mutants relative to the wild type and the better absolute growth can easily be seen at 0 μM EDTA. Figure 8 shows gametophyte area at 12 DFT as a percent of the 0 μM control. The wild type shows a steep decline in growth above 100 μM EDTA, but enhanced growth relative to the control and to the mutants at 100 μM. The mutant HαAT7 grows as well,

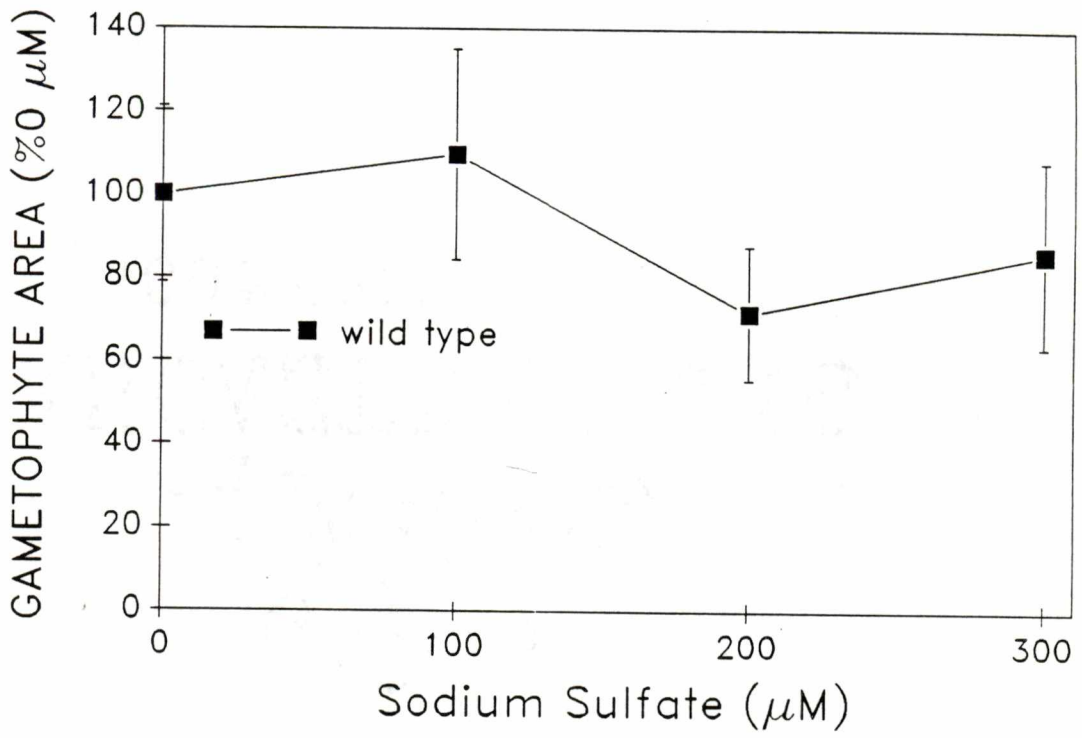


Figure 5. Gametophyte area, in multispore sowings, of surface growing cordates of the wild type as a function of sodium sulfate treatment, presented as a percent of the 0 μM control at 14 DFS, at pH 4.4. (Symbols represent means ± S.D., n = 40). Data from duplicate experiments were combined.

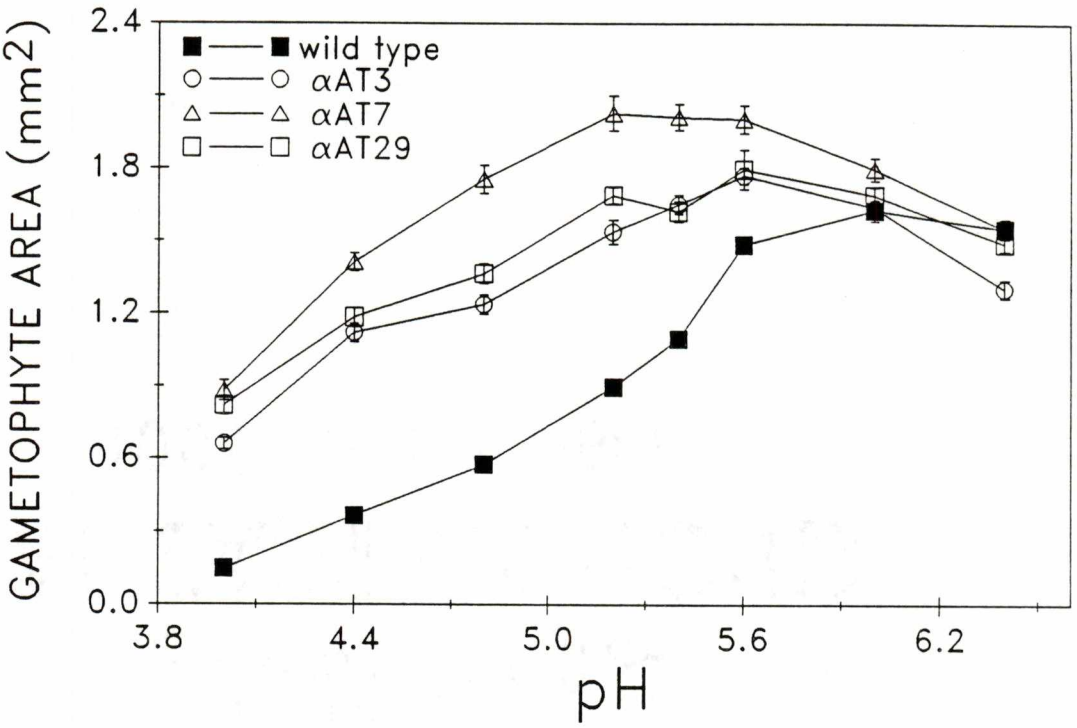


Figure 6. Gametophyte areas, in multispore sowings, of surface growing cordates of the wild type and three mutants at 12 DFS over a range of pH (Symbols represent means ± S.E.M., n = 60 except at pH 4.0 where n > 29). Data from duplicate experiments were combined.

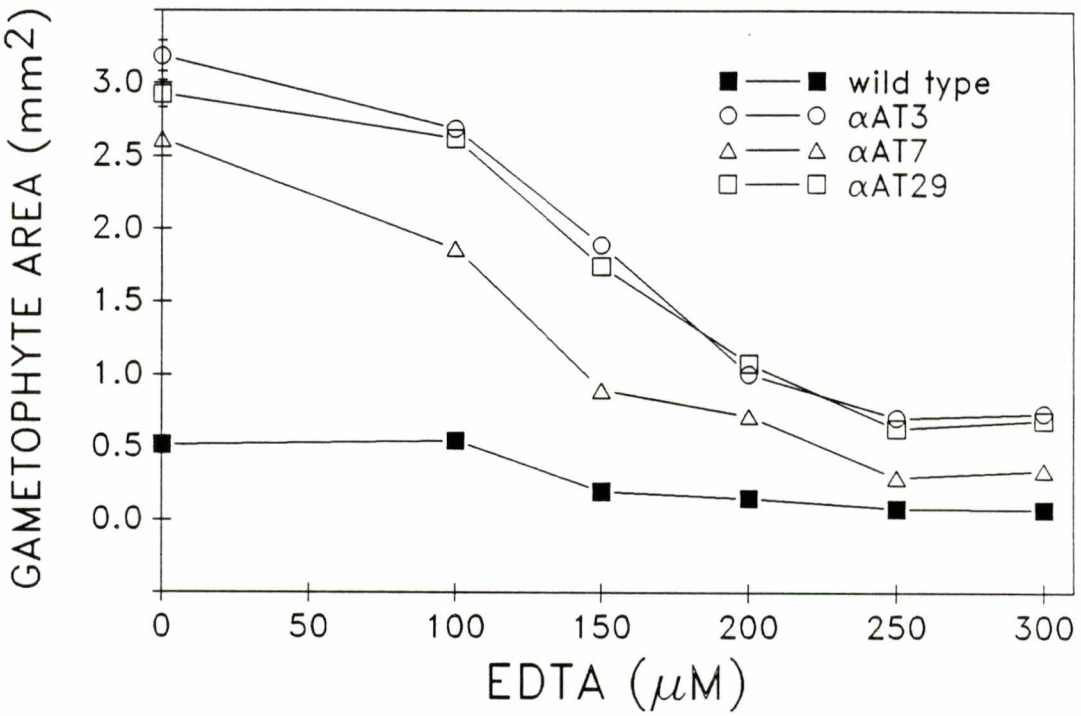


Figure 7. Gametophyte areas, of the wild type and three mutants, 12 DFT over a range of EDTA concentrations. (Symbols represent means ± S.E.M., n ≥ 30 from 0 -150 μM, above 150 μM n ranged from 5 - 51). Data from triplicate experiments were combined.

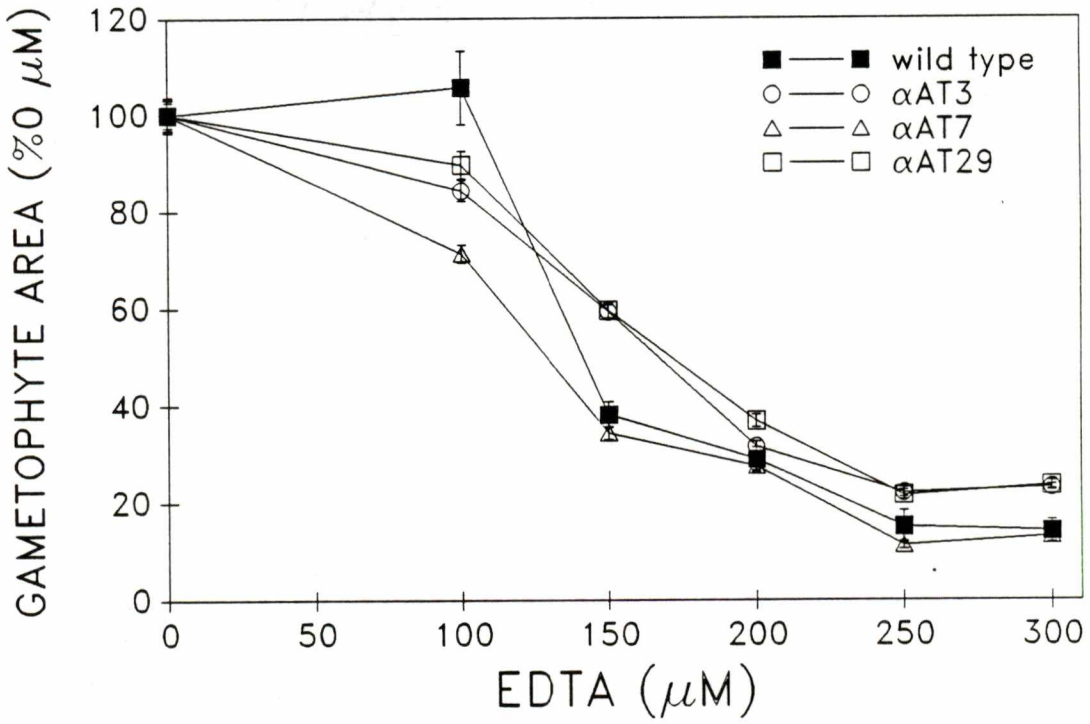


Figure 8. Gametophyte areas, of the wild type and three mutants, 12 DFT over a range of EDTA concentrations as a percent of the 0 μM control. (Symbols represent means ± S.E.M., n ≥ 30 from 0 -150 μM, above 150 μM n ranged from 5 - 51). Data from triplicate experiments were combined.

or slightly less well than the wild type, at all treatment levels. Tukey's test of the means (Table 3) indicates that the means of HαAT3 and HαAT29 are not statistically different from each other, but are statistically different from the wild type. At 150 μM HαAT3 and HαAT29 grow about 20 percent better than the wild type and slightly better above 150 μM.

Gametophytic Genetic Analysis

Segregation analyses were performed to determine the genetic basis of tolerance exhibited by the mutants. Gametophytes from F1 sporophytes were assayed for tolerance with the appropriate parental stocks used as a reference.

HαAT3

Because of different germination rates, precordates representing the wild type were isolated in one day while other stocks were isolated over a range of days. In all cases slides were prepared at 8 DFT to 24-well plates. Figure 9 shows parental response on 300 μM AL-EDTA. The wild type has a mean of 1.45 mm^2 and the mutant has a mean of 2.94 mm^2. A wider range of response is observed for the mutant than for the wild type. Figure 10 shows the response of the gametophytes from the reciprocal crosses, F1(Hn-n x HαAT3)7 and F1(HαAT3 x Hn-n)4. The reciprocal crosses exhibit similar behavior, indicating a nuclear gene basis for tolerance. The distribution is bimodal, suggesting the segregation of two response types, one with a mean of ca. 1.6 mm^2 and one with a mean near 2.8 mm^2. The presence of only two response types suggests a single gene basis for tolerance, with one class representing

Table 3. Tukey's Studentized Range (HSD) Test

Stock Comparisons	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit	
Hn-n - HαAT3	-8.173	-4.495	-0.818	*
Hn-n - HαAT7	3.647	7.328	11.009	*
Hn-n - HαAT29	-10.502	-6.733	-2.965	*
HαAT3 - Hn-n	0.818	4.495	8.173	*
HαAT3 - HαAT7	9.173	11.823	14.473	*
HαAT3 - HαAT29	-5.008	-2.238	0.532	
HαAT7 - Hn-n	-11.009	-7.328	-3.647	*
HαAT7 - HαAT3	-14.473	-11.823	-9.173	*
HαAT7 - HαAT29	-16.835	-14.061	-11.287	*
HαAT29 - Hn-n	2.965	6.733	10.502	*
HαAT29 - HαAT3	-0.532	2.238	5.008	
HαAT29 - HαAT7	11.287	14.061	16.835	*

Note: This test controls type I experimentwise error rate.

Alpha = 0.05 Confidence = 0.95 df = 462 MSE = 78.43207
Critical Value of Studentized Range = 3.646

Comparisons significant at the 0.05 level are indicated by '*'.

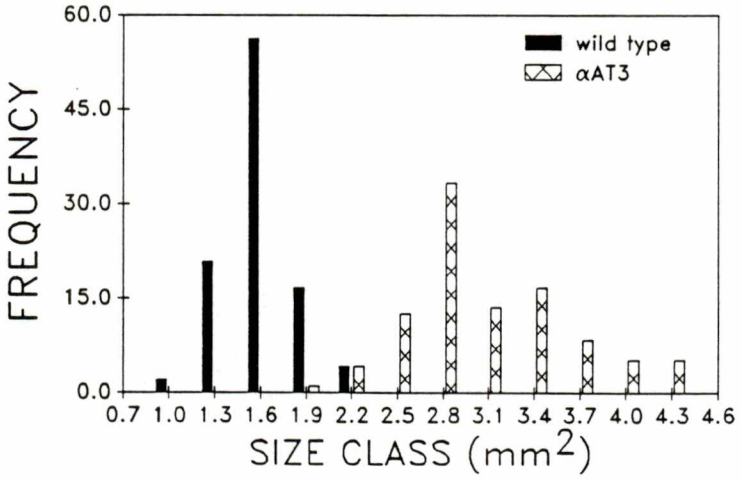


Figure 9. Growth of M2 gametophytes from parental stocks on medium supplemented with 300 µM Al-EDTA at pH 4.4, 8 DFT of precordates. (n = 48 for the wild type, and n = 96 for HαAT3). Data from duplicate experiments were combined.

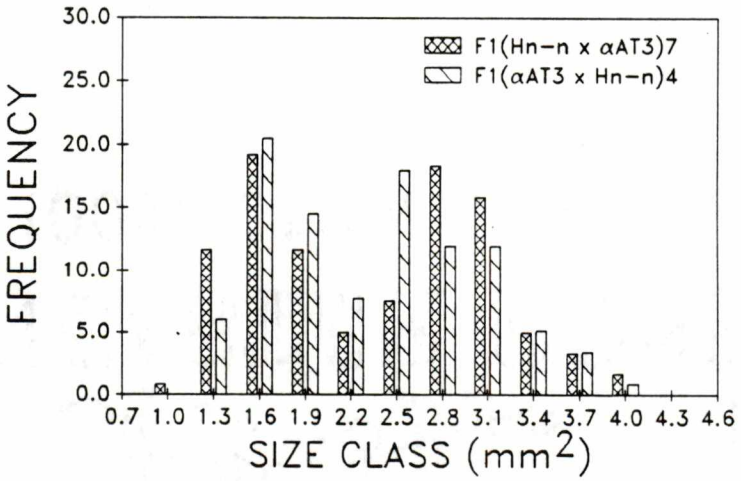


Figure 10. Segregation pattern of gametophytes from F1 individuals grown on medium supplemented with 300 µM AL-EDTA at pH 4.4, 8 DFT of precordates. (for F1 (Hn-n x HαAT3)7 n =120, n = 117 for F1 (HαAT3 x Hn-n)4). Data from duplicate experiments were combined.

segregation for the wild type allele and the other class representing segregation for the mutant allele.

A chi-square analysis was done by eliminating the 2.2 mm^2 size class because of overlap of the mutant and wild type response (Figure 9). One HαAT3 gametophyte of the remaining 92 was still present in the wild type class, but it was ignored. Of 114 gametophytes from F1(Hn-n x HαAT3)7, 52 were in the wild type response class of 0.7 - 1.9 mm^2. Chi-square analysis, using Yates correction, indicated that the data are in agreement with a 1:1 ratio (X^2 = 0.71, $p > 0.20$). Chi-square analysis, of the reciprocal cross F1(HαAT3 x Hn-n)4 was also performed using Yates correction. Of the 108 gametophytes, 48 were in the size range (0.7 - 1.9 mm^2) expected for the wild type. This is in agreement with a 1:1 ratio (X^2 = 1.12, $p > 0.20$).

HαAT7

For this analysis, gametophytes were isolated and immersed in the agar, in an attempt to more clearly resolve responses. Gametophytes from the parental types were isolated in one day, and the gametophytes from the F1 hybrids were isolated over two days, based on the germination response. The parental response is shown in Figure 11. The wild type has a mean area of 0.24 mm^2. In contrast, HαAT7 has a mean of 0.87 mm^2. Overlap of the mutant response into the wild type response is evident with a roughly normal distribution. The mean size for both the wild type and the mutant are smaller than for surface growing gametophytes (cf., Fig. 13). The response of the reciprocal hybrids is shown Figure 12. The results are similar suggesting that the genetic basis is of nuclear origin. Although considerable overlap is evident, only two

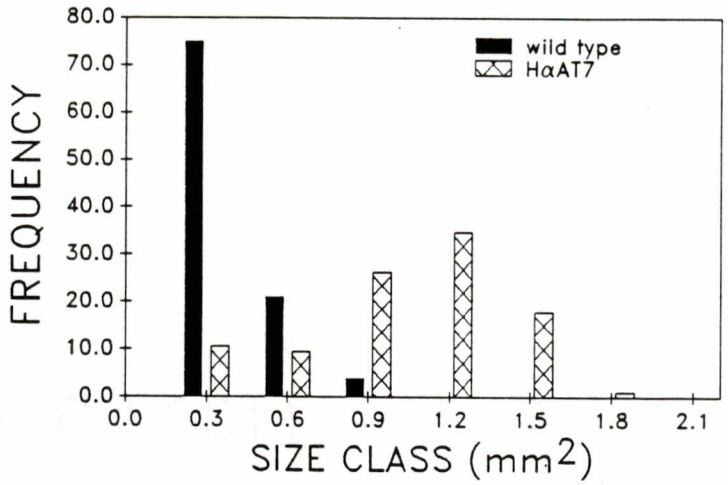


Figure 11. Growth of M2 gametophytes from parental stocks immersed in medium supplemented with 200 µM Al-EDTA at pH 4.4, 11 DFT of precordates. (n = 100 for the wild type, n = 95 for HαAT7). Data from duplicate experiments were combined.

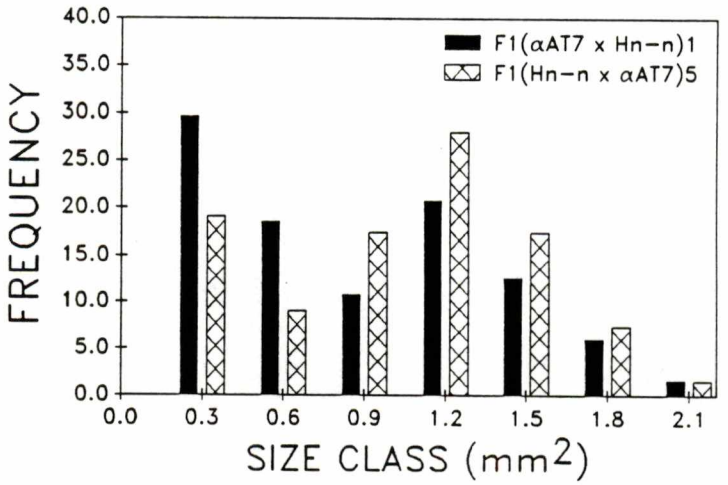


Figure 12. Segregation pattern of gametophytes from F1 individuals immersed in media supplemented with 200 µM AL-EDTA at pH 4.4, 11 DFT of precordates. (n = 170 for F1(HαAT7 x Hn-n)1, n = 177 for F1(Hn-n x HαAT7)5). Data from duplicate experiments were combined.

segregation types that approximate the parental response are apparent; one response type with a mean of 0.3 - 0.6 mm^2 and the other with a mean of about 1.2 mm^2. This would indicate a single gene basis for tolerance. However, because of the significant overlap, chi-square analysis was not performed.

Analysis of the HαAT7 parental response indicated a sizable number of sensitive gametophytes in the population. Because this could suggest the possible segregation of both tolerant and sensitive types from the presumed homozygous mutant, two M2 gametophytes representing the highest tolerance class were isolated from a separate experiment and selfed to produce homozygous M2 sporophytes, HαAT7H-T1 and HαAT7H-T2.

Tests for segregation were performed using gametophytes from the M1 and M2 sporophytes, and wild type gametophytes. Precordate gametophytes of the wild type were isolated in one day, M2 precordates were isolated over two days, and M3 precordates, from αAT7H-T1 and αAT7H-T2, were isolated over several days. The growth of the wild type and the M2 gametophytes on 24-well plates supplemented with 300 μM Al-EDTA is shown in Figure 13. The wild type has a mean of 2.81 mm^2, whereas HαAT7 has a mean of 4.52 mm^2. The mutant shows a roughly normal distribution with overlap into all size classes representing the wild type. No segregation is evident. The growth of the M3 gametophytes is shown in Figure 14. The M3 gametophytes have means of between 3.2 and 3.6 mm^2, which is less than the M2 gametophytes (Figure 13). This may be related to the immaturity of the spore collection relative to all other collections used in the study. A nearly normal distribution is evident.

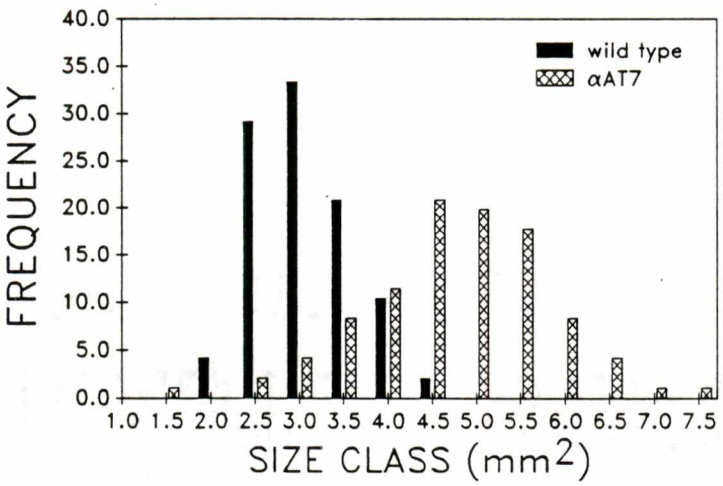


Figure 13. Growth of wild type and M2 gametophytes from parental stocks on medium supplemented with 300 μM Al-EDTA at pH 4.4, 11 DFT of precordates. (n = 48 for the wild type , n = 96 for HαAT7). Data from duplicate experiments were combined.

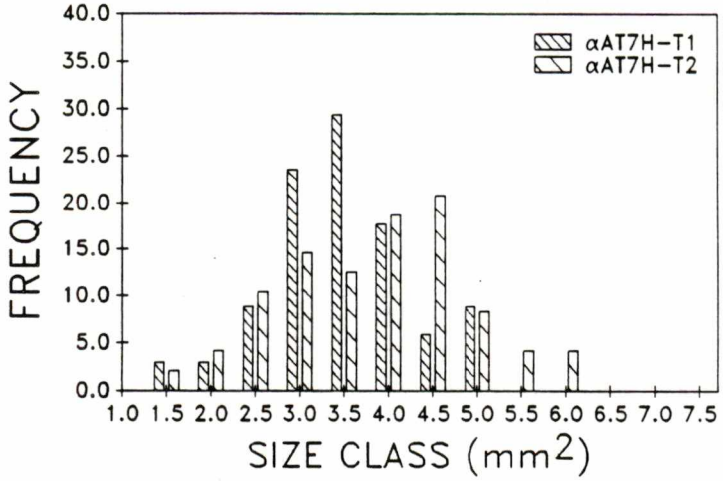


Figure 14. Growth of M3 gametophytes from parental stocks on medium supplemented with 300 μM Al-EDTA at pH 4.4, 11 DFT of precordates. (n = 34 for αAT7H-T1, n = 48 for αAT7H-T2).

HαAT29

A preliminary segregation analysis of this mutant suggested that a more tolerant class may have been segregating from the F1 hybrids. The reciprocal hybrids exhibited similar behavior, indicating a nuclear basis of tolerance, but segregation of two or more distinct types was not evident. Because of limited spore collections, further segregation tests only used one of the reciprocal crosses.

An experiment was performed to determine if the more tolerant class could be observed at Al-EDTA levels lower than that used in the preliminary test, and to possibly clarify the genetic basis of tolerance. The wild type and the mutant were isolated in one day, and spores from the hybrid were isolated over three days based on their germination responses. The response at 30 μM Al-EDTA is shown in Figure 15. The wild type has a mean of 2.2 mm^2. The mutant has a mean of 3.7 mm^2. The F1 is intermediate between the wild type and HαAT29 with a mean of about 3.5 mm^2. No distinct segregation types from the F1 are evident. The largest F1 size classes have a lower frequency than what was actually present because the five visually largest gametophytes were isolated and selfed to produce sporophytes for future experiments. Comparison of the growth response of gametophytes from these sporophytes to the growth response of HαAT29 may show whether the gametophytes in the largest size class represent a genotypically distinct class.

Figure 16 shows the size classes obtained from media at pH 4.4 containing no Al-EDTA. The wild type has a mean of 3.1 mm^2, and HαAT29 has a mean of 3.7 mm2. The hybrid shows no apparent segregation of distinct types, therefore, little information of the genetic basis of

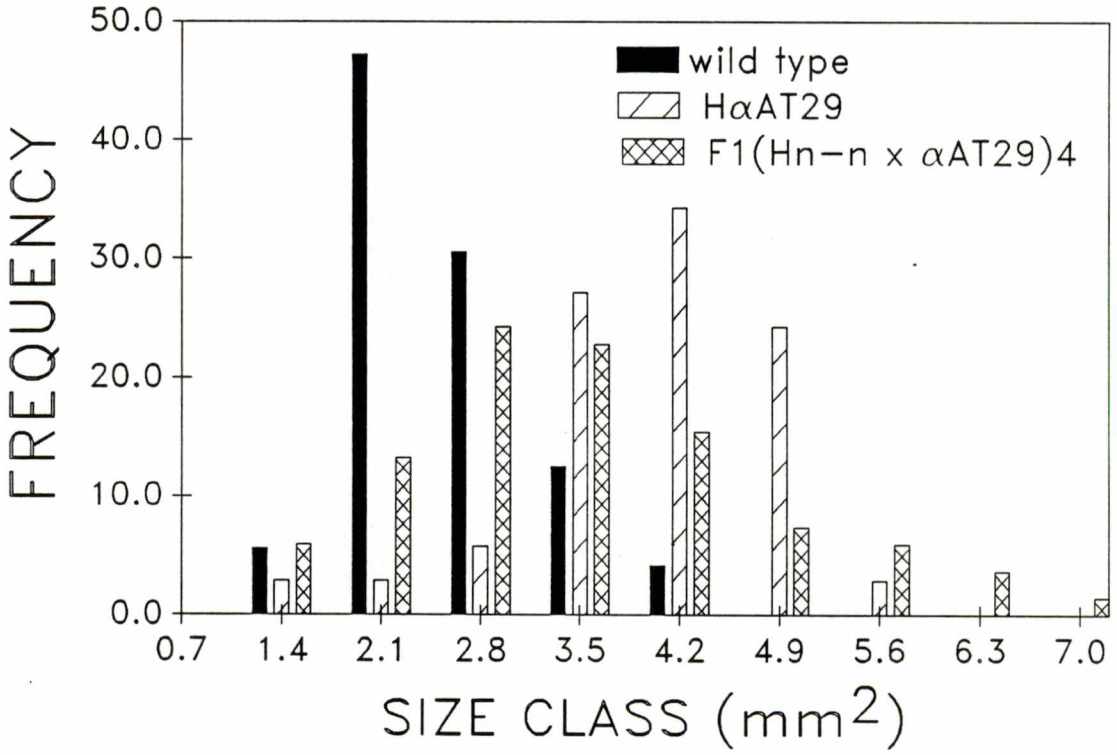


Figure 15. Growth of M2 gametophytes, from the wild type and HαAT29, and segregation of gametophytes from F1(Hn-n x HαAT29)4, 9 DFT of precordates to 30 μM Al-EDTA.(For HαAT29 n = 70, n = 72 for Hn-n, and n = 136 for F1(Hn-n x HαAT29)4). Data from triplicate experiments were combined.

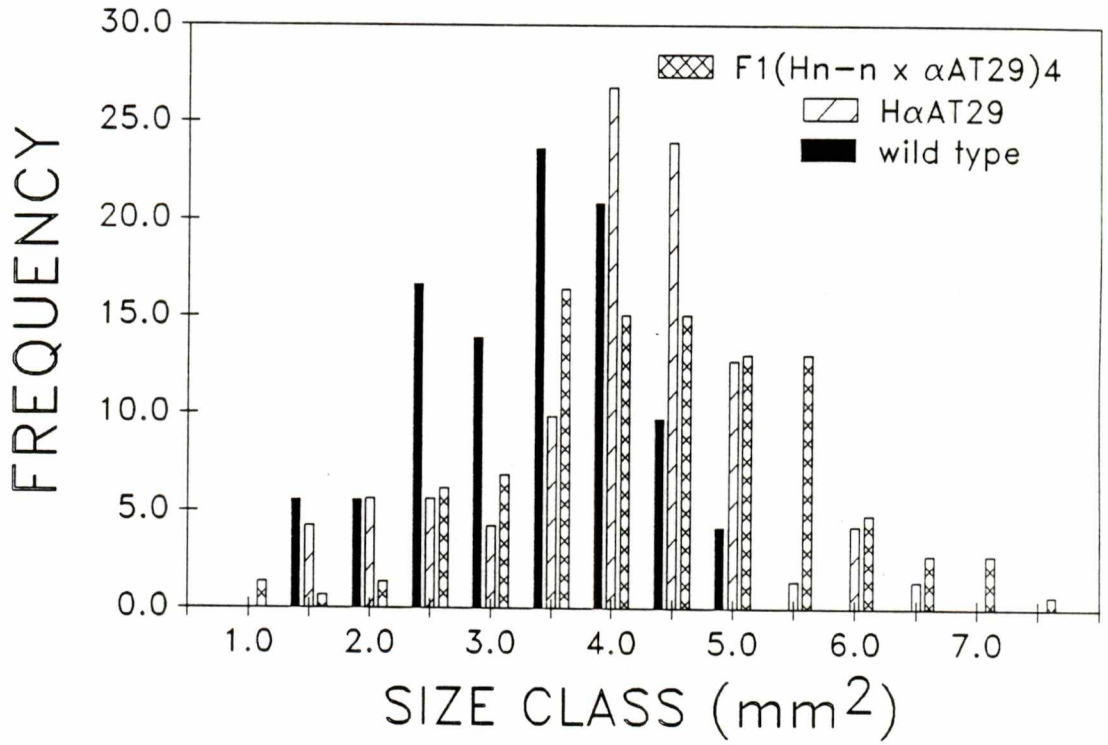


Figure 16. Growth of M2 gametophytes, from the wild type and HαAT29, and segregation of gametophytes from F1(Hn-n x HαAT29)4, 9 DFT of precordates to pH 4.4 media. (For HαAT29 n = 71, n = 72 for Hn-n and , n = 146 for F1(Hn-n x HαAT29)4). Data from triplicate experiments were combined.

of tolerance can be obtained. The five visually largest gametophytes were isolated and selfed as in the 30 μM treatment.

Tests using higher levels of Al-EDTA were also attempted. Gametophytes of HαAT29 and F1(HαAT29 x Hn)7 were isolated over a range of days to well plates containing 100, 300, and 500μM Al-EDTA. Wild type gametophytes were not isolated. No apparent segregation of distinct types from the F1 hybrid was observed at any level. Figure 17 shows the results obtained from the 100 μM treatment. A class that may represent the wild type might be evident at 1.75 - 3.25 mm^2. The mean of HαAT29 was 4.43. A class representing the more tolerant class may be present at 7.75 mm^2. Figure 18 shows the results from the 300 μM treatment. The mean of HαAT29 is 3.50 mm^2. A class possibly representing the more tolerant class (5.2 - 6.0 mm^2) is more evident than at the 100 μM treatment. The responses of the mutant gametophytes and the gametophytes from the hybrid at 500 μM are shown in Figure 19. HαAT29 had a mean of 2.76 mm^2. One size class possibly representing the more tolerant class is present at 4.4 mm^2.

A model illustrating how the more tolerant class could have arisen is shown in Figure 20. Although tolerance appears to be inherited from a nuclear gene, no information on the number of genes associated with the response in this strain can be obtained from the segregation tests performed.

Sporophyte Characterization

Sporophyte characterization was not completed for this study, but a method that will produce a response in the wild type has been developed. The result of one such experiment is shown in Figure 21. The wild type

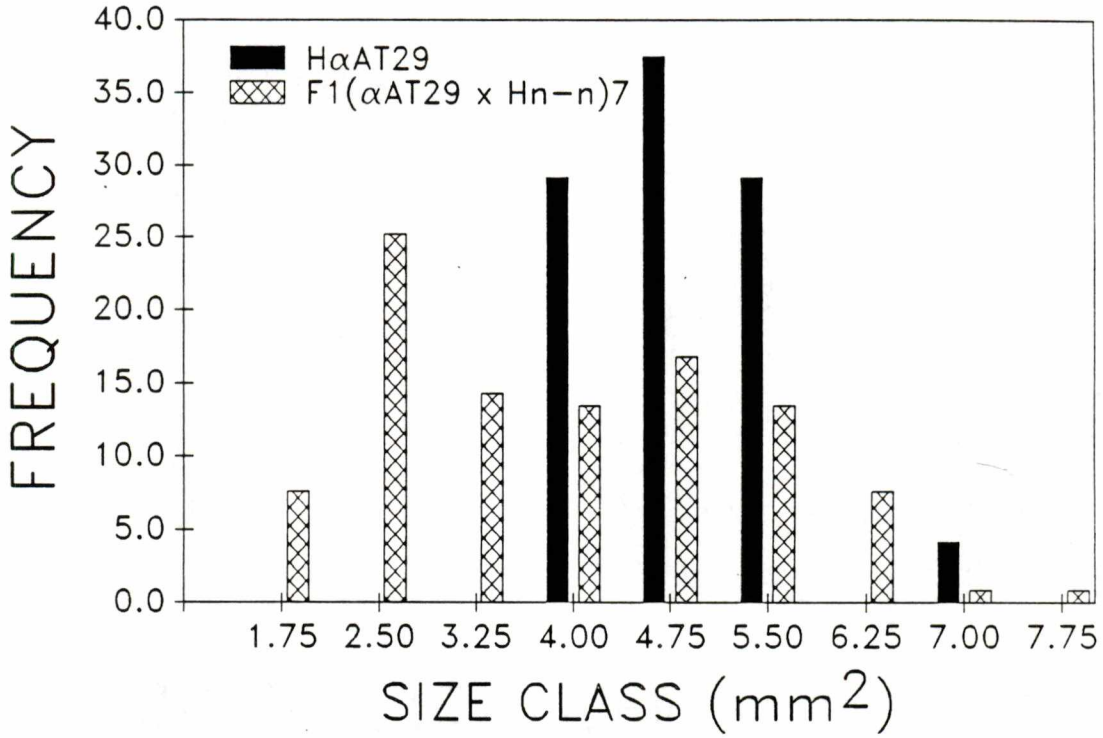


Figure 17. Growth of M2 gametophytes, from parental stock HαAT29, and segregation of gametophytes from F1(HαAT29 x Hn-n)7, 9 DFT of precordates to 100 μM Al-EDTA. (For HαAT29 n = 24, for F1 (HαAT29 x Hn-n)7 n = 119).

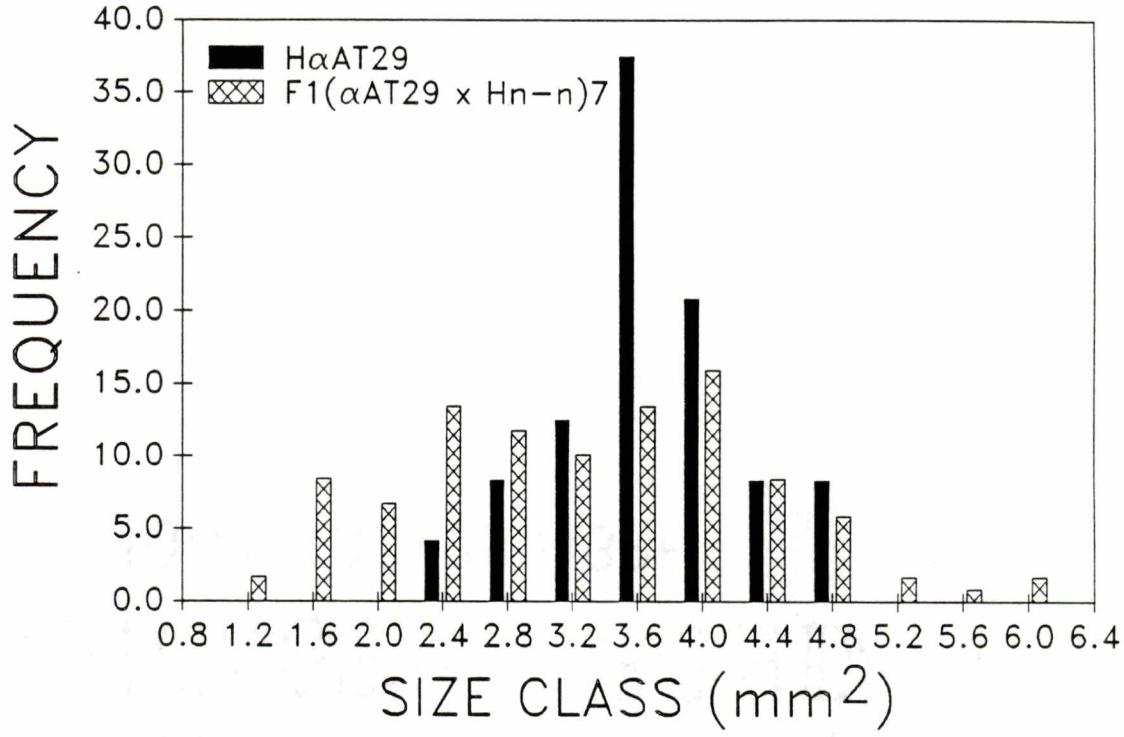


Figure 18. Growth of M2 gametophytes, from parental stock HαAT29, and segregation of gametophytes from F1(HαAT29 x Hn-n)7, 9 DFT of precordates to 300 μM Al-EDTA. (For HαAT29 n = 24, for F1 (HαAT29 x Hn-n)7 n = 119).

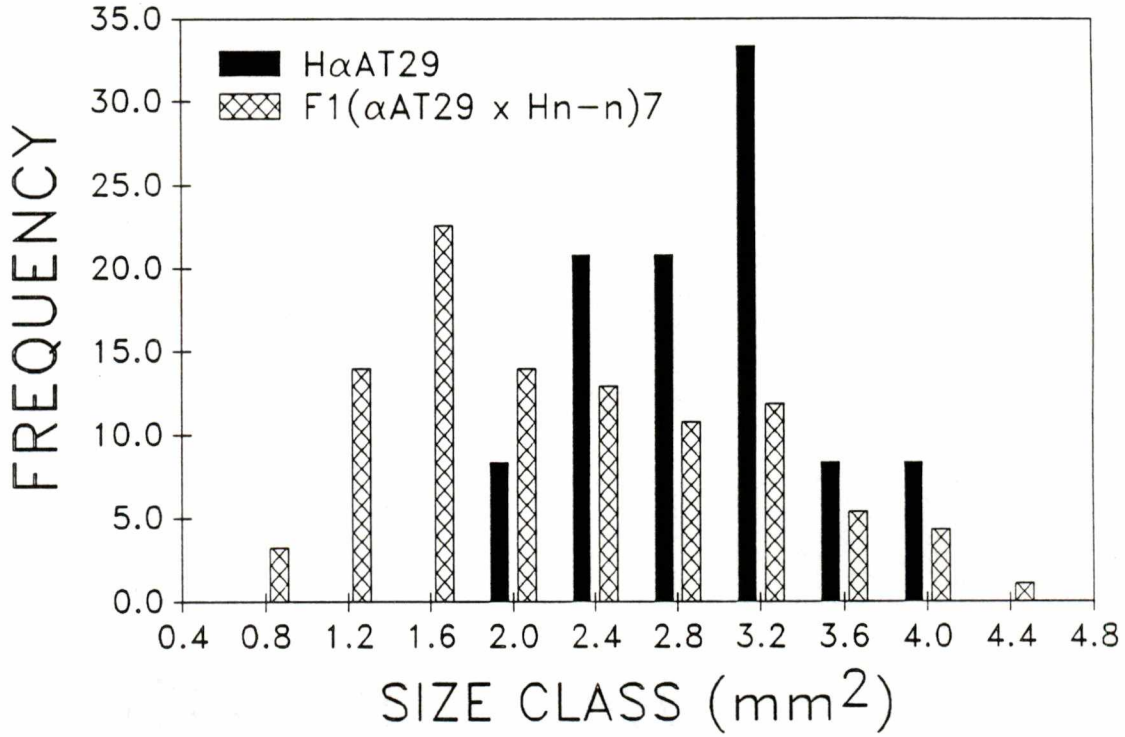


Figure 19. Growth of M2 gametophytes, from parental stock HαAT29, and segregation of gametophytes from F1(HαAT29 x Hn-n)7, 9 DFT of precordates to 500 μM Al-EDTA. (For HαAT29 n = 24, for F1 (HαAT29 x Hn-n)7 n = 93).

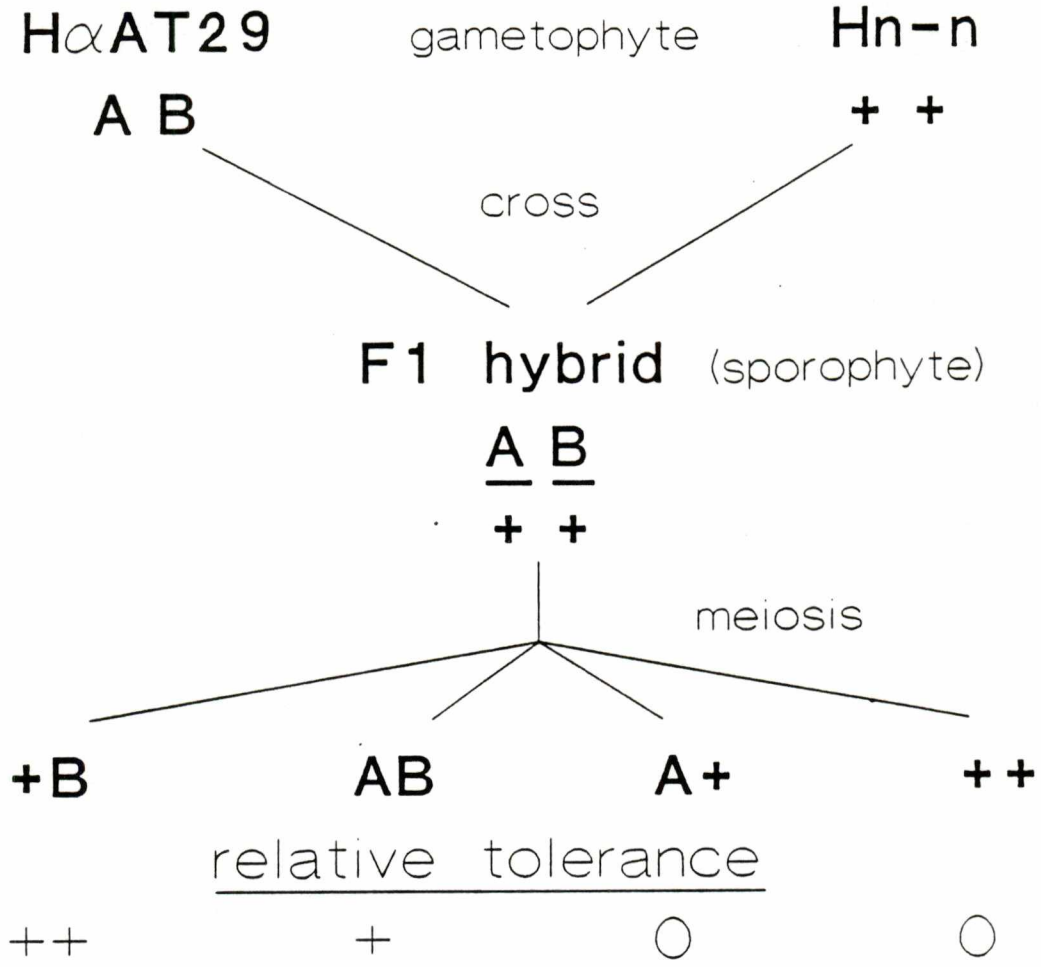


Figure 20. Model illustrating segregation of a high tolerance group from F1 hybrids. The presence of the A allele in HαAT29 prevents full expression of tolerance. (+ indicates HαAT29 response, ++ indicates high tolerance response, and 0 represents wild type response)

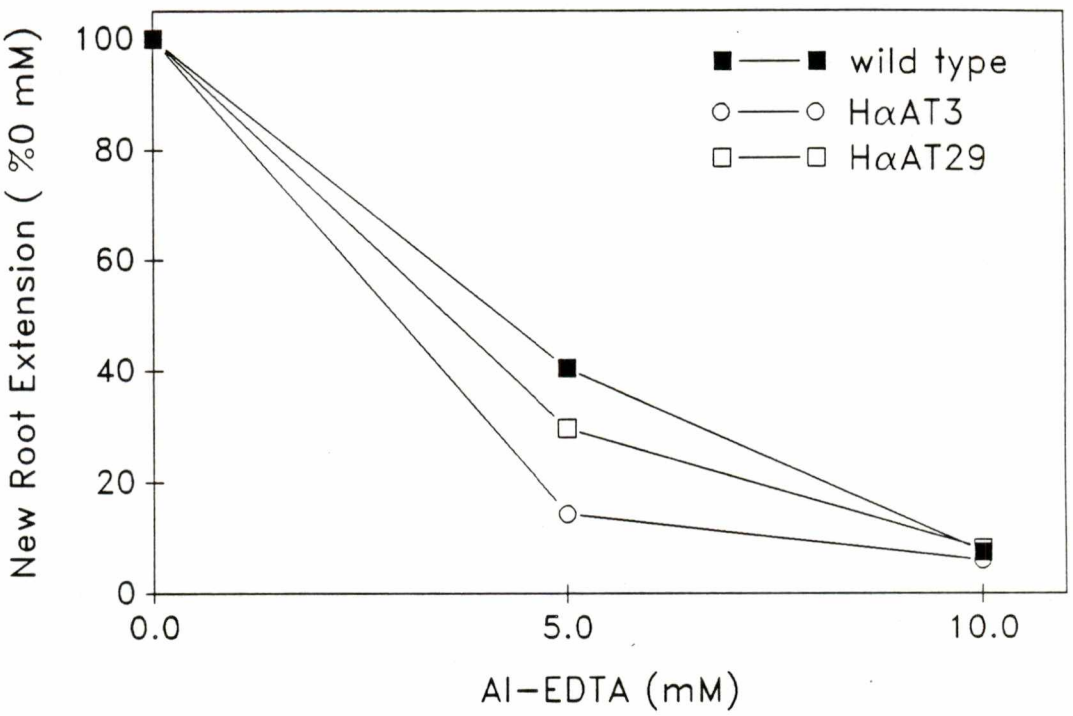


Figure 21. Mean new root extension of the wild type and two mutants, as a function of Al-EDTA treatment, presented as a percent of the 0 mM control after seven days of treatment. (Symbols represent means, n = 4 - 6, with 1 to 6 new roots produced per plant.)

produced 40.5% as much new root at 5 mM Al-EDTA as at 0 mM after seven days of treatment. In contrast, HαAT29 produces ca. 29.7 and HαAT3 produces 14.4%.

IV. DISCUSSION

Characterization of the three mutant lines indicated enhanced tolerance to the selection conditions relative to the wild type. Variable responses were observed over the range of Al-EDTA treatments and relative tolerance levels were not assigned.

The basis of tolerance in the gametophytes is not related to obvious morphological differences. Two mutants, HαAT3 and HαAT29, demonstrate the wild type morphology. One mutant, HαAT7, has fewer rhizoids (Figure 4, p. 26) and does not produce a primary rhizoid at germination. The lack of the primary rhizoid and lower absolute number of rhizoids could be presumed to be providing tolerance only if rhizoids had specific uptake for aluminum. If rhizoid uptake was not specific for aluminum, the mutant would be expected to have a reduced growth compared to the wild type, but it consistently grew larger than the wild type. Also, it is doubtful that the rhizoid has much importance in nutrient uptake in *Ceratopteris richardii* relative to the remainder of the gametophyte surface.

The original selection medium was more complex than standard culture media. Therefore, tolerance testing of individual components was considered important to determine if the mutants were tolerant to aluminum and/or some other condition present in the selection medium. Component tolerance testing is seldom reported in the literature. It is doubtful if it is even performed in the whole plant studies, because most screenings of agricultural crops are done in soil, and soil is an inherently complex medium with various physical, chemical and biological properties.

Additional sulfate was present in the selection medium, because the Al-EDTA was made using aluminum sulfate. The effect of the additional sulfate was tested by measuring the growth of the wild type on media supplemented with sodium sulfate. The sodium sulfate was added so that the sulfate at the highest treatment level was equal to the amount added in the selection media. The growth response of the wild type varied with the treatment level (Figure 5, p. 28), but even at its lowest level of growth it did not show the same response as in the original screening. A large part of the response with sodium sulfate was probably due to the sodium, because the total amount of sulfur/liter of medium was 0.88 mM. Murashige and Skoog (1962) reported elemental concentrations of nine different plant tissue culture media, and only two had sulfur concentrations below 0.88 mM. The mutants' response to sulfate was not tested because the tolerance they expressed in the original screening was not due soley to the sulfate.

The importance of acidity on plant growth, through its relationship to nutrient availability, is recognized by researchers (Blamey et al., 1987; Jackson, 1967) but seldom examined in aluminum studies. Islam et al. (1980) studied pH responses in six species, but Ryan et al. (1986a,b) presented the only example of acid tolerance testing known to this author. Because the original screening was performed at pH 4.5, a suboptimal pH for growth of the wild type, a dose response over a range of pH was performed to determine if the mutants were tolerant to low pH. All mutants showed a lower pH optimum for growth than the wild type, and appeared to be less effected by additional increases in the acidity of the media. Because aluminum solubility increases

significantly with acidity, this may indicate that the mutants are also tolerant to aluminum. However, this can not be directly tested. Bilski and Foy (1987) investigated differential tolerance of oat cultivars to aluminum nutrient solutions. The tolerant cultivars were also grown in pots and field plots of soil over a pH range of 3.8 to 5.5. Aluminum saturation in the soil was not stated. They determined that the order of tolerance to aluminum in nutrient solution agreed with the order of tolerance to acid soil.

EDTA tolerance testing was done because of its increased concentration in the selection media relative to the basal medium. The effect of EDTA, and its use in plant nutrient studies, has been discussed in several studies (Bartlett et al., 1972, Decock and Mitchell, 1957, Hern et al., 1988, Martell, 1957, and Wallace et al., 1957). Conner and Meredith (1985c) state that Al-EDTA should never be used in aluminum studies because the effects of the aluminum are difficult to separate from the effects of the EDTA. However, by doing the proper testing these effects may be separated. Also, chelating agents exist in the soil, and the use of these compounds in aluminum research has been studied (Arp and Ouimet, 1986, and Muchovej et al., 1988). The better absolute growth of the mutants at the 0 µM treatment (Figure 7, p. 30) may be related to the mutants' tolerance to low pH. The wild type had an increase in growth at 100 µM relative to the control and the mutants (Figure 8, p. 31). This can be explained by the fact that the pH of the growth medium increased with increasing treatment levels (e.g., the pH of the 0 µM treatment was 4.31, and the pH of the 100 µM treatment was 4.57). The mutants would not be

expected to respond as greatly to a pH stress reduction and above 100 μM the amelioration of pH stress can not offset the effect of the EDTA. HαAT7 is not tolerant to EDTA and its larger size (Figure 7, p. 30) is due to absolute growth differences. HαAT3 and HαAT29 are EDTA tolerant at 150 μM and express slight tolerance relative to the wild type above 200 μM EDTA.

The growth response of gametophytes from F1 hybrids was analyzed relative to the wild type and the proper parental stock to determine the genetic basis of tolerance. Segregation analysis of HαAT3 x Hn-n reciprocal crosses showed segregation for two response types. This suggests that tolerance is controlled by a single gene, with one response representing the wild type allele and the other representing the mutant allele response. Reciprocal crosses exhibited similar segregation patterns, indicating a nuclear gene basis of tolerance.

Analysis of HαAT7 x Hn-n reciprocal crosses also showed segregation for two response types, although a significant overlap in the ranges of the parents prevented chi-square analysis. The reciprocal crosses showed a similar pattern of segregation, indicating a nuclear gene basis of tolerance. Because of the significant overlap in the ranges of Hn-n and HαAT7, tests were done to ensure that the presumed HαAT7 homozygote was not heterozygous and segregating for both sensitive and tolerant types. M3 generation gametophytes derived from the most highly tolerant M2 gametophytes showed responses similar to the pattern expressed in the M2 generation. Therefore, the HαAT7 strain was in fact homozygous. The broad range of responses and the overlap into the wild type response range is likely associated epigenetic factors.

Segregation analysis was attempted for HαAT29 at five treatment levels. One preliminary test showed similar behavior for the reciprocal crosses, indicating a nuclear basis of tolerance. A class exhibiting more tolerance than the wild type also appeared to be segregating from the hybrids. Further tests were performed to determine if the clear resolution of this more tolerant class could be accomplished at a higher or lower level of treatment. No level was obtained that provided this resolution, although the presence of a more tolerant class was suggested in all of the tests. A two gene hypothesis could explain this behavior, if one mutation in HαAT29 was inhibitory to full expression of tolerance associated with another mutation (Figure 20, p. 46). No determination of how many genes are associated with the response of HαAT29 could be derived from the segregation data. The five visually largest gametophytes from two treatment levels were isolated, and sporophytes generated. These may be useful in further studies to help clarify the genetic basis of tolerance.

In nature, multiple genetic changes may be necessary for enhanced levels of aluminum tolerance to be observed. All mutants investigated in this study showed only low to moderate levels of tolerance relative to the wild type in the gametophyte stage. Because, the *Ceratopteris* system is sensitive when comparisons of gametophyte growth are made, it is possible that the levels of tolerance observed in this study would not have been detected in whole plant or tissue culture approaches. Further characterization of the mutants' method of tolerance to low pH may prove useful and, the EDTA tolerance of HαAT3 and HαAT29 could indicate a greater nutrient use efficiency.

Sporophyte characterization was begun to determine if the mutations are dominant or recessive. If more than one mutant had a recessive mutation, complementation testing could be performed. Root regeneration was used as an indicator of aluminum tolerance. Pagnelli et al. (1987) has also used root regeneration as an indicator of aluminum toxicity. In contrast to expected results, the wild type produced more new roots as a function of the 0 μM control than the mutants (Figure 21, p. 48). It is possible that the mutation controlling tolerance is not expressed in the sporophyte. Alternatively it is possible that other mutations related to the more complex development of the sporophyte are present and not expressed in the gametophyte.

Poole et al. (1978) used a qualitative method involving hematoxylin staining to evaluate aluminum tolerance in wheat seedlings. Seedlings were grown in nutrient solution and treated with aluminum chloride. The roots of the seedlings were then stained with hematoxylin and the the degree of stain taken up by the roots observed. Poole et al. (1978) stated that the degree of aluminum tolerance based on hematoxylin staining correlated with aluminum tolerance based on root elongation or field trials. The more tolerant cultivars took up less stain than sensitive cultivars at a given treatment level. This may also prove useful in characterization of the mutant sporophytes obtained in this study, but has not been attempted to date.

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