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Generating Autotetraploid Sporophytes and Their Use in Analyzing Mutations Affecting Gametophyte Development in the Fern Ceratopteris

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Manuscript received April 3, 1997
Accepted for publication June 19, 1997

ABSTRACT

The haploid gametophytes of the fern *Ceratopteris richardii* are autotrophic and develop independently of the diploid sporophyte plant. While haploid genetics is useful for screening and characterizing mutations affecting gametophyte development in Ceratopteris, it is difficult to assess whether a gametophytic mutation is dominant or recessive or to determine allelism by complementation analysis in a haploid organism. This report describes how apospory can be used to produce genetically marked polyploid sporophytes whose gametophyte progeny are heterozygous for mutations affecting sex determination in the gametophyte and a known recessive mutation affecting the phenotype of both the gametophyte and sporophyte. The segregation ratios of wild-type to mutant phenotypes in the gametophyte progeny of polyploid sporophyte plants indicate that all of the mutations examined are recessive. The presence of many multivalents and few univalents in meiotic chromosome preparations of spore mother cells confirm that the sporophyte plants assayed are polyploid. The DNA content of the sperm of their progeny gametophytes was also found to be approximately twice that of sperm from wild-type haploid gametophytes.

THE life cycles of all sexually reproducing vascular plants alternate between a haploid gametophyte phase and diploid sporophyte phase of development. Although the gametophyte became more reduced in size and more dependent on the sporophyte as vascular plants evolved, the function of the gametophyte, to produce the male and female gametes, remains essential to all sexually reproducing plants (Dickinson 1994). Unlike the gametophytes of flowering plants, which are entirely surrounded and protected by maternal tissues of the flower throughout most of their development, the gametophytes of ferns are autotrophic and entirely independent of the sporophyte. All aspects of fern gametophyte growth and development, from spore germination to fertilization of the egg, can be easily observed and studied in ferns in a nondestructive way. Of the ferns, *Ceratopteris richardii* has been developed as a model genetic system for studying genes involved in gametophyte growth and development (Hickok *et al.* 1995).

The haploid spores of Ceratopteris develop as either male or hermaphroditic gametophytes. The sex of the gametophyte is determined by the pheromone antheridiogen (or A_{CE}), which is secreted by the hermaphrodite and induces male development of gametophytes that are exposed to the pheromone continuously from the time of spore germination (Banks *et al.* 1993). To identify genetic factors that are also involved in sex determination in Ceratopteris, mutations affecting the sex of the gametophyte have been isolated and characterized (Warne *et al.* 1989; Banks 1994). The epistatic interactions among these genes have been studied and used to derive models that describe how A_{CE} may affect the activities of these interacting genes in determining the sex of the gametophyte (Eberle and Banks 1996; Banks 1997). These models assume that the sex-determining mutations are the result of recessive, loss-of-function mutations. While haploid genetics has been useful for identifying such gametophytic mutations, it is impossible to assess whether a mutation is dominant or recessive or to determine allelism by complementation analysis in a haploid organism. One way to test the assumption that sex-determining mutants are recessive is to double the ploidy level of the sporophyte and its gametophyte progeny such that the ability of a wild-type gene to complement a mutant allele in the gametophyte can be determined.

Druery (1884) first reported that gametophytes could be regenerated from the diploid cells of intact sporophyte leaves of the fern *Adiantum felix-femina* without meiosis, a process termed apospory. This early finding demonstrated that gametophytes could be haploid or diploid and that ploidy level alone could not sufficiently explain the dramatic differences between the diploid sporophyte and haploid gametophyte phases of the fern life cycle. In theory, apospory can be used to generate tetraploid sporophytes of Ceratopteris, which then produce diploid gametophytic progeny. If the resulting tetraploid sporophyte is heterozygous for gametophytic mutations and meiosis is normal, the segrega-

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tion ratios of mutant and wild-type phenotypes in the diploid gametophyte progeny can be used to determine whether gametophytic mutations are dominant or recessive. This report describes how apospory can be incorporated into the life cycle of Ceratopteris to generate genetically polyploid sporophytes heterozygous for a known recessive mutation and each of four *hermaphroditic* (*her*), sex-determining mutations. The results of this study indicate that the four *her* mutations are recessive.

MATERIALS AND METHODS

The origins of the wild-type Hnn strain and the *her1, her3, her10,* and *her17* mutants of *C. richardii,* the conditions for gametophyte and sporophyte culture, and the methods for self-fertilizing and out-crossing gametophytes are described in BANKS (1994). The *clumped chloroplast2* (*cp2*) mutant was originally selected from *her1* spores mutagenized with EMS according to BANKS (1994). The *cp2* mutation causes the chloroplasts to aggregate to one corner of the cell and is similar to a *cp* allele previously isolated and described (VAUGHN *et al.* 1990). The *cp* phenotype is easily observed in individual cells of gametophytes and homozygous sporophytes. Because the *cp2* mutation is recessive in the sporophyte and visible in the gametophyte, it serves as an internal control in these experiments.

Diploid sporophyte plants heterozygous for *cp2* and each of the four *her* mutations were generated by crossing *her cp2* hermaphrodites by wild-type males. The female was used as a gamete donor of mutant alleles in these crosses to confirm hybridity of the cross. The leaves of the young sporophyte plants (genotypically *HER her/CP2 cp2* and phenotypically wild-type) that had formed no more than five leaves were removed and placed on fern medium (FM; containing 0.5× Murishege Skoog salts, pH 6.0, solidified with 0.7% washed agar). After about 1 month, gametophytic outgrowths that had regenerated from the sporophyte leaf were isolated. Although all of the putative diploid gametophytic outgrowths were hermaphrodites, it is unknown whether A_{CE} is present in cells of the sporophyte or if the aposporously derived, developing gametophytes are competent to respond to A_{CE} even if A_{CE} is present. For these reasons, the aposporously derived hermaphrodites were self-fertilized and the phenotype of their progeny gametophytes was assessed. Intra-gametophytic fertilization of aposporously derived gametophytes should result in tetraploid sporophytes that are genotypically *her her HER HER/cp2 cp2 CP2 CP2.* These sporophytes were transferred to soil and grown for 6–10 months in a greenhouse. Of the putative tetraploid plants generated in this manner, only about one fourth produced viable spores. The remaining plants were small, displayed an irregular, disorganized growth pattern, and did not survive to produce fertile sporophylls. If possible, each sporophyte was vegetatively propagated by transferring the plantlets that formed along the margins of the leaves into soil. The sexual phenotypes of the progeny of putative tetraploid sporophytes were scored after growth on FM medium containing antheridiogen (or A_{CE}) prepared as described in BANKS (1994). Because it is difficult to assess the *cp2* phenotype in male gametophytes, the *cp2* phenotype was scored from hermaphroditic members of the population.

Meiotic chromosome squashes of spore mother cells were obtained from the sporangia on unfurling fronds of greenhouse-grown plants, harvested between 1100 and 1300 hours. Tissue was prepared, stained, and mounted for cytological examination according to HICKOK (1977).

To measure the DNA content of sperm cells, sperm were harvested in liquid FM from haploid and putative diploid gametophytes then stained with propidium iodide following the protocol of ARUMUGANATHAN and EARLE (1991). Chicken red blood cells (CRBC) were used as an internal standard and added to an aliquot of sperm before staining. The fluorescence of cells (sperm and sperm plus CRBC) was measured using a Coulter Elite cytometer (Coulter Electronics, Hialeah, FL) with a 15 mW air-cooled, argon-ion laser operating at a wavelength of 488 nm and with a 550-nm dichroic filter.

RESULTS

The segregation of *cp2* and *her* alleles in the gametophyte progeny of heterozygous diploid sporophyte plants: Diploid sporophyte plants that are heterozygous for *her1, her3, her10,* and *her17* segregate hermaphrodite and male progeny in a 1:1 ratio when grown on medium containing A_{CE} (Table 1). Under the same conditions, wild-type spores develop as males (data not shown). The progeny of sporophytes heterozygous for *cp2* are male in the presence of A_{CE} and segregate *cp2* and *CP2* hermaphrodite gametophytes in a 1:1 ratio when grown in the absence of A_{CE} (Table 1). The progeny of sporophytes heterozygous for *cp2* and each of the *her* alleles segregate *CP2* hermaphrodites, *cp2* hermaphrodites, and males (either *CP2* or *cp2*) in a 1:1:2 ratio on medium containing A_{CE} (Table 1). These segregation ratios indicate that each mutation (*her1, her3, her10, her17,* and *cp2*) behaves as a single Mendelian trait and that *cp2* segregates independently of all *her* alleles.

The segregation of *cp2* and *her* alleles in the gametophyte progeny of putative heterozygous tetraploid sporophyte plants: To generate tetraploid plants that are genotypically *her her HER HER/cp2 cp2 CP2 CP2,* diploid hermaphroditic gametophytes were derived by apospory from diploid sporophyte leaves, genotypically *CP2 cp2/HER her* (see MATERIALS AND METHODS). Intra-gametophytic fertilization of these diploid gametophytes should yield a tetraploid sporophyte plant. The progeny spores of such fertile, putative tetraploids were plated on medium containing A_{CE}. If the sporophyte is diploid, the progeny gametophytes should segregate *her* hermaphrodites to *HER* males, and *her cp2* hermaphrodites to *her CP2* hermaphroditic gametophytes, in a 1:1 ratio on medium containing A_{CE}. If the sporophyte is tetraploid and the mutations are recessive, the segregation of the same should be 1:>1. The null hypothesis tested by these crosses is that the progeny of the aposporously derived plants segregate mutant to wild-type phenotypes in a 1:1 segregation ratio; *i.e.,* the parent sporophyte is diploid and produces haploid progeny. Rejection of the null hypothesis would indicate that these sporophytes do not segregate wild-type and mutant phenotypes in a ratio expected of a diploid sporophyte plant, and, therefore, are not diploid.

The segregation ratios of the phenotypes in the progeny gametophytes of the putative tetraploids are shown in Table 2. Of 33 independently derived putative tetraploid plants, the null hypothesis could not be rejected

TABLE 1

Segregation of *cp2* and *her* alleles in the haploid gametophyte progeny of diploid sporophytes

Genotype of parent sporophyte	No. of progeny			Expected ratio[a]	
	CP2 herm	*cp2* herm	Male[b]	*CP2* herm:*cp2* herm:male[b]	χ^2; *P*
HER1 her1	206	0	195	1:0:1	0.31; >0.50
HER3 her3	151	0	153	1:0:1	0.01; >0.90
HER10 her10	122	0	115	1:0:1	0.21; >0.50
HER17 her17	147	0	150	1:0:1	0.03; >0.70
CP2 cp2	53[c]	50[c]	0[c]	1:1:0[c]	0.09; >0.70
HER1 her1/CP2 cp2	108	98	195	1:1:2	0.80; >0.30
HER3 her3/CP2 cp2	133	123	237	1:1:2	1.14; >0.20
HER10 her10/CP2 cp2	26	27	55	1:1:2	0.06; >0.70
HER17 her17/CP2 cp2	94	111	195	1:1:2	1.70; >0.10

[a] The ratios are that expected for the null hypothesis of assortment from a diploid sporophyte.
[b] Gametophytes were grown on medium containing A_{CE}.
[c] Gametophytes were grown individually in medium lacking A_{CE}; in the presence of A_{CE}, all gametophytes are male.

only four times (see plants 1724-2, 1724-11, 1742-3 and 1733-1). In the remaining 29 plants, the observed ratios of *cp2 her* hermaphrodites to *CP2 her* hermaphrodites ranged from 1:2.0 to 1:9.3, while the observed ratios of *her* hermaphrodites to *HER* males in the same populations ranged from 1:2.4 to 1:6.3. In no instance did the number of gametophytes with a mutant phenotype exceed the number of gametophytes with wild-type phenotypes in these plants, which would be expected if either the *her* or *cp2* mutations were dominant. Spore viability, assessed by determining percent germination, ranged from 20 to 57% in the progeny of putative fertile autopolyploid plants; more than 70% of wild-type spores germinate (data not shown). The decrease in viability of polyploid spores may affect the segregation ratios of gametophyte phenotypes. These results indicate that most of the parent sporophytes are not diploid but polyploid, and that the *her* and *cp2* mutations are recessive.

Independent measures of ploidy level: The ploidy level of genetically polyploid sporophytes was confirmed by examining meiotic chromosomes. The chromosomes of autotetraploid plants should form multivalents (pairing of more than two homologous chromosomes) during meiosis. The chromosomes of spore mother cells, isolated from the sporangia of wild-type diploid and five plants genetically polyploid for each of the alleles, were examined. Each autotetraploid plant should have 156 chromosomes (n = 39) and form 39 tetravalents at metaphase I, whereas the diploid should form 39 bivalents. In all cases, the number of chromosomes in the polyploid sporophytes was much greater than 78, and a few univalents and many multivalents were observed in spore mother cells of the same plants undergoing meiosis (illustrated in Figure 1). Although it is not possible to resolve trivalents from tetravalents in these chromosome preparations, all of the putative tetraploids examined contained more than two sets of homologous chromosomes, although each set may not be complete due to aneuploidy.

Cells of the diploid gametophyte progeny of an autotetraploid sporophyte should have twice the DNA content of normal haploid gametophytes. The relative amount of DNA in wild-type haploid and putative diploid sperm was assessed by staining sperm samples, with and without chick red blood cells (CRBC), with propidium iodine (PI), a fluorescent DNA stain. The PI fluorescence of each sperm or cell was assessed by cell flow cytometry. As shown in Table 3, the mean PI fluorescence of the sperm of gametophytes whose sporophyte parents are autopolyploid was approximately twice that of haploid sperm obtained from wild-type (Hnn) male gametophytes. The variance of PI fluorescence in a population of sperm was also greater in these sperm samples compared to the wild-type, haploid sperm (Table 3). The mean PI fluorescence of sperm from gametophytes derived from plant 1742-3, whose segregation of mutant and wild-type phenotypes is consistent with the parent sporophyte being diploid, is similar to the mean PI fluorescence of sperm from wild-type, haploid sperm (Table 3).

DISCUSSION

Several mutations of Ceratopteris that affect the sex of the gametophyte have been isolated and characterized (EBERLE *et al.* 1996). The *HER* loci examined in this study are thought to encode proteins that are involved in the perception and/or transduction of A_{CE}, the primary sex-determining signal in this species, since *her* mutants are insensitive to the pheromone. Whether this signal transduction pathway is inactive or active ultimately determines the sex of the gametophyte, which is either hermaphroditic when A_{CE} is absent or

TABLE 2

Segregation ratios of *her* and *cp* phenotypes in the progeny gametophytes of putative tetraploid sporophytes

her allele	Plant no.[b]	No. of progeny: *cp* herm	No. of progeny: *CP* herm	No. of progeny: Male[c]	Expected ratio[a]: *cp* herm:*CP* herm:male[c]	χ^2; *P*	Observed ratio *cp2* herm:herm	Observed ratio herm:male
her1	1724-1	19	38	268	1:1:2	139.02; <0.001	1:2.00	1:4.70
	1724-2	70	90	159	1:1:2	2.51; >0.20	1:1.29	1:1
	1724-7	12	62	300	1:1:2	149.94; <0.001	1:5.17	1:5.05
	1724-8	12	57	300	1:1:2	154.43; <0.001	1:4.75	1:4.35
	1724-11	65	68	168	1:1:2	3.01; >0.20	1:1.05	1:1.26
	1740-5	9	52	300	1:1:2	168.47; <0.001	1:5.78	1:4.92
	1741-2	11	41	300	1:1:2	143.73; <0.001	1:3.72	1:5.77
	1741-5	13	79	404	1:1:2	213.82; <0.001	1:6.08	1:4.39
	1741-7	22	94	615	1:1:2	354.82; <0.001	1:4.27	1:5.30
	1743-1	9	46	300	1:1:2	176.80; <0.001	1:5.11	1:5.45
	1743-7	9	42	300	1:1:2	182.84; <0.001	1:4.67	1:5.88
	1746-5	11	51	326	1:1:2	187.87; <0.001	1:4.64	1:5.26
her3	1726-1	11	51	301	1:1:2	166.19; <0.001	1:4.64	1:4.85
	1726-2	6	46	388	1:1:2	237.28; <0.001	1:9.33	1:6.26
	1727-4	5	29	135	1:1:2	67.18; <0.001	1:5.80	1:3.97
	1728-6	12	53	234	1:1:2	106.77; <0.001	1:4.42	1:3.60
	1728-9	10	65	234	1:1:2	101.39; <0.001	1:6.50	1:3.12
	1739-4	6	40	254	1:1:2	153.74; <0.001	1:6.67	1:5.52
	1742-3	43	50	107	1:1:2	1.47; >0.30	1:1.16	1:1.15
	1742-6	11	58	373	1:1:2	219.08; <0.001	1:5.27	1:5.41
	1745-4	12	71	344	1:1:2	175.84; <0.001	1:5.92	1:4.14
	1745-5	37	154	664	1:1:2	236.68; <0.001	1:4.16	1:3.48
	1745-7	18	62	300	1:1:2	137.55; <0.001	1:3.40	1:3.75
	1745-9	12	61	346	1:1:2	189.33; <0.001	1:5.08	1:4.74
her10	1733-1	27	26	55	1:1:2	0.06; >0.70	0.96:1	1:1.04
	1734-1	23	94	283	1:1:2	94.10; <0.001	1:4.09	1:2.42
	1734-4	10	64	350	1:1:2	193.41; <0.001	1:6.4	1:4.73
	1734-6	11	57	323	1:1:2	177.13; <0.001	1:5.18	1:4.75
	1744-5	14	70	400	1:1:2	219.28; <0.001	1:5.0	1:4.76
	1744-2	19	39	302	1:1:2	167.60; <0.001	1:2.05	1:5.21
her17	1731-1	28	33	318	1:1:2	174.40; <0.001	1:1.18	1:5.21
	1747-1	59	65	367	1:1:2	120.41; <0.001	1:1.10	1:2.96
	1747-2	14	74	365	1:1:2	185.27; <0.001	1:5.29	1:4.45

[a] The ratios are that expected for the null hypothesis of assortment from a diploid sporophyte.

[b] Each plant listed represents a putative tetraploid plant independently derived by apospory and self-fertilization of the gametophyte. Plants with the same first four digit numbers were different aposporous gametophytes but derived from the same leaf.

[c] Gametophytes were grown on medium containing A_{CE}.

male when A_{CE} is present. The four *her* alleles are not linked to one another, indicating that they represent four different genetic loci (Eberle *et al.* 1996), nor are they linked to the *CP2* locus (this study). Three *her* alleles (*her1, her3,* and *her10*) are completely penetrant and expressive while *her17* is incompletely penetrant in that progeny gametophytes of sporophytes homozygous for *her17* produce some (<5%) males (Eberle *et al.* 1996). Since diploid sporophytes that are homozygous or heterozygous for each of the *her* mutations are indistinguishable from each other and from wild-type plants (Banks 1994), these mutations have no effect on sporophyte development. The difficulty in studying gametophytic mutations in Ceratopteris is that complementation analysis to determine their dominance, recessiveness, or allelism cannot be performed. The purpose of this study was to determine if this problem could be overcome by producing diploid gametophytes heterozygous for mutations known to affect only gametophyte development.

The ploidy levels of fern sporophytes and gametophytes can be artificially altered by two processes, referred to as apospory and apogamy (reviewed by Walker 1979; White 1979). In apospory, diploid gametophytic tissue is produced from diploid sporophytic tissue without an intervening meiotic division or the production of spores. In apogamy, sporophytic tissue is induced from gametophytic tissue without the intervention of fertilization. Apospory has been used in several species of ferns to generate synthetic autotetraploid

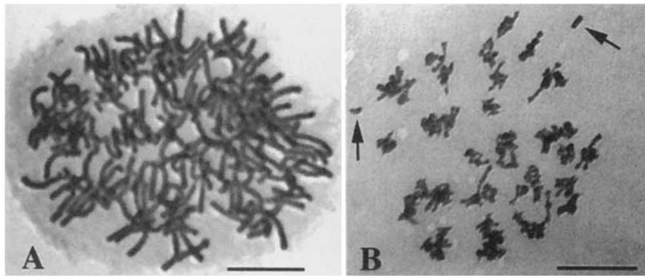

FIGURE 1.—Mitotic (A) and meiotic (B) chromosomes, stained with acetocarmine, of plant 1724-8. The approximately 150 chromosomes apparent in A pair during meiosis to form tetravalents, trivalents, or univalents (indicated by arrows) shown in B. Scale bars, A, 25 nm; B, 50 nm.

sporophytes by self-fertilizing aposporous diploid gametophytes. However, the segregation of mutant alleles in synthetic autotetraploids has not been reported in any fern.

In this study, synthetic autopolyploids that are heterozygous for each of four *her* alleles and *cp2*, a known recessive mutation, were produced via apospory. A majority of these plants do not segregate wild-type and mutant (*cp2* or *her*) progeny gametophytes in a 1:1 ratio, indicating that the parent sporophyte plants are not diploid but polyploid, at least for the *CP2* and *HER* loci. The proportion of wild-type to mutant gametophytes derived from genetically polyploid sporophyte plants was always greater than one, indicating that the *cp2* and all *her* mutations are recessive. Four sporophytes segregated wild-type and mutant gametophytes in a 1:1 ratio, indicating that the sporophyte parent was diploid. It is possible that in the generation of these four plants, the gametophyte derived by apospory underwent apogamy, thus resulting in a diploid sporophyte.

The examination of meiotic chromosomes of five genetically identified polyploid plants revealed the presence of a few univalents and many mulivalents in all samples. Aneuploidy is frequently observed in tetraploid maize, indicating that chromosomes may be gained or lost during meiosis following the event that led to polyploidy, even though the actual number of chromosomes is four times the monoploid number (RANDOLPH 1935). Although the presence of univalents in tetraploid spore mother cells of Ceratopteris sporophytes indicates that the spores and gametophytes derived from them may be aneuploid, the actual number of chromosomes in the genetically polyploid sporophytes is much greater than twice the number of chromosomes in a haploid set ($n = 39$).

A direct way of confirming the ploidy level of the gametophyte is by measuring and comparing DNA content in individual cells. The sperm of Ceratopteris are single-celled, motile, and easily collected from male gametophytes. For these reasons, their DNA content can be readily determined by cell flow cytometry. As predicted, the amount of DNA in sperm of gametophytes derived from polyploid sporophytes was found to be about twice that of wild-type haploid sperm. However, the variation in DNA content in putative diploid sperm was large compared to haploid sperm. There are two possible explanations for the greater variance observed in the sperm of gametophytes derived from polyploid

TABLE 3

Measurements of PI fluorescence in haploid and diploid sperm by flow cytometry

Sample	Mean fluorescence[a] Sperm/CRBC	Ratio of mean sperm/CRBC	No. of sperm	No. of CRBC
Hnn	299 (10)/NA		19,314	
Hnn + CRBC	284 (11)/69 (3)	4.1	1,974	16,728
1724-7 (*her1*)	599 (37)/NA		2,252	
1724-7 + CRBC	593 (37)/79 (3)	7.5	253	4,292
1724-8 (*her1*)	596 (33)/NA		1,222	
1724-8 + CRBC	595 (34)/78 (3)	7.6	242	7,483
1741-2 (*her1*)	609 (36)/NA		1,189	
1741-2 + CRBC	607 (34)/81 (3)	7.6	880	18,955
1726-2 (*her3*)	536 (100)/NA		5,076	
1726-2 + CRBC	591 (43)/78 (3)	7.7	2,391	17,771
1742-3 (*her3*)	293 (12)/NA		19,973	
1742-6 (*her3*)	570 (65)/NA		674	
1742-6 + CRBC	568 (68)/79 (3)	7.2	456	19,428
1745-5 (*her3*)	599 (36)/NA		498	
1745-5 + CRBC	593 (37)/79 (3)	7.5	531	19,282
1744-2 (*her10*)	579 (68)/NA		265	
1744-2 + CRBC	562 (50)/73 (5)	7.7	174	18,501
1747-2 (*her17*)	551 (83)/NA		1,641	
1747-2 + CRBC	582 (50)/76 (4)	7.8	1,137	18,875

[a] Values in parentheses are SD. CRBC, chick red blood cells; NA, not available.

parents. First, 2:2 disjunction of chromosomes during anaphase I of meiosis may not always occur for all homologous chromosomes in a tetraploid spore mother cell. The progeny gametophytes and sperm produced by them may thus be aneuploid for different chromosomes. A second factor to be considered is that the differences in variation may reflect differences in sample size. Since the gametophyte progeny of polyploid sporophytes produce far fewer sperm than wild-type haploid gametophytes, the number of sperm analyzed per sample was less than optimal for this technique (ARUMUGANATHAN and EARLE 1991).

By three different criteria, we have demonstrated that autopolyploid sporophytes can be generated by inducing diploid gametophytes by apospory, followed by self-fertilization of the diploid gametophyte. The segregation ratios of mutant and wild-type phenotypes in the gametophyte progeny of heterozygous polyploid sporophytes indicate that the mutants examined are recessive. Although it takes approximately 10–12 months to go from single haploid spore to mature diploid gametophyte, the technique is very simple and can be applied to most mutations affecting gametophyte growth and development in Ceratopteris.

We thank the Purdue Cell Flow Cytometry Lab for technical assistance and CHI KUANG WEN and CRISTINA JUAREZ for helpful discussion. This research was supported by the National Science Foundation and the Purdue Agricultural Experimental Station. This is journal paper no. 15356 of the Purdue University Agricultural Experimental Station.

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Communicating editor: J. A. BIRCHLER