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Characterization of Mutations That Feminize Gametophytes of the Fern Ceratopteris

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ABSTRACT

Gametophytes of the fern Ceratopteris are either male or hermaphroditic. Their sex is epigenetically determined by the pheromone antheridiogen, which is secreted by the hermaphrodite and induces male and represses female development in other young, sexually undetermined gametophytes. To understand how antheridiogen represses the development of female traits at the genetic level, 16 new mutations that feminize the gametophyte in the presence of antheridiogen were identified and characterized. Seven are very tightly linked to the *FEM1* locus previously described. Nine others define another locus named *NOTCHLESS1* (*NOT1*), as several of the *not1* mutants lack a meristem notch. Some *not1* mutations also affect sporophyte development only when homozygous, indicating that the *not1* mutations are recessive and that *NOT1* is also required for normal sporophyte development. The epistatic interactions among *FEM1*, *NOT1*, and other sex-determining genes are described. This information was used to expand the genetic model of the sex-determining pathway in Ceratopteris. On the basis of this model, we can say that the presence of antheridiogen leads to the activation of the *FEM1* gene, which not only promotes the differentiation of male traits, but also represses female development by activating the *NOT1* gene. *NOT1* represses the *TRA* genes necessary for the development of female traits in the gametophyte.

THE haploid gametophyte generation of the plant life cycle begins with the production of haploid spores through meiotic divisions of diploid spore mother cells. Like many nonseed plants, the fern *Ceratopteris richardii* produces only one type of spore (*i.e.*, is homosporous), yet each Ceratopteris spore has the potential to develop as a free-living haploid gametophyte that produces only sperm or produces both egg and sperm. The sex of the gametophyte is epigenetically determined in many species of homosporous ferns, including Ceratopteris, by the pheromone antheridiogen (Döpp 1950; Näf 1959, 1979; Näf *et al.* 1975; Warne and Hickok 1991; Banks 1997a; Yamane 1998). When the antheridiogen of Ceratopteris (or A_{CE}) is absent, a Ceratopteris spore develops as a hermaphroditic gametophyte, which produces and secretes the pheromone once it becomes insensitive to its male-inducing effects (Banks *et al.* 1993). In the presence of A_{CE}, a spore develops as a male gametophyte. This mechanism of sex determination allows the ratio of males and hermaphrodites in a population to vary depending on population density rather than on genetic predisposition, such that the proportion of males increases as the population size increases.

This article is dedicated to the memory of DeLill Nasser.

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The male and hermaphroditic gametophytes of Ceratopteris are morphologically distinct, which aids in genetic screens for sex-determining mutants. Hermaphroditic gametophytes have a distinct lateral multicellular meristem from which most cells of the hermaphrodite prothallus are derived (Figure 1A). This meristem forms a meristem notch that gives the hermaphrodite its heart-shaped appearance. A cell within the meristem has four possible cell fates. It can remain within the meristem as a stem cell to maintain the meristem, differentiate as an egg-forming archegonia or as a sperm-forming antheridia, or enlarge to form a photosynthetic, vegetative cell of the prothallus. Male gametophytes never develop a multicellular meristem or archegonia. Growth of the male gametophyte occurs by divisions of a single apical initial. Derivatives of the initial quickly differentiate as antheridia such that at maturity, almost all cells of the male gametophytic prothallus have differentiated as antheridia (Figure 1B). Antheridiogen therefore acts to repress meristem and archegonia formation as well as to promote the rapid differentiation of antheridia during gametophyte development.

To understand how A_{CE} regulates the sex of the fern gametophyte, a genetics approach has been used to identify genes that are involved in the sex-determining process in Ceratopteris (reviewed in Banks 1997a). Four phenotypic classes of mutations that alter the sex of the gametophyte have been characterized and include the *hermaphroditic* (*her*) mutants, which are hermaphrodites in the presence or absence of A_{CE}; the *transformer* (*tra*) mutants, which are male in the presence or absence of

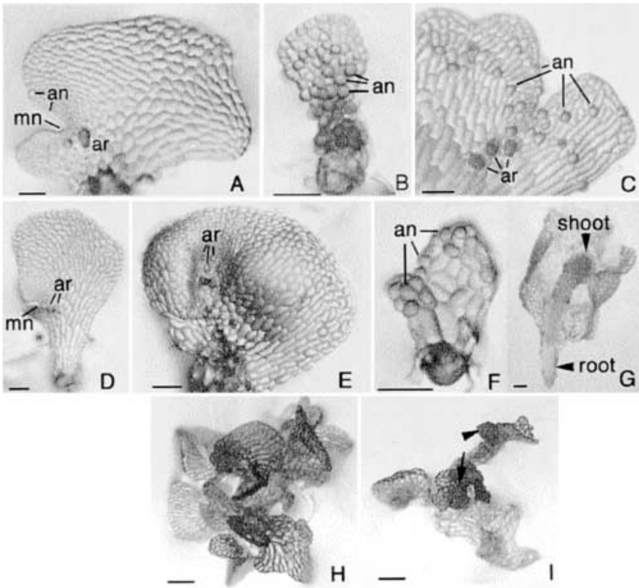

Figure 1.—Wild-type and mutant phenotypes. (A) An 11-day-old wild-type hermaphrodite that is similar in appearance to *her* hermaphrodites. (B) An 11-day-old wild-type male that is similar in appearance to the *tra2* male. (C) The meristematic region of an 11-day-old *man1* gametophyte, which produces many more antheridia than wild-type or *her* hermaphrodites. (D) A 10-day-old *fem1-1* female, which produces a meristem and archegonia but no antheridia. (E) A 16-day-old *not1-7* (allele 15) gametophyte showing the centralized meristem and notchless phenotype. (F) An 11-day-old male gametophyte mutant for *not1-4* (allele 8) and *tra2*, which is smaller and produces fewer antheridia than wild-type or *tra2* males grown in the presence of A_{CE}. (G) An 8-week-old sporophyte homozygous for *not1-7* (allele 15) produced by self-fertilizing a *not1-7* female; this sporophyte has a normal root but the shoot develops as a ball from which no leaves form. (H) A 4-week-old *fem1-1 tra2* intersexual gametophyte. Although ruffled in appearance due to multiple but shallow meristems, the prothallus consists of a single layer of cells. (I) A 4-week-old asexual gametophyte mutant for *fem1-1*, *not1-4* (allele 8), and *tra2*. Arrows point to the meristematic regions of the gametophyte that are growing in three dimensions. mn, meristem notch; ar, egg-forming archegonia; an, sperm forming antheridia. Bars: A–F and H–I, 0.1 mm; G, 2 mm.

A_{CE}; the *feminization1* (*fem1*) mutant, which is female in the presence or absence of A_{CE}; and the *many-antheridia1* (*man1*) mutant, which is male in the presence of A_{CE} and a hermaphrodite that produces ~10 times more antheridia than wild-type hermaphrodites when A_{CE} is absent. A model of the sex-determining pathway in Ceratopteris, based upon the epistatic interactions observed among these mutants (Banks 1997b), has been developed. In the present study, we have identified additional sex-determining mutants that specifically address the function of the *FEM1* gene. On the basis of the phenotype of the previously described *fem1* mutant (always female), *FEM1* has two conceivable wild-type functions, both of which are consistent with the model of the sex-determining pathway. One function would be to promote the differentiation of male traits (antheridia) and the other would be to repress the differentiation of female traits (meristem and archegonia). If the *FEM1* gene fulfills the latter function by activating a repressor of the *TRA* genes that are required for the development of female traits, we predict that a mutation that is defective only in its ability to repress the *TRA* genes could exist. If the current model of the sex-determining pathway is correct, such a mutant would have a *fem* phenotype but would be genetically unlinked to the *FEM1* locus and have different but predictable epistatic interactions with other sex-determining genes. By isolating and characterizing 16 new feminizing mutant gametophytes, we have identified additional mutations that are tightly linked to *fem1* as well as mutations that define at least one additional *fem1*-like locus called *NOTCHLESS1* (*NOT1*), so named because many *not1* gametophytes do not form a characteristic meristem notch. Tests of epistasis between the *not1* mutants and other sex-determining mutants confirm that the function of the *NOT1* gene is to repress the *TRA* genes. The function of the *FEM1* gene in the sex-determining pathway is therefore not only to promote the differentiation of antheridia, but also to repress meristem and archegonia development indirectly by activating the *NOT1* gene.

MATERIALS AND METHODS

The origins of Hnn, the wild-type strain of Ceratopteris, and the *tra*, *her*, *fem1*, and *man1* mutants used in this study are described in Banks (1997a,b). The conditions for gametophyte and sporophyte culture were the same as those described by Banks (1994). To isolate new *fem* mutants, 2×10^6 Hnn spores were mutagenized with EMS according to Banks (1994) and plated on medium containing A_{CE}, conditions that promote the development of male gametophytes in wild-type spores. Mutagenized gametophytes that developed a multicellular meristem but no or very few (fewer than five) antheridia (putative *fem* mutants) were transferred to fresh A_{CE}-containing medium and grown for 20 days. Those that formed no or few antheridia were backcrossed by Hnn twice and then crossed by other sex-determining mutants to test linkage and epistasis. In crosses using a wild-type hermaphrodite as the female parent or a *her* hermaphrodite as a male parent, the hermaphrodite carried the *clumped chloroplast2* (*cp2*) mutation. Since the recessive *cp2* mutation is visible in cells of the gametophyte and sporophyte, it is a useful marker for confirming the hybridity of a cross.

The phenotypes of progeny gametophytes were scored after 2 weeks of growth on A_{CE}-containing medium prepared by conditioning the medium with the growth of wild-type gametophytes. The phenotypes of gametophytes grown for the same period of time in the absence of A_{CE} were tested by pipetting individual spores suspended in nonconditioned fern medium into individual microtiter wells. When scoring gametophytes grown in the presence of A_{CE}, hermaphrodites were ignored if they occurred at a frequency of $<1\%$. These hermaphrodites are likely to be wild-type gametophytes that developed as hermaphrodites even though A_{CE} was present, as the proportion of wild-type spores that develop as males in the presence of A_{CE} is always $<100\%$ but $>95\%$.

The number of antheridia present on mutant and wild-type hermaphroditic gametophytes was determined by counting

TABLE 1

Results of crosses showing that all feminizing mutations segregate as single Mendelian traits

Cross	No. of females: males[a]	Expected ratio[a]	$\chi^{2\,b}$	No. of antheridia/ female[a,c]	Able to self	Sporophyte phenotype[d]
Mutant 2 × Hnn	96:106	1:1	0.50	0	No	
Mutant 3 × Hnn	128:140	1:1	0.54	0–8	Yes	Normal
Mutant 4 × Hnn	118:104	1:1	0.88	0	No	
Mutant 5 × Hnn	94:108	1:1	0.97	0	No	
Mutant 6 × Hnn	133:147	1:1	0.70	0–3	Yes	Dwarf, sterile
Mutant 7 × Hnn	104:100	1:1	0.08	0	No	
Mutant 8 × Hnn	148:162	1:1	0.63	0	No	
Mutant 9 × Hnn	98:104	1:1	0.17	0–1	No	
Mutant 10 × Hnn	96:95	1:1	0.01	0	No	
Mutant 11 × Hnn	103:96	1:1	0.25	0	No	
Mutant 12 × Hnn	159:145	1:1	0.64	0	No	
Mutant 13 × Hnn	96:104	1:1	0.32	0	No	
Mutant 14 × Hnn	168:156	1:1	0.44	0–1	Yes	Dwarf, sterile
Mutant 15 × Hnn	153:151	1:1	0.01	0	No	
Mutant 16 × Hnn	106:94	1:1	0.72	0–3	Yes	Dwarf, sterile
Mutant 17 × Hnn	151:151	1:1	0	0	No	
Hnn × Hnn	1:200			20–51	Yes	Normal

[a] Grown in the presence of A_{CE}.
[b] All P values are >0.05.
[c] Antheridia were counted on 20-day-old gametophytes.
[d] Homozygous sporophyte formed by self-fertilizing gametophyte.

the number of antheridia on each of 40 gametophytes after 20 days of growth. The ability of a *fem* gametophyte to self-fertilize was determined by adding water to 40 individual gametophytes that had been placed individually into microtiter wells before reaching sexual maturity. Gametophytes were kept submerged until embryos could be observed or, if no embryos formed, until 25 days of growth.

RESULTS

Identification of new feminizing mutants: By virtue of their large size and presence of a meristem compared to the smaller, ameristic male members of the population, putative *fem* mutants are easy to identify in a population of mutagenized gametophytes grown in the presence of A_{CE}. After selecting and backcrossing such gametophytes, each putative *fem* mutant was crossed by wild-type sperm to test the heritability of the *fem* phenotype. As shown in Table 1, diploid sporophytes heterozygous for each of 16 new mutations produced haploid progeny that segregated male and female or hermaphroditic gametophytes with fewer than nine antheridia in a 1:1 ratio, confirming that the partial or complete feminizing phenotypes segregate as single Mendelian traits. For convenience, these feminizing mutants were temporarily referred to as mutants 2–17.

Eleven of the mutants (2, 4, 5, 7, 8, 10, 11, 12, 13, 15, and 17) did not form visible antheridia nor did they self-fertilize, while five mutants (3, 6, 9, 14, and 16) formed between zero and eight antheridia (Table 1). Other aberrant gametophyte phenotypes were observed among the group of mutants that could form antheridia. Three mutants (2, 9, and 15) lost their meristem notch after 2 weeks of growth and eventually formed tubes through a centralized rather than marginal meristem. The notchless phenotype is illustrated in Figure 1E. Mutant 16 formed archegonia along the marginal cell layer of the meristem notch rather than a few cell layers below the margin of the notch (data not shown). Collectively, these phenotypes indicate that the wild-type functions of some of these genes are not only to promote antheridia development, but also possibly to organize patterns of cell division and differentiation within the meristem.

Among the five mutants that formed antheridia, four (3, 6, 14, and 16) could be self-fertilized to form sporophytes. Of these, only the sporophytes homozygous for mutation 3 developed normally. Sporophytes homozygous for mutations 6, 14, or 16 were abnormally small and either failed to form leaves (Figure 1G, for example) or formed up to 10 small leaves before dying. None of these sporophyte plants produced fertile sporophylls bearing sporangia. The abnormal sporophyte phenotypes associated with these gametophyte mutations indicate that these genes are also necessary for normal sporophyte development. Since sporophytes heterozygous for mutations 6, 14, or 16 are indistinguishable from wild-type sporophytes (data not shown), these mutations are likely to be recessive, complete, or partial loss-of-function mutations.

Seven mutations are linked to *FEM1*: Although tests

TABLE 2

Results of crosses showing that some feminizing mutations are tightly linked to *fem1* and have an intersex double-mutant phenotype when also mutant for *tra2* when grown in the presence of A_{CE}

Cross	No. of females	No. of males	No. of intersexes	Expected ratio of females:males intersexes	χ^{2} [a]
fem1 × *tra2*	112	196	110	1:2:1	1.14
fem1 × *fem1 tra2*	51	0	50	1:0:1	0.01
Mutant 4 × *tra2*	73	150	90	1:2:1	2.38
Mutant 4 × *fem1 tra2*	99	0	101	1:0:1	0
Mutant 5 × *tra2*	71	162	75	1:2:1	0.94
Mutant 5 × *fem1 tra2*	96	0	86	1:0:1	0.55
Mutant 7 × *tra2*	80	148	72	1:2:1	1.02
Mutant 7 × *fem1 tra2*	51	0	53	1:0:1	0.04
Mutant 10 × *tra2*	96	190	114	1:2:1	4.72
Mutant 10 × *fem1 tra2*	115	0	110	1:0:1	0.11
Mutant 11 × *tra2*	65	149	88	1:2:1	3.56
Mutant 11 × *fem1 tra2*	102	0	110	1:0:1	0.30
Mutant 12 × *fem1 tra2*	86	0	88	1:0:1	0.02
Mutant 13 × *tra2*	72	152	76	1:2:1	0.16
Mutant 13 × *fem1 tra2*	101	0	110	1:0:1	0.38

[a] All P values are >0.05 except for the progeny of the mutant 10 × *tra2* cross where $P > 0.01$.

of allelism cannot be determined in the haploid gametophyte, it is possible to establish whether the new feminizing mutations are linked to the *FEM1* locus previously characterized by crossing each mutant by the sperm of a *fem1 tra2* intersexual gametophyte (illustrated in Figure 1H). As shown in Table 2, sporophytes heterozygous for *tra2*, *fem1*, and seven feminizing mutations (4, 5, 7, 10, 11, 12, and 13) segregated females and intersexes in a 1:1 ratio. The absence of males in >2000 progeny per cross (data not shown) indicates that no recombination between *fem1* and this group of seven mutant alleles occurred. Although the absence of males does not prove that these new mutants are allelic to *fem1*, the data do indicate that they are very tightly linked and may be allelic. For this reason, these seven feminizing mutations are collectively referred to as the fem1 group of mutations. Sporophytes homozygous for *fem1*, generated by crossing a *fem1* female by the sperm of a *fem1 tra2* intersex, are indistinguishable from wild-type sporophytes, as are sporophytes mutant for both *fem1* and each of eight new mutations in the *fem1* group (data not shown).

To confirm that gametophytes mutant for both *tra2* and each of the *fem1* group mutations are intersex, each of the eight *fem1* group mutant females was crossed by the sperm of a *tra2* male. As shown in Table 2, the resulting heterozygous sporophytes produced progeny that segregated female, male, and intersexual progeny gametophytes in a 1:2:1 ratio as expected. This indicates that all gametophytes mutant for *tra2* and each of the *fem1* group mutations are phenotypically the same (intersex) and that all of the *fem1* group mutations segregate independently of *tra2*. Similar results were obtained using other *tra* mutations (data not shown). The intersex, double-mutant gametophyte phenotype is novel, indicating a lack of epistasis between the *FEM1* group genes and the *TRA* genes.

Epistasis and linkage among the remaining feminizing mutants: Eight of the nine remaining mutants isolated (2, 3, 6, 8, 9, 15, 16, and 17) could be genetically distinguished from the *fem1* mutants on the basis of the phenotypes of gametophytes also mutant for *TRA2*. Mutant females, when crossed by the sperm of *tra2* males, formed heterozygous sporophytes whose progeny segregated females and males in a 1:3 ratio when grown in the presence of A_{CE} (Table 3). Among the male class of progeny, two phenotypically distinct types were observed: one indistinguishable from wild-type and *tra2* males and the other having fewer antheridia than wild-type males (compare in Figure 1, B and F). The two types of males segregated in 1:2 ratios of small to wild-type males (data not shown), indicating that the odd male phenotype occurs in gametophytes mutant for these feminizing mutations and *tra2*. In the absence of A_{CE}, sibling gametophytes derived from the same crosses segregated females, hermaphrodites, and males in a 1:1:2 ratio. These ratios indicate that gametophytes mutant for *tra2* and each of the 2, 3, 6, 7, 8, 15, 16, and 17 mutations are male in the absence or presence of A_{CE}. The *TRA2* gene is therefore epistatic to the locus or loci defined by these feminizing mutations.

To examine the linkage between the 2, 3, 6, 7, 8, 15, 16, and 17 mutations and *fem1*, mutant females were crossed by the sperm of *fem1 tra2* intersexual gametophytes. With one exception (mutant 3), the resulting heterozygous sporophytes produced progeny that segregated female, male, intersex, and asexual gametophytes

TABLE 3

Results showing that eight mutant alleles (renamed *not1-1* to *not1-8*) are not linked to *fem1* and have, with one exception, a male phenotype when also mutant for *tra2*

Cross	A_{CE}	No. of females	No. of hermaphrodites	No. of males[a]	No. of intersexes	No. of asexuals	Expected ratio of females:hermaphrodites: males:intersexes:asexuals	$\chi^{2\,b}$
Mutant 2 (*not1-1*) × *fem1 tra2*	+	257	0	227	88	82	3:0:3:1:1	2.35
Mutant 2 (*not1-1*) × *tra2*	ND							
Mutant 3 (*not1-2*) × *fem1 tra2*	+	262	0	486	124	125	3:0:3:1:1	67
Mutant 3 (*not1-2*) × *tra2*	+	83	0	224	0	0	1:0:3:0:0	0.63
Mutant 6 (*not1-3*) × *fem1 tra2*	+	173	0	183	69	75	3:0:3:1:1	1.91
Mutant 6 (*not1-3*) × *tra2*	+	90	0	210	0	0	1:0:3:0:0	1.74
Mutant 8 (*not1-4*) × *fem1 tra2*	+	297	0	271	82	93	3:0:3:1:1	2.69
Mutant 8 (*not1-4*) × *tra2*	+	69	0	232	0	0	1:0:3:0:0	0.74
	−	46	38	89	0	0	1:1:2:0:0	0.75
Mutant 9 (*not1-5*) × *fem1 tra2*	+	213	0	230	73	87	3:0:3:1:1	2.79
Mutant 9 (*not1-5*) × *tra2*	+	71	0	229	0	0	1:0:3:0:0	1.23
	−	85	76	134	0	0	1:1:2:0:0	2.96
Mutant 14 (*not1-6*) × *fem1 tra2*	+	182	0	255	107	65	3:0:2:2:1	24.03
	+	196	0	230	92	73	3:0:2:2:1	37.06
Mutant 14 (*not1-6*) × *tra2*	+	71	0	166	75	0	1:0:2:1:0	1.39
Mutant 15 (*not1-7*) × *fem1 tra2*	+	245	0	229	88	92	3:0:3:1:1	3.25
Mutant 15 (*not1-7*) × *tra2*	+	86	0	216	0	0	1:0:3:0:0	1.95
	−	67	57	120	0	0	1:1:2:0:0	0.80
Mutant 16 (*not1-8*) × *fem1 tra2*	+	174	0	172	60	61	3:0:3:1:1	0.27
Mutant 16 (*not1-8*) × *tra2*	+	76	0	241	0	0	1:0:3:0:0	0.15
	−	68	60	125	0	0	1:1:2:0:0	0.57
Mutant 17 (*not1-9*) × *fem1 tra2*	+	182	0	210	69	73	3:0:3:1:1	2.27
Mutant 17 (*not1-9*) × *tra2*	+	77	0	225	0	0	1:0:3:0:0	0.03
	−	31	34	67	0	0	1:1:2:0:0	0.17

ND, not determined.

[a] Includes all males regardless of their phenotype.

[b] All P values are >0.05 except for the *not1-6* × *fem1 tra2* and *not1-2* × *fem1 tra2* crosses.

TABLE 4

Results of testcrosses or backcrosses to confirm the genotypes of intersex, female, and male progeny gametophytes grown in the presence of A_{CE} and derived from the *not1-4* × *fem1 tra2* cross

Cross	No. of females	No. of males	No. of intersexes	No. of asexuals	Expected ratio of females:males: intersexes:asexuals	χ^2 P[a]
fem1 × intersex	111	0	89	0	1:0:1:0	2.42
	99	0	101	0	1:0:1:0	0.02
	88	0	112	0	1:0:1:0	2.88
	90	0	110	0	1:0:1:0	2.00
	91	0	109	0	1:0:1:0	1.62
	98	0	99	0	1:0:1:0	0.02
	107	0	93	0	1:0:1:0	0.98
	93	0	108	0	1:0:1:0	1.13
	104	0	105	0	1:0:1:0	0.01
	101	0	99	0	1:0:1:0	0.02
Female × Hnn[b]	99	111	0	0	1:1:0:0	0.69
	147	153	0	0	1:1:0:0	0.12
	139	160	0	0	1:1:0:0	1.48
	233	68	0	0	3:1:0:0	0.87
	240	91	0	0	3:1:0:0	1.03
	214	87	0	0	3:1:0:0	2.60
	229	73	0	0	3:1:0:0	0.11
not1-4 × male	80	75	0	0	1:1:0:0	0.16
	79	71	0	0	1:1:0:0	0.43
	87	94	0	0	1:1:0:0	0.27
	113	97	0	0	1:1:0:0	1.22
	51	152	0	0	1:3:0:0	0
fem1 × male	270	321	108	103	3:3:1:1	5.20

[a] All P values are >0.05.
[b] Hnn is a wild-type male.

in a 3:3:1:1 ratio when grown in the presence of A_{CE} (Table 3). This ratio indicates that *fem1* is not linked to any of these mutations. Crosses using the females harboring mutation 3 as the female parent and *fem1 tra2* intersexes as the male parent also resulted in the same four phenotypic classes of progeny (Table 3). However, they did not segregate in a 3:3:1:1 ratio for reasons that are unknown at this time.

The asexual gametophytes resulting from all crosses produced no antheridia or archegonia and did not form a marginal meristem typical of the female or hermaphroditic gametophytes, which is a novel phenotype. In the hermaphrodite and *fem1* females, cell divisions occur in two planes (anticlinal and periclinal) such that the gametophyte prothallus consists of a single layer of cells in these individuals (see Figure 1, A and D). While the asexual gametophyte forms a distinct meristematic region, divisions within the meristem often occur in three planes and parts of the gametophyte prothallus are consequently three-dimensional, as illustrated in Figure 1I.

Eight possible progeny genotypes result from each of the crosses described above (*i.e.*, mutant 2, 3, 6, 7, 8, 15, 16, or 17 females × *fem1 tra2* intersex). The phenotypes associated with only two genotypes are unknown, including double mutants harboring *fem1* and each of the feminizing mutations and triple mutants harboring *fem1*, *tra2*, and each feminizing mutation. On the basis of the uniform segregation ratios of phenotypes resulting from these crosses and the known phenotypes of the remaining six genotypes, we deduced that the double-mutant gametophytes are likely to be female and the triple-mutant gametophytes are likely to be asexual when grown in the presence of A_{CE}. To confirm the genotypes of the phenotypic classes resulting from the mutant 2, 3, 6, 7, 8, 15, 16, and 17 × *fem1 tra2* intersex crosses, backcrosses or testcrosses were performed on the progeny gametophytes derived from the mutant 8 × *fem1 tra2* cross (Table 4). All progeny intersexes, when crossed to *fem1* females, formed sporophytes that segregated females and intersexes in a 1:1 ratio, indicating that the intersexes are genotypically *fem1 tra2*. Females crossed by wild-type sperm formed sporophytes that segregated females and males in a 3:1 or a 1:1 ratio. The 3:1 ratio indicates that the female parent harbored both *fem1* and mutation 8. The 1:1 ratio indicates that the female parent carried either *fem1* or mutation 8. Male progeny, when crossed to mutant 8 females, formed sporophytes whose progeny segregated females and males in either a 1:1 or a 1:3 ratio. One male crossed

TABLE 5

Results of crosses to determine the *fem1 her19* and *not1 her19* double-mutant phenotypes in the presence of A_{CE}

Cross	No. of meristics[a]	No. of males	Hermaphrodites: females[b]	Expected ratio of meristics:males	$\chi^{2\,c}$
fem1 × *her19*	226	79	18:31	3:1	0.16
not1-1 × *her19*	226	78	25:58	3:1	0.07
not1-2 × *her19*	229	73	11:30	3:1	0.11
not1-3 × *her19*	217	88	29:56	3:1	2.52
not1-4 × *her19*	310	110	40:110	3:1	0.32
not1-5 × *her19*	ND				
not1-6 × *her19*	230	80	22:53	3:1	0.11
not1-7 × *her19*	ND				
not1-8 × *her19*	225	75	40:73	3:1	0
not1-9 × *her19*	233	68	20:42	3:1	0.87

ND, not determined.
[a] Includes females and hermaphrodites.
[b] Numbers of hermaphrodites and females among the meristic gametophytes.
[c] All P values are >0.05.

to a *fem1* female resulted in a segregation ratio of three females:three males:one intersex:one asexual, which is the same as that observed from the original mutant 8 × *fem1-1 tra2* cross. The results of the crosses using the males as sperm donors indicate that the male parent was wild type, a *tra2* mutant, or doubly mutant for *tra2* and mutation 8. The one of eight genotypes not accounted for (the mutation 8 *fem1-1 tra2* triple mutant) is likely to result in an asexual gametophyte phenotype. Since the asexual gametophyte does not form antheridia or archegonia, crosses to confirm its genotype could not be performed.

To establish the linkage relationships among mutations 2, 3, 6, 7, 8, 15, 16, and 17, >100 females of each mutant were crossed by the sperm of small males derived from several of the mutant 2, 3, 6, 7, 8, 15, 16, and 17 × *tra2* crosses, *i.e.*, males carrying both *tra2* and a feminizing mutation. Either no embryos or abnormal embryos giving rise to abnormal and sterile plants developed from these mutant females (data not shown). Since these plants produced no progeny, we could not establish the linkage relationships among these mutations.

Each mutant 2, 3, 6, 7, 8, 15, 16, and 17 female, when crossed by wild-type or *tra2* males, formed sporophytes that were indisinguishable from wild-type sporophytes (data not shown). However, only abnormal sporophytes developed when sibling females were crossed by other males harboring one of the feminizing mutants of this group. Because no combination of any two mutations of this group could restore a wild-type sporophytic phenotype to the sporophyte, each of these feminizing mutations is likely to be recessive in the sporophyte. The inability of these mutations to complement one another *in trans* in the diploid sporophyte provides strong evidence that mutants 2, 3, 6, 7, 8, 15, 16, and 17 are allelic. Since they are allelic and behave similarly in their epistatic interactions with *fem1*, *tra2*, and other sex-determining mutations, as will be shown, these mutations are collectively referred to as *notchless1* (or *not1-1* to *not1-9*) alleles, so named because some of them (not1-2, -7, and -9) lack a meristem notch at maturity.

The *not1-6* (mutant 14) mutant is unique. Sporophytes heterozygous for *not1-6* and *tra2* produced progeny that segregated females, males, and intersexes in a 1:2:1 ratio in the presence of A_{CE}, indicating that the *not1-6 tra2* double mutant is intersex (Table 3). It resembles the *fem1* group mutants in this regard. When crossed by the sperm of a *fem1 tra2* male, however, its progeny gametophytes segregated females, males, intersexes, and asexuals, but not in a 3:2:2:1 ratio, as would be expected if the *not1-6 tra2* double-mutant gametophyte were intersex (Table 3). Although the reasons for the observed segregation distortion are unknown, the presence of males and asexual gametophytes in this population is typical of the *not1* mutants. We group this mutant with the *not1* alleles until evidence showing that it represents another *fem*-like locus is obtained.

Epistasis among the *FEM1* group, *NOT1*, and *HER* genes: All mutant females were crossed by the sperm of *her19* hermaphrodites to determine the phenotypes of the double mutants. As shown in Table 5, sporophytes heterozygous for *fem1* or each of the *not1* alleles and *her19* produced progeny that segregated meristic gametophytes (which included hermaphrodites and females) and males in a 3:1 ratio in the presence of A_{CE}. After scoring, the meristic gametophytes were segregated and permitted to grow further to allow more time for antheridia to develop before determining their sex. Of the meristic gametophytes, hermaphrodites and females segregated in a 1:2 ratio. The segregation ratios of meristics to males and hermaphrodites to females

TABLE 6

Results of crosses to determine the *fem1 man1* and *not1 man1* double-mutant phenotypes

Cross	A_{CE}	No. of females	No. of hermaphrodites	No. of *man1*	No. of males	Expected ratio of females:hermaphrodites: *man1*:males	$\chi^{2\,a}$
fem1 × *man1*	+	160	0	0	164	1:0:0:1	0.20
	−	41	20	17	0	2:1:1:0	0.43
not1-2 × *man1*	+	80	0	0	229	1:0:0:3	0.16
	−	55	44	110	0	1:1:2:0	1.64
not1-3 × *man1*	+	77	0	0	228	1:0:0:3	0.01
	−	35	32	66	0	1:1:2:0	0.15
not1-4 × *man1*	+	77	0	0	223	1:0:0:3	0.07
	−	56	53	100	0	1:1:2:0	0.57
not1-8 × *man1*	+	66	0	0	225	1:0:0:3	0.44
	−	13	17	35	0	1:1:2:0	0.86

[a] All P values are >0.05.

indicate that the *fem1 her19* and *not1 her19* double mutants are female. Similar results were obtained using other *fem1* group mutants (data not shown). On the basis of the double-mutant phenotypes, we conclude that the *FEM1* group and *NOT1* genes are epistatic to *HER19*.

Epistasis among the *FEM1* group, *NOT1*, and *MAN1* genes: Several mutant females were crossed by the sperm of a *man1* hermaphrodite to determine the phenotypes of the *fem1* group *man1* and *not1 man1* double mutants (Table 6). The *man1* phenotype is illustrated in Figure 1C. The *fem1* × *man1* cross resulted in progeny that segregated females and males in a 1:1 ratio in the presence of A_{CE} (Table 6). Similar results were obtained using other *fem1* group mutants (mutants 4, 5, 10, and 11) as females (data not shown). In the absence of A_{CE}, sibling gametophytes segregated females, hermaphrodites, and *man1* gametophytes in a 2:1:1 ratio (Table 6). These results indicate that the *fem1* group *man1* double-mutant gametophytes are female in the presence or absence of A_{CE}. The *not1-2*, *1-3*, *1-4*, and *1-8* × *man1* crosses resulted in progeny that segregated females and males in a 1:3 ratio in the presence of A_{CE}; in the absence of A_{CE}, sibling gametophytes segregate females, hermaphrodites, and *man1* gametophytes in a 1:1:2 ratio (Table 6). These results indicate that the phenotypes of the *not1 man1* double mutants are the same as the *man1* single mutant, *i.e.*, a hermaphrodite with many antheridia in the absence of A_{CE} and male in its presence. On the basis of the double-mutant phenotypes, we can say that the *FEM1* group genes are epistatic to *MAN1*, while *MAN1* is epistatic to *NOT1*.

DISCUSSION

The feminizing mutants of Ceratopteris: Sixteen independently derived mutations of Ceratopteris that completely or partially feminize the gametophyte have been isolated and characterized. Seven of them are very tightly linked and morphologically indistinguishable from the *fem1* mutant previously characterized (Banks 1994). None of these seven *fem* mutants form antheridia, even in the presence of A_{CE}, and all display the same double-mutant phenotype when combined with other sex-determining mutations (*i.e.*, *tra*, *her*, and *man*). For these reasons, they are likely to be alleles of the *FEM1* locus, although the possibility that they are alleles of other very closely linked loci cannot be ruled out. Sporophytes homozygous or heterozygous for these feminizing mutations are indistinguishable from wild-type sporophytes, indicating that they are not necessary for normal sporophyte development. On the basis of the mutant gametophyte phenotypes of these mutants, we conclude that the *FEM1* gene(s) is an important regulator of sexual development that functions to promote the development of antheridia and/or to repress the development of the meristem and archegonium in the gametophyte.

Eight other feminizing mutants characterized define at least one other *fem*-like locus that is unlinked to *FEM1*. Unlike the *fem1* mutants, all but one mutation affected both the gametophyte and sporophyte when homozygous. The lack of complementation among these alleles in the sporophyte indicates that all are alleles of another *FEM*-like locus. To distinguish them from *fem1*, they are referred to as *notchless1* (*not1*) alleles because several of them lack the meristem notch typical of wild-type and *her* hermaphrodites and *fem1* females. All of the *not1* alleles had similar gametophytic phenotypes when combined with other sex-determining mutants, which is expected if they are alleles of the same locus.

The recessive *not1* mutants vary in phenotype, depending on the allele, indicating that they form an allelic series. The strongest *not1* alleles (*not1-1*, *1-4*, *1-7*, and *1-9*) include those that form no antheridia, cannot be self-fertilized, and have aberrant notchless meristems.

TABLE 7

Summary of single-, double-, and triple-mutant phenotypes

Genotype	Phenotype + A_{CE}	Phenotype − A_{CE}	Additional references
Wild type	Male	Hermaphrodite	
feminization1 (*fem1*)	Female	Female	Banks (1994)
notchless1 (*not1*)[a]	Mostly female	Mostly female	
transformer (*tra*)	Male	Male	Banks (1994, 1997b)
hermaphroditic (*her*)	Hermaphrodite	Hermaphrodite	Warne *et al.* (1988); Eberle and Banks (1996)
many antheridia1 (*man1*)	Male	Many antheridia	Banks (1997b)
fem1 tra	Intersex	Intersex	Eberle and Banks (1996)
not1 tra	Male	Male	
fem1 man1[b]	Intersex-female	Female	Banks (1997b)
not1 man1	Male	Many antheridia	
her man1[c]	Many antheridia	Many antheridia	Banks (1997b)
her tra	Male	Male	Eberle and Banks (1996)
tra man1	Male	Male	Banks (1997b)
fem1 her	Female	Female	Eberle and Banks (1996)
not1 her	Female	Female	
fem1 not1	Female	Female	
fem1 man1 tra	Intersex	Intersex	Banks (1997b)
fem1 not1 tra	Asexual	Asexual	

[a] Several *not1* alleles form antheridia, but the number formed is much less than the number formed on wild-type hermaphrodites.

[b] The *fem1 man1* double mutant is intersex early in development but eventually forms a completely female prothallus.

[c] Some leaky *her* alleles are male in the presence of A_{CE} if also mutant for *man1*.

Gametophytes carrying the weakest allele, *not1-2*, produce a normal meristem and several antheridia (but much less than wild-type hermaphrodites) and, when self-fertilized, form normal sporophytes. Alleles with intermediate phenotypes, including *not1-3*, *1-6*, and *1-8*, can produce at least one antheridium, yet after self-fertilization, form abnormal, sterile sporophytes. The pleiotropic effects of most *not1* mutations reveal that the *NOT1* gene is an important regulator of sex expression in the gametophyte and of sporophyte development.

Interactions among the *fem1*, *not1*, and other sex-determining mutants: The epistatic relationships among the new feminizing mutants and previously characterized sex-determining mutants were determined by comparing the phenotypes of double- or triple-mutant gametophytes to single-mutant gametophytes. These results are summarized in Table 7 and have been used as a basis for expanding the model of the sex determination pathway in Ceratopteris, illustrated in Figure 2. Since this model is based solely on genetic analyses, no assumptions regarding the molecular mechanisms involved are implied. According to this model, in Ceratopteris two major groups of sex-determining genes are responsible for the development of male traits (antheridia) or female traits (multicellular meristem and archegonia) during the sex-determining process. One group includes the *TRA* genes, of which there are at least two loci (Banks 1997b), and the other is the *FEM1* locus (Banks 1994). The *TRA* genes have two functions. One is to promote the development of female traits and the other is to repress the development of male traits. To demonstrate that these two functions could be genetically separated, a screen for mutants defective in their ability to repress maleness, but wild type in their ability to promote female traits, was carried out and resulted in the isolation of the *man1* mutant (Banks 1997b). Since the *man1* gametophyte is incapable of repressing antheridia development when A_{CE} is absent, it produces an order of magnitude of more antheridia than normal hermaphrodites. The identification and further characterization of this mutant led to the conclusion that in addition to its female-promoting function, *TRA* represses male development, but does so indirectly by activating the *MAN1* gene. Once activated, *MAN1* represses the gene responsible for male development (*i.e.*, *FEM1*).

The other major sex-determining gene in Ceratopteris is the *FEM1* gene. If *FEM1* functions both to promote maleness and to repress female development, it should be possible to genetically uncouple these two functions. To test this, we sought a *fem*-like mutant that was defective in its ability to repress female development (*i.e.*, able to repress the *TRA* genes), but wild type in its ability to promote the development of male traits. Although able to promote the development of antheridia, this mutant was expected to have no or few

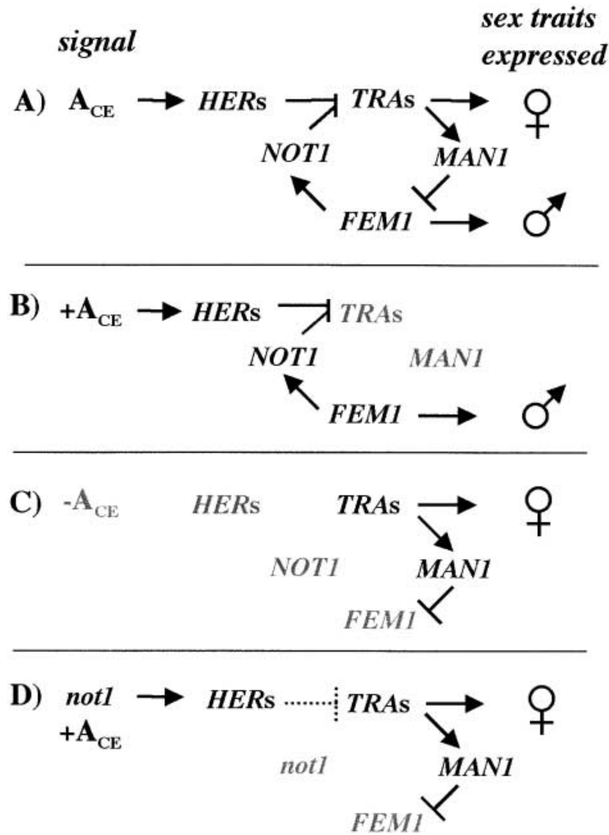

Figure 2.—A model of the sex-determining pathway in Ceratopteris. The potential functions and interactions among the sex-determining genes are shown in A. The activities of the sex-determining genes in wild-type gametophytes grown in the presence of A_{CE} are shown in B, and, in the absence of A_{CE}, in C. (D) The activities of the sex-determining genes in mutant *not1* gametophytes grown in the presence of A_{CE}. The line ending in a bar between the *HER* and *TRA* genes in D is dotted to indicate that although the *HER* genes are active and can repress the *TRA* genes, the *TRA* genes are not repressed. The *TRA* genes are not repressed because repression of *TRA* also requires *FEM1* (discussed in Eberle and Banks 1996) and the *NOT1* genes (this study). Female sex traits include the multicellular meristem and archegonia; the one male sex trait in this species is antheridia. Lines ending in arrows indicate positive regulation (activation) and lines ending in bars represent negative regulation (repression).

antheridia (a *fem1* phenotype) because the *TRA* genes would not be repressed in this mutant, which would lead to the repression of *FEM1*, the gene required for the development of male traits. The results of this study show that, on the basis of their phenotypes and epistatic interactions with other sex-determining mutations, the *not1* mutants fulfill these predictions. We conclude from these results that in addition to its male-promoting function, *FEM1* also functions to repress female development, but does so indirectly by activating the *NOT1* gene, which, in turn, represses the *TRA* genes.

Given that the *FEM1* and *TRA* genes mutually exclude each other's expression via the *NOT1* and *MAN1* genes, respectively, the sex of the Ceratopteris gametophyte ultimately depends on which of two genes (*FEM1* or *TRA*) is activated early in the sex-determining process. This is determined by A_{CE}, the male-inducing pheromone. According to the model, the presence of A_{CE} activates the *HER* genes, which repress the *TRA* genes (Figure 2B). Since the *TRA* genes are not active, *MAN1* is also not active. The absence of active *MAN1* leads to the activation (lack of repression) of *FEM1*. *FEM1* in turn activates *NOT1*, which, together with the active *HER* genes (Eberle and Banks 1996), continues to repress the *TRA* genes. Since the *FEM1* gene is active and the *TRA* genes are not when A_{CE} is present, the young gametophyte develops as a male in the presence of A_{CE}. When A_{CE} is absent (Figure 2C), the activities of the sex-determining genes are reversed; the *TRA* genes are not repressed, which ultimately leads to the repression of *FEM1* by *MAN1*. The gametophyte therefore develops female traits when A_{CE} is absent.

This simple model of the sex-determining pathway in Ceratopteris is inherently flexible, allowing the sex of the gametophyte to be determined by its environment (specifically the presence or absence of pheromone in its surrounding medium) as the spore begins to germinate (Banks 1994) rather than by genetic predetermination. Although consistent with all of the available genetic data thus far, this model does not explain the hermaphrodite, *i.e.*, how antheridia form on an otherwise female gametophyte. One hypothesis is that the *TRA* and/or *MAN1* gene products form a gradient within the meristem, the highest levels being in the center of the meristem where archegonia initiate, and the lowest levels in cells as they exit the meristem, where antheridia are know to initiate. As some cells exit the meristem, the levels of *MAN1* or *TRA* would fall below a level necessary to repress *FEM1*. In this case, *FEM1* would become active, *TRA* would be repressed, and the cell(s) would consequently differentiate as an antheridium. An alternative hypothesis is that another signal emanating from the hermaphrodite prothallus directly or indirectly activates the *NOT1* gene in some cells as they exit the meristem, causing a change in gene activity that ultimately leads to the activation of the *FEM1* gene. Testing of these and other hypotheses to explain how antheridia form on an otherwise female gametophyte will require cloning and molecular analyses of the sex-determining genes in Ceratopteris.

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