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Genetic characterization of a mutation that enhances paraquat tolerance in the fern *Ceratopteris richardii*

L. G. Hickok and O. J. Schwarz
Department of Botany, The University of Tennessee, Knoxville, TN 37996-1100, USA

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Summary. Three nuclear mutations that affect tolerance to the herbicide paraquat have been selected in the fern *Ceratopteris richardii*. Two of the mutations, *pq2* and *pq45* are allelic and confer low and moderate tolerance, respectively. A third mutation, *pqa6*, is not linked to the other two and significantly enhances the level of tolerance when in combination with either *pq2* or *pq45*. The *pqa6* mutation does not independently confer tolerance in the absence of the other mutations.

Key words: *Ceratopteris richardii* – Fern – Herbicide-tolerant – Paraquat – Selection

Introduction

The gametophyte generation of the fern *Ceratopteris richardii* is useful in a wide variety of studies involving the selection and characterization of mutants with altered responses to natural and synthetic chemical agents (Hickok et al. 1987). Hickok and Schwarz (1986a) described the selection and characterization of two recessive nuclear mutations, *pq2* and *pq45*, that confer tolerance to the bipyridinium herbicide, paraquat. Subsequently, these mutations were shown to be allelic, with *pq2* conferring a low level of tolerance and *pq45* conferring a moderate level of tolerance (Hickok and Schwarz 1986b). Relative tolerance levels were assayed by the effects of paraquat on growth in the gametophyte generation and on chlorophyll loss in both gametophytes and sporophytes.

Attempts were also made to enhance the level of paraquat tolerance by mutagenizing spores containing the *pq45* mutation and selecting for strains exhibiting higher levels of tolerance. One such strain, HaPQ45a-6, was shown to have significantly enhanced tolerance as well as more vigorous growth in the absence of the herbicide (Hickok and Schwarz 1986b). This report further documents the enhanced tolerance and characterizes the HaPQ45a-b strain genetically, by analysis of hybrid segregation patterns.

Materials and methods

The derivations of the strains used in this study have been previously described (Hickok and Schwarz 1986a, b). General culture methods and procedures are described in Hickok et al. (1987). All cultures and assays reported here were carried out at a constant thermo-photoperiod of 27 ± 2°C and 90 ± 7 μmol s^{-1} m^{-2} photon flux density (PAR).

Gametophyte cultures for all experiments were established by axenically sowing spores obtained from homozygous or hybrid sporophytes onto agar-solidified minimal medium and, upon germination (emergence of the primary rhizoid), isolating the gametophytes in individual wells of 24-well culture plates containing medium supplemented with 1.5 μ*M* paraquat. Generally, isolations were carried out over a 48 h period. Slides of stained gametophytes were prepared as previously described (Hickok and Schwarz 1986b) 21 days following the transfer to paraquat-supplemented medium. Measurements of gametophyte area were taken from the prepared slides under magnification using the Bioquant IV image analysis system.

Comparison of paraquat tolerance in 27–34-day-old clones of homozygous and hybrid sporophyte material were made by measuring chlorophyll retention in leaves following their exposure to 0.5 μ*M* paraquat in one-half-strength liquid nutrient medium for 42 h. Procedures for extraction and chlorophyll determinations were as described previously (Hickok and Schwarz 1986b).

Results

Growth responses on 1.5 μ*M* paraquat of gametophytes containing either the wild type (sensitive), *pq2* (low toler-

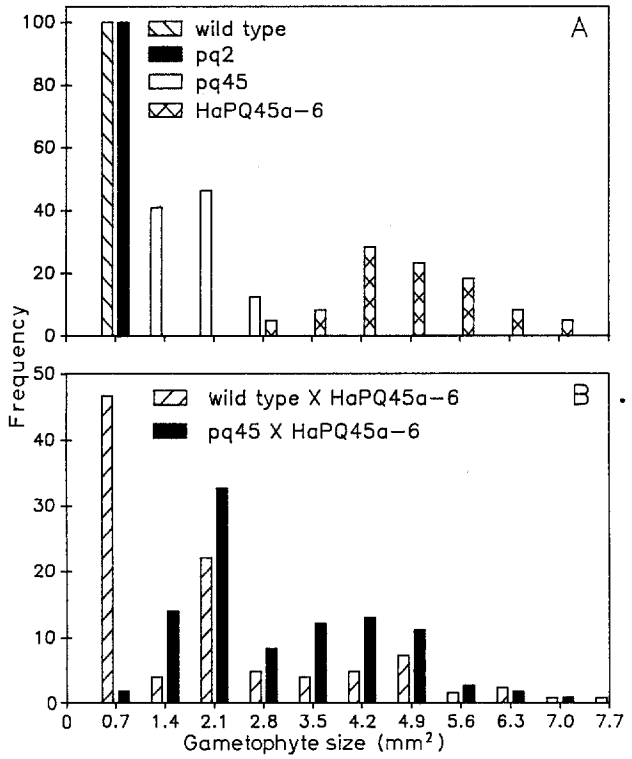

Fig. 1. **A** Comparison of gametophyte growth on 1.5 μ*M* paraquat of the wild type (N = 48) and mutant strains *pq2* (N = 37), *pq45* (N = 56) and HaPQ45a-6 (N = 60). **B** Segregation patterns from crosses of HaPQ45a-6 with the wild type (N = 126) and with *pq45* (N = 116)

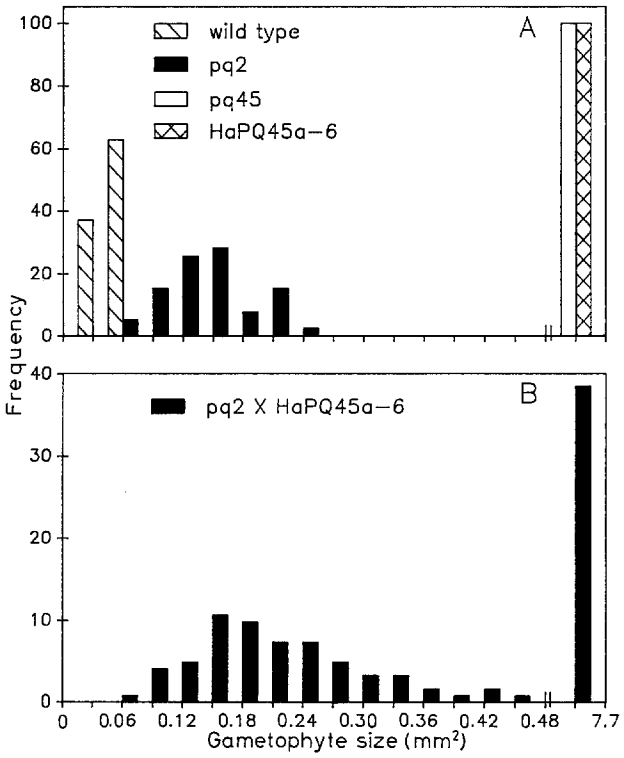

Fig. 2. **A** Comparison of gametophyte growth on 1.5 μ*M* paraquat of the wild type (N = 35) and *pq2* (N = 39). Strains *pq45* (N = 62) and HaPQ45a-6 (N = 62) are combined in one large (> 0.48 mm²) size category. **B** Segregation pattern from a cross of HaPQ45a-6 with strain *pq2* (N = 122). Note scale difference from Fig. 1

ance), or *pq45* (moderate tolerance) allele are compared to gametophytes from the homozygous HaPQ45a-6 strain (high tolerance) in Fig. 1 A. With the exception of an overlap in the 2.8 mm² category, gametophytes from the HaPQ45a-6 strain can be readily distinguished from gametophytes with the *pq45* mutation. In addition, *pq45* genotypes are clearly distinguished from *pq2* and wild-type genotypes. Although Fig. 1 A does not quantitatively distinguish between *pq2* and the wild type, these genotypes can be distinguished at lower concentrations of paraquat or when the data are plotted at a higher level of resolution (Fig. 2 A and Hickok and Schwarz 1986 b). Visual distinction of all four phenotypes is also possible. On 1.5 μ*M* paraquat, the wild type dies after only a few cell divisions and *pq2* grows until the attainment of a cordate morphology (Hickok et al. 1987) and then dies. Although both *pq45* and HaPQ45a-6 gametophytes remain viable when cultured on 1.5 μ*M* paraquat, *pq45* gametophytes die after extended culture (> 30 days) and can be readily distinguished from HaPQ45a-6 types.

Segregation patterns from the F1 hybrids involving the HaPQ45a-6 strain as the male parent were determined from both visual and quantitative assessments. Visual determinations of gametophytes from the F1 [wild type × HaPQ45a-6] hybrid sporophyte showed segregation of 58 sensitive, 39 moderate tolerance, and 29 high tolerance types, approximating a 2:1:1 ratio ($X^2 = 2.39$, $P > 0.20$). Visual determinations of gametophytes from the F1 [*pq45* × HaPQ45a-6] hybrid sporophyte showed segregation of 64 moderate tolerance and 52 high tolerance types, approximating a 1:1 ratio ($X^2 = 1.04$, $P > 0.20$). Quantitative comparisons (Fig. 1 B) also suggest these respective segregation ratios, although statistical analysis was not done because of an overlap in the 2.8 mm² class in both Figs. 1 A and 1 B.

The segregation patterns evident in Fig. 1 B fit a model involving two unlinked genes: *pq45* as previously defined and a new mutation, *pqa6*. In this model, gametophytes of the homozygous strain HaPQ45a-6 contain both mutations, *pq45 pqa6*, while gametophytes of the wild-type and *pq45* strain are, respectively, + + and *pq45* +. Therefore, the F1[wild type × HaPQ45a-6] hybrid sporophyte (+/*pq45* +/*pqa6*) segregates as follows: 1 + + : 1 + *pqa6* : 1 *pq45* + : 1 *pq45 pqa6*. However, the + + and + *pqa6* classes appear to be indistinguishable at 1.5 μ*M* paraquat, resulting in a 2:1:1 phenotypic ratio. Because the *pqa6* mutation apparently shows no tolerance when present in a wild-type background, the en-

hanced tolerance observed in the HaPQ45a-6 strain most likely results from the combination of *pq45* and *pqa6*. The F1[*pq45* × HaPQ45a-6] hybrid sporophyte (*pq45/pq45* +/*pqa6*) segregates in a 1:1 ratio of *pq45* + :*pq45 pqa6*.

To determine the effect of the *pqa6* mutation when in combination with *pq2*, which is allelic to *pq45*, a separate experiment was performed and comparisons of the wild type and mutants were made (Fig. 2A, B). Figure 2A shows quantitatively the difference between the wild type and gametophytes carrying the *pq2* mutation. At this level of resolution, *pq45* and HaPQ45a-6 were grouped into a single large size class (>0.48 mm^2, note scale break). Figure 2B shows the segregation from an F1[*pq2* × HaPQ45a-6] hybrid. Two size categories are evident: 0.06–0.45 mm^2 (75 individuals) and >0.48 mm^2 (47 individuals).

Based on the model generated from data shown in Fig. 1B, it would be expected that the F1[*pq2*+ × *pq45 pqa6*] hybrid sporophyte (*pq2*/*pq45* + / *pqa6*) should segregate as follows: 1*pq2*+ : 1 *pq2 pqa6* : 1 *pq45*+ : 1 *pq45 pqa6*. The *pq2*+ and *pq2 pqa6* classes cannot be clearly distinguished in Fig. 2B. However, the extreme skewness and extended upper limit of the smaller class segregating from the hybrid, as compared to the distinct *pq2*+ class in Fig. 2A, suggests that both classes are present. This indicates that the combination of *pqa6* with *pq2* enhances tolerance in comparison to *pq2* by itself, but that the level of enhancement does not permit clear distinction between the two types. The grouping of these two classes (N = 75) along with the grouping of *pq45*+ and *pq45 pqa6* (N = 47) approach the value expected for a 1:1 segregation. However, the deviation from expected is significant at the 5% level ($X^2 = 5.98$, $P < 0.05$). This deviation is probably related to different rates of germination associated with low versus high tolerance types, which could lead to sampling error during the isolation of individual spores from the F1 hybrid. For instance, in a related experiment, the segregation of tolerant to sensitive types ranged from 41 : 31 for early isolations to 27 : 45 for isolations made 12 h later. Other independent experiments (Table 1) involving similar hybrid combinations have consistently shown an overall 1:1 segregation of tolerant and sensitive types.

Because the above experiments did not distinguish between the wild-type and +*pqa6* individuals, additional segregation analyses of the F1[*pq2* × HaPQ45a-6] hybrid were carried out using lower (0.2 and 0.05 μ*M*) concentrations of paraquat. These tests also failed to distinguish either type (data not shown). This further suggests that the *pqa6* mutation alone confers no tolerance to paraquat, even at relatively low concentrations.

A further test of the genetic model was made by sowing spores from the F1[wild type × HaPQ45a-6] hybrid sporophyte (+/*pq45* +/*pqa6*) on medium containing 0.5 μ*M* paraquat in order to distinguish sensitive and tolerant types. Sensitive types, which would be either + + or +*pqa6* according to the model, were transferred to medium without paraquat, allowed to recover and backcrossed as females with strain HaPQ45a-6 gametophytes (*pq45 pqa6*). Thus, two hybrid combinations were possible, [+ + × *pq45 pqa6*] and [+*pqa6* × *pq45 pqa6*]. These backcrosses should be distinguishable on the basis of their segregation behavior; i.e., 2 sensitive (+ + and +*pqa6*) : 1 moderate tolerance (*pq45*+) : 1 high tolerance (*pq45 pqa6*) or 1 sensitive (+*pqa6*) : 1 high tolerance (*pq45 pqa6*), respectively. As shown in Table 1, both types of hybrids were recovered from the crosses. This supports the prediction that two genotypically distinct sensitive gametophyte types (+ + and +*pqa6*) segregated from the F1[wild type × HaPQ45a-6] hybrid sporophyte.

The *pqa6* mutation was subsequently isolated as a pure paraquat sensitive line by selecting sensitive gametophytes segregating from the [+*pqa6* × *pq45 pqa6*] hybrid, transferring them as isolates to medium without paraquat, and self-fertilizing them to produce homozygous sporophytes (+/+ *pqa6*/*pqa6*). In addition, some of the sensitive gametophytes from this hybrid were crossed as females with *pq45* gametophytes as males. Segregation analysis of this hybrid combination, [+*pqa6* × *pq45*+], indicated a 2:1:1 ratio for sensitive (+ + and +*pqa6*), moderate (*pq45*+), and high (*pq45 pqa6*) tolerance types, as predicted by the model ($X^2 = 1.085$, $P > 0.05$).

Comparison of tolerance levels in diploid homozygous and hybrid sporophytes are shown in Table 2. The data are in agreement with the previous demonstration of the recessive nature of the *pq2* and *pq45* mutations (Hickok and Schwarz 1986b). The resolution of the sporophyte assays is considerably less than the assay of growth responses in gametophytes (Hickok and Schwarz

Table 1. Segregation for paraquat tolerance in backcross hybrids[1]

Hybrid combination	Sensitive (+ + or +*pqa6*)	Moderate tolerance (*pq45* +)	High tolerance (*pq45 pqa6*)
+ + × *pq45 pqa6*[2]	37	19	14
+*pqa6* × *pq45 pqa6*[3]	68	0	71

[1] Visual distinction between the segregating types (sensitive, moderate and high tolerance) was made after >21 days culture on 1.5 μ*M* paraquat and in comparison with cultures derived from the parental strains

[2] Segregation approximates a 2:1:1 ratio ($\chi^2 = 0.943$, $P > 0.5$). The model predicts that the sensitive class is composed of equal numbers of + + and +*pqa6* types, which are indistinguishable

[3] Segregation approximates a 1:1 ratio ($\chi^2 = 0.029$, $P > 0.8$). The model predicts that all sensitive types are +*pqa6* individuals

Table 2. Comparison of paraquat tolerance (chlorophyll retention) in homozygous and heterozygous diploid sporophytes. Chlorophyll values represent the mean ±SD

Sporophyte genotype			N	Chlorophyll a+b (mg/g fresh wt.)		
				Control	Treatment	% of control
Homozygotes						
1	+ / +	+ / +	5	1.61±0.08	0.19±0.07	11.8
2	*pq2* / *pq2*	+ / +	5	1.78±0.18	1.68±0.18	94.4
3	*pq45* / *pq45*	+ / +	4	1.95±0.54	1.65±0.05	84.6
4	*pq45* / *pq45*	*pqa6* / *pqa6*	5	1.69±0.30	1.73±0.11	102.4
5	+ / +	*pqa6* / *pqa6*	2	0.89±0.02	0.15±0.06	16.3
Heterozygotes						
6	*pq45* / *pq45*	+ / *pqa6*	5	1.49±0.17	1.60±0.16	107.4
7	+ / *pq45*	+ / *pqa6*	5	1.70±0.22	0.20±0.09	11.8
8	*pq45* / +	+ / *pqa6*	2	1.48±0.28	0.22±0.03	14.6

1 – wild type, 2 – *pq2*, 3 – *pq45*, 4 – HaPQ45a-6, 5 – *pqa6*, 6 – *pq45* × HaPQ45a-6, 7 – wild type × HaPQ45a-6, 8 – *pq45* × *pqa6*

1986b), e.g., compare homozygotes 2 and 3. Accordingly, no distinctions were made among the different sporophyte genotypes in the tolerant category.

Discussion

Studies of paraquat-resistant genotypes in other genera have shown a variety of inheritance patterns. Polygenic inheritance was documented in a study of an outbreeding population of *Lolium perenne* (Faulkner 1974). In tomato plants regenerated from callus cultures selected for tolerance to paraquat, inheritance was described as possibly polygenic and associated with dominant or semidominant nuclear mutations (Thomas and Pratt 1982). Single gene nuclear dominant or semidominant mutations have been described in *Conyza bonariensis* (Shaaltiel et al. 1988), *Erigeron philadelphicus* (Itoh and Miyahara 1984), and *Hordeum glaucum* (Islam and Powles 1988). In *C. bonariensis*, constitutively elevated levels of superoxide dismutase, ascorbate-peroxidase, and glutathione-reductase were inherited along with resistance, indicating probable pleiotropic control by the single dominant mutation (Shaaltiel et al. 1988).

The *pq2* and *pq45* resistant mutations in *Ceratopteris* represent the only reported examples of inheritance as a recessive trait in sporophytes. Since selection in the other examples involved diploid material, as opposed to the use of haploid gametophytes in *Ceratopteris*, the likelihood of identifying recessive mutants was low. In addition, the ability to resolve even subtle differences in tolerance by analysis of growth in gametophytes of *Ceratopteris* allowed not the identification of two allelic variants at a single locus (*pq2* and *pq45*), but also the isolation of the *pqa6* enhancer mutation. The availability of this mutation in a pure line will allow further assessment of its effects and mode of action. For instance, it should be possible to determine if another mutant line (HaB50) that exhibits cross tolerance to both paraquat and acifluorfen (Hickok et al. 1987) can be enhanced by addition of the *pqa6* mutation.

Comparative biochemical studies of the wild-type and the *pq2* and *pq45* mutants indicate that the physiological basis for tolerance does not lie in differences in the oxygen radical scavenging system, in whole plant (gametophyte) uptake, or in an inducible stress response (Carroll et al.1988). Cross-tolerance studies of *pq2* and *pq45* singly and in combination with *pqa6*, using structurally different chemicals that are known to produce various toxic oxygen species, have shown cross tolerance only to diquat, which is structurally similar to paraquat. This further suggests that the mechanism of tolerance is not associated with general tolerance to toxic oxygen species (Schwarz and Hickok, unpublished results). In addition, the possibility that the mutants sequester paraquat, either within the cell or in the cell wall, away from the site of its reduction by PSI is currently being examined.

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