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An in vitro whole plant selection system: paraquat tolerant mutants in the fern *Ceratopteris*

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Summary. A whole plant selection system using the haploid gametophyte generation of the fern *Ceratopteris richardii* has been developed to select for mutations that confer resistance or tolerance to various selection pressures. The expression of the mutations can be analyzed and characterized in both the haploid gametophyte and diploid sporophyte generations. Genetic analyses are facilitated by the fern's rapid life cycle and the ease of manipulating the gametophyte generation. Selection for tolerance to the herbicide paraquat has yielded two mutants which have an increased tolerance to the herbicide in both the gametophyte and sporophyte generations. Both mutants exhibit single nuclear gene inheritance patterns and appear to be closely linked or allelic.

Key words: *Ceratopteris richardii* – Fern – Herbicide tolerant – In vitro selection – Paraquat

Introduction

Cell suspension and protoplast cultures of higher plants have been effectively used to select for specific traits such as herbicide resistance (Chaleff and Ray 1984).

However, the usefulness of plant cell cultures is often restricted by several factors, including: 1) the inability to regenerate adequate numbers of plants from selected cell lines; 2) the genetic and chromosomal instabilities associated with plant cell culture; 3) the limitations imposed by restricting selection to undifferentiated and nonphotosynthetic single cells; and 4) the limited application of cell culture selection techniques to plant varieties that can be maintained in the proper developmental mode for a prolonged period of time (Chaleff 1983; Meredith and Carlson 1982; Negrutiu et al. 1984). Additional approaches would greatly expand the capabilities of in vitro selection. Because current technology is circumventing natural barriers to gene exchange between species, other organisms (e.g., bacteria, yeast, algae) are a source of agriculturally useful genes (Barton and Brill 1983; Drummond 1983). Although microbial systems can provide more efficient selection systems than higher plant cell cultures, the usefulness of these organisms is limited by their genetic distance from higher plants. Furthermore, the expression of a selected microbial gene in a higher plant system cannot be assessed until after isolation and characterization of the gene, transformation of cell cultures, and subsequent regeneration of plants and their growth under greenhouse or field conditions. These processes involve a large commitment of time and resources.

By contrast, the fern *Ceratopteris* provides an alternative system that combines the use of whole plants with the ability to efficiently and rapidly screen large numbers of haploid individuals. Recent research with this fern has demonstrated that the gametophyte phase can be effectively utilized to screen for recessive or dominant mutations conferring resistance to growth regulators and other chemical agents (Hickok 1985 a, b). This system provides the ability to quickly and directly assess the effects of the resistance traits in morphologically differentiated gametophytes and subsequently in homozygous vascular sporophytes.

Ceratopteris reproduces sexually through the meiotic production of single-celled haploid spores. Upon germination, spores differentiate into photosynthetic haploid gametophytes that have the potential to develop into cordate bisexual individuals or into smaller spatulate males (Hickok 1983). Sexual differentiation is hormonally controlled. Through self-fertilization, cordate gametophytes produce completely homozygous diploid sporophytes. Mature spore-bearing plants can be produced within 12–16 weeks of spore sowing. These features of the life cycle provide several advantages for the purpose of mutant induction, selection, and characterization. Spores can be conveniently

mutagenized and cultured axenically on petri plates containing mineral nutrient medium. Gametophytes, which are initially microscopic, develop into mature gametophytes of 1–2 mm diameter within 3 weeks of sowing. When sown on a medium containing appropriate concentrations of a selection agent, most gametophytes fail to develop normally (Hickok 1985 a, b). The few gametophytes with mutations that confer resistance, however, exhibit normal or near-normal levels of growth and can be seen within 2–3 weeks as macroscopic cordate gametophytes. Each gametophyte, in essence, represents a "colony" of cells that has been derived mitotically from a single mutant spore. Cultures containing over a million individuals can be maintained in an area of less than 1.0 m^2. The entire screening process requires less than 3 weeks. This system provides a rapid and powerful means of screening large numbers of photosynthetic, haploid plants for both dominant and recessive mutations. All selections (putative mutants) can be immediately selfed to produce homozygous diploid plants that provide an unlimited supply of genetically identical spores carrying the selected mutation. Subsequent confirmation and characterization of the putative mutants can be carried out in both the gametophyte and sporophyte generations. To illustrate the utility of this selection system, we describe here the use of *Ceratopteris* to isolate and characterize mutants tolerant to the herbicide paraquat.

Materials and methods

Paraquat-tolerant mutants were selected by culturing spores of a homozygous diploid stock of *Ceratopteris richardii* (strain Hn-n) on nutrient medium (Hickok 1983) containing 0.5 μM paraquat, which was filter sterilized and added after autoclaving the medium. Wild-type spores, which are highly sensitive to paraquat, germinate but fail to grow at this concentration and subsequently die. Both mutagenized and nonmutagenized spores were utilized. All spores were soaked for 24 h in distilled water and surface sterilized (Hickok 1978). Spores were mutagenized by exposure to X-irradiation (400 R/min, 28 min). A total of approximately 6.25×10^6 mutagenized spores (5.0 g) and ca. 3.75×10^6 nonmutagenized spores (3.0 g) were screened by sowing them axenically on the selection medium and monitoring gametophyte development. Sowing density was approximately 1.25×10^3 spores/plate. All cultures were maintained under continuous illumination (6.2 $W \cdot m^{-2}$) at 25° ± 2 °C.

Putative mutants were identified as macroscopic green cordate gametophytes of various sizes and were isolated into individual culture dishes containing standard medium without paraquat. Homozygous M1 sporophytes were obtained by adding sterile water to the isolate cultures in order to release swimming spermatozoids to allow self fertilizations of the putative mutant gametophytes (Hickok 1983). These sporophytes were subsequently transferred to greenhouse culture. Certain M1 sporophytes (see below) were vegetatively cloned by surface sterilizing small plantlets that are present on the leaves of mature sporophytes and culturing them on standard medium without paraquat. Such cultures were used as sources of vegetative leaf material for characterizing the mutants and as a means to generate additional greenhouse material for the production of M2 generation spores and gametophytes.

Confirmation and characterization of the mutants were accomplished by comparative studies with the wild-type. Tolerance was assayed in the M1 sporophyte and M2 gametophyte generations by measuring chlorophyll retention in response to exposure to paraquat. Each sporophyte sample consisted of all of the leaves from a 4–5 week-old sporophyte clone grown axenically on standard mineral nutrient medium. Gametophyte samples (0.1 g fr.wt., approximately 100 individuals) were taken from populations of 23-day-old gametophyte cultures. Both sporophyte and gametophyte samples were floated on aqueous solutions of paraquat and maintained at 25 °C under fluorescent lights at 8.6 and 6.2 $W \cdot m^{-2}$, respectively. At the end of each experiment, chlorophyll content was determined spectrophotometrically and expressed as mg chlorophyll/g fr.wt. (Bruinsma 1963). In addition, growth assays for paraquat tolerance were carried out in the M2 gametophyte generation. Gametophytes were grown for 15 or 19 days on basal medium and paraquat-supplemented medium. Gametophytes sampled from each experiment were stained and mounted on slides using a 2 : 1 mixture of Hoyer's medium and 0.5% acetocarmine. Areas of the gametophytes, which are essentially two dimensional, were determined with a "Bioquant" (TM) image analysis system.

Results

No mutants were obtained from the approximately 3.75×10^6 nonmutagenized spores. The approximately 6.25×10^6 irradiated spores yielded a total of 138 putative mutants (M1 generation), which were evident after 3 weeks' culture. Nineteen of the selections have been tested in the M2 gametophyte generation for expression of paraquat tolerance. Seventeen of these have been confirmed as stable mutants in that tolerance was maintained after passage through an entire sexual cycle. Two of the confirmed mutants (strains HαPQ2 and HαPQ45) have been subjected to further characterization and analysis in comparative studies with the wild-type. Quantitative comparisons between the mutant and wild-type strains were made by assaying chlorophyll loss in response to exposures to aqueous solutions of paraquat in both the M1 sporophyte and M2 gametophyte generations (Fig. 1). In addition, growth assays were conducted with wild-type and M2 generation gametophytes of the two mutants (Fig. 2 A). Figures 1 A–D and 2 A clearly show increased paraquat tolerance in the mutants compared to the wild type. Distinctions between the mutants were not consistent, although some indications that HαPQ45 is somewhat more tolerant than HαPQ2 are evident (Fig. 1 B, C). To further characterize the mutants, 40 gametophytes of each strain were cultured on 2.5 μM paraquat and measured after 15 days (Fig. 2 B). This analysis indicated a clear difference between the mutants.

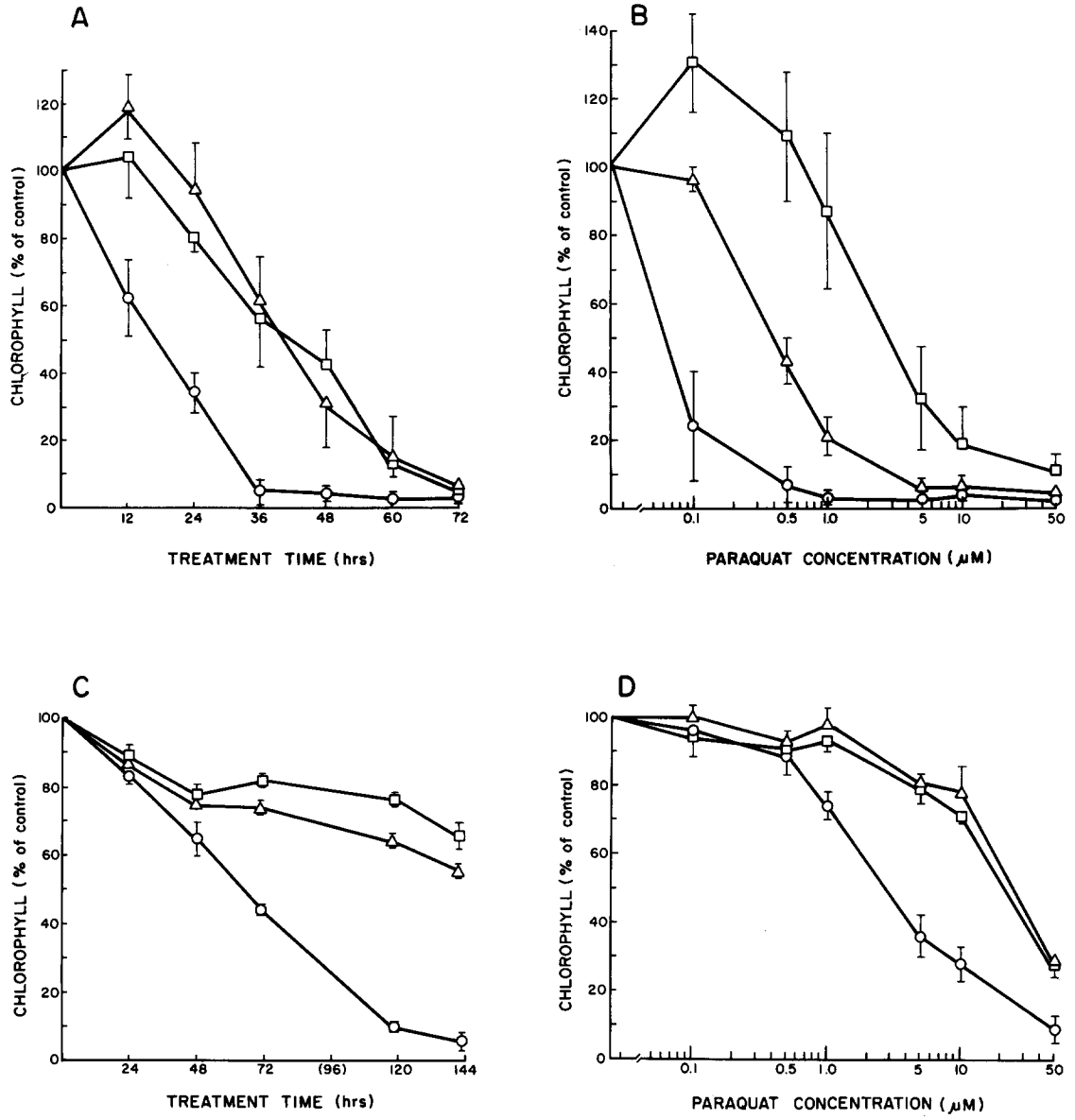

Fig. 1A–D. Chlorophyll retention bioassays for comparisons of paraquat tolerance in wild-type and mutant sporophytes and gametophytes. Values represent the mean ± SD for three sporophyte replicates and two gametophyte replicates. Comparisons of wild-type and mutant responses were made using least significant differences based on analysis of variance. Wild-type ○; HαPQ45 □, HαPQ2 △. **A** Chlorophyll retention over time in sporophyte leaves exposed to 0.75 μM paraquat. The mutants are clearly distinguished from the wild-type ($P < 0.01$) but not from each other ($P > 0.05$). **B** Chlorophyll loss in sporophyte leaves floated on paraquat solutions for 48 h. All three strains are clearly distinguished ($P < 0.05$). **C** Chlorophyll retention over time in gametophytes exposed to 2.5 μM paraquat. All three strains are clearly distinguished ($P < 0.05$). **D** Chlorophyll loss in gametophytes floated on paraquat solutions for 72 h. The mutants are clearly distinguished from the wild-type ($P < 0.01$) but not from each other ($P > 0.05$)

Initial genetic characterizations of the mutants show single nuclear gene patterns of inheritance for each strain. This is evident in the data shown in Table 1 in which segregations closely approximating 1 : 1 ratios were obtained from all hybrid combinations, including reciprocal crosses for all except the HαPQ45 × Hn combination. The lack of wild type or double mutant segregates from the crosses between the two mutant strains (HαPQ2 × HαPQ45 and the reciprocal) indicates that the two mutations are either closely linked or allelic. Additional tests to determine expression in heterozygous sporophytes have shown that both mutants are recessive and functionally allelic (Hickok and Schwarz, unpublished).

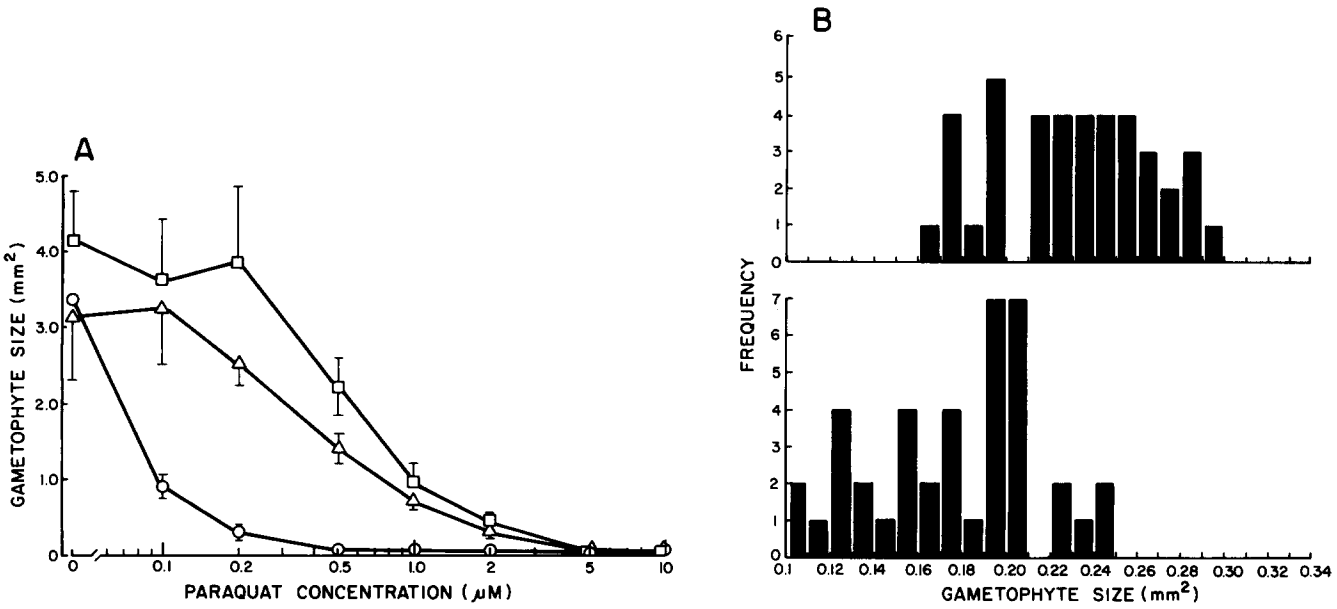

Fig. 2A, B. Growth assays for paraquat-tolerance as expressed in M2 gametophytes. **A** Gametophyte growth as a function of paraquat concentration. Populations of gametophytes were grown for 19 days on culture medium containing various concentrations of paraquat. Areas of 10 randomly selected cordate gametophytes were determined as described in the text. Values represent the mean ± SD. Both mutants are clearly different from the wild-type and from each other ($P < 0.01$). **B** Comparison of the two mutants. Gametophytes of each of the mutant strains were cultured for 15 days on medium containing 2.5 μM paraquat. The wild-type did not grow under these conditions. Areas of 41 cordate gametophytes from each mutant strain were measured as above. Because of asynchronous development within the cultures, a range in size is evident for each mutant. The mutants are highly significantly different ($P < 0.01$), indicating that HαPQ45 (*top*) is more tolerant to paraquat than HαPQ2

Table 1. Segregation of paraquat-tolerant and sensitive gametophyte types from hybrid sporophytes[a]

Hybrid combination	Segregation[b]			
	Wild-type	HαPQ2-type	HαPQ45-type	Ratio
Hn × HαPQ2	103	97	–	1:1 ($P > 0.8$)
HαPQ2 × Hn	98	102	–	1:1 ($P > 0.8$)
HαPQ45 × Hn	107	–	93	1:1 ($P > 0.2$)
HαPQ2 × HαPQ45	–	253	247	1:1 ($P > 0.8$)
HαPQ45 × HαPQ2	–	235	264	1:1 ($P > 0.5$)

[a] Spores were sown onto plain medium and immature gametophytes were subsequently transferred to medium containing 0.75 μM paraquat and cultured as isolates

[b] Wild-type segregates were evident as small gametophytes that died after approximately 1 week in culture. HαPQ2-types were evident as medium sized gametophytes that turned pale green and died only after extended culture (> 45 days). HαPQ45-types were larger than HαPQ2-types and remained green and viable in extended culture. Probabilities were determined from chi-square analysis

Discussion

Paraquat tolerant forms of weeds have been found under field conditions in certain strains of perennial ryegrass (*Lolium perenne*) (Faulkner 1974, 1982) and *Conyza linifolia* (Youngman and Dodge 1981). In cell cultures, paraquat-resistant calli have been isolated from tobacco, soybean and tomato selections (Miller and Hughes 1980; Hughes 1979; Thomas and Pratt 1982). Soybean studies were limited to callus cultures, but whole plants were regenerated in some of the tobacco and tomato lines. Most regenerated tobacco plants showed no resistance, although callus cultures from these plants generally exhibited either partial or complete resistance. Further studies have shown that the trait is sexually transmitted (Hughes et al. 1984). Tolerance in the tomato selections was also generally limited to the original or regenerated calli. One regenerated plant exhibited slight tolerance. Genetic studies showed the tolerance to be associated with an

undetermined number of dominant nuclear mutations (Thomas and Pratt 1982).

The rapid effects of paraquat and the availability of substantial information concerning its mode of action and possible defense mechanisms make it an attractive experimental system (Fridovich and Hassan 1979; Youngman and Dodge 1981; Lewinsohn and Gressel 1984; Fuerst et al. 1985; Rabinowitch and Fridovich 1985; Vaughn and Fuerst 1985). Furthermore, because paraquat toxicity is associated with the production of superoxide free radicals, the selection and characterization of paraquat-tolerant mutants may provide important information on the cellular mechanisms associated with the mediation of oxygen toxicity in aerobic organisms (Fridovich and Hassan 1979). The successful isolation and characterization of single-gene nuclear mutations for paraquat tolerance in *Ceratopteris* illustrates the ease and efficiency of this selection system. At least two mutations have been recovered in which the phenotypes can be clearly distinguished from each other (Figs. 1 B and 2 B). The ability to characterize the mutations in sporophytes allows a direct assessment of the expression of the trait in a vascular plant.

In addition, the ability to monitor the responses of gametophytes in laboratory cultures allows precise control of experimental conditions. Such control is essential for studies of the basis of the resistance, which are currently in progress.

The *Ceratopteris* selection system has also been successfully employed to select for resistance to a number of other compounds (e.g., abscisic acid, glyphosate, 5-flurodeoxyuridine, 2-aminoethyl-L-cysteine, sodium chloride, L-azetidine-2-carboxylate, hydroxyproline; Hickok 1985 a, b and unpublished). This success with a diverse group of selection agents illustrates the broad applicability of the system. The selection system can complement cell culture and microbial approaches in that whole plants are utilized and the trait is expressed in photosynthetic vascular plants. At the same time, mutagenesis and selection are carried out on single celled haploid spores in numbers approaching microbial or cell culture system capabilities. In addition, although ferns are vascular plants with many of the same morphological and biochemical features of flowering plants, they do represent a distinct phylogenetic line and provide opportunities for the expansion of germplasm resources that may be of benefit in the genetic improvement of crops.

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