Sex-determining genes in the homosporous fern *Ceratopteris*

Jo Ann Banks

Department of Botany and Plant Pathology, Lilly Hall, Purdue University, West Lafayette, IN 47907, USA

SUMMARY

Haploid *Ceratopteris* gametophytes are either hermaphroditic or male. The determinate of sex type is the pheromone antheridiogen (ACE) which is secreted by the meristic hermaphrodite and promotes ameristic male development of sexually undetermined gametophytes. Several mutations affecting the sex of the haploid gametophyte have been isolated and are described. The hermaphroditic (her) mutations are insensitive to ACE and develop as meristic hermaphrodites. These mutations effect ameristic male development in the presence of ACE but have no effect on hermaphroditic development. While most her mutations also have no effect on diploid sporophyte development, some partially ACE-insensitive her mutations have profound effects on sporophyte development. The transformer (tra) mutation effects both meristem and archegonia formation and causes the gametophyte to be an ameristic male under conditions that promote hermaphroditic development. The feminization (fem) mutation effects antheridia development in both male and hermaphroditic gametophytes and causes the gametophyte to develop as a meristic female in the absence or presence of the pheromone. The her1 tra1 double mutant is male in the absence or presence of ACE, indicating that tra1 is epistatic to her1. The phenotypes of her1, tra1 and fem1 single gene mutant phenotypes and the her1 tra1 double mutant phenotype are used to deduce a model suggesting how the products of these genes might interact in a regulatory pathway to control sex determination.

Key words: sex-determination; sexual dimorphism; *Ceratopteris*; antheridiogen; pheromone

INTRODUCTION

Haploid gametophytes of many homosporous ferns, including *Ceratopteris richardii*, develop as males or hermaphrodites. The choice between these alternative developmental fates is determined by the pheromone antheridiogen (Döpp, 1950; Näf, 1979; Näf et al., 1975). In the absence of antheridiogen, single spores germinate and develop as hermaphroditic gametophytes, which produce egg-forming archegonia, sperm-forming antheridia and a well-defined meristem (are meristic). Hermaphrodites produce and secrete antheridiogen into their surroundings when they are no longer competent to respond to it. Spores that germinate and develop in the presence of antheridiogen become males lacking a defined meristem (are ameristic). Thus, in a population, spores that germinate and develop rapidly become hermaphrodites which secrete antheridiogen, while those that develop more slowly become males under the influence of the pheromone.

Physiological studies (Banks et al., 1993) have shown that the *Ceratopteris* gametophyte is competent to respond to its own antheridiogen (ACE) for only a brief period early in development (three to four days after spore inoculation, stage 2) when the spore wall cracks and the gametophyte is between one and four cells in size. Exposure to ACE during this and subsequent stages of gametophyte development is required for male development. If ACE is removed from the supporting medium, sexually undetermined or undifferentiated cells of the male gametophyte revert to the hermaphroditic form. Thus, the male program of expression is reversible indicating that antheridiogen is necessary for both initiating and maintaining the male program of expression. In contrast, the hermaphroditic program of expression is stable in that, once established by the absence of ACE during the critical early stage of development, it cannot be reversed by the addition of ACE at any time thereafter.

Although the structure of ACE is unknown, antheridiogens of other ferns are gibberellins (GAs) (Corey et al., 1987; Yamane et al., 1987; Furber et al., 1988; Takeno et al., 1989). The effectiveness of the GA biosynthesis inhibitors AMO-1618, CCC and ancymidol in blocking the biological response to ACE suggests that the biosynthesis of ACE may have steps in common with the biosynthesis of GA (Warne and Hickok, 1989). Abscisic acid (ABA), a known antagonist of GA responses in flowering plants (Walton, 1980), also blocks the antheridiogen response in *Ceratopteris* (Hickok, 1983). Homosporous ferns are well suited for the study of sex determination in plants primarily because the determinant of sex type is known, as is when and where during development sex determination takes place. Of the homosporous ferns, *Ceratopteris* was chosen for study because it has many features of a model genetic organism (Chasen, 1992; Hickok et al., 1987). Given that each genetically identical *Ceratopteris* spore has the potential to develop as either a hermaphroditic or male gametophyte, the sex of the gametophyte is determined by an epigenetic, hormonally regulated mechanism that acts after sporogenesis and during the gametophyte generation. In order to
define and understand this mechanism, we set out to identify mutations of Ceratopteris with altered responses to ACE, resulting in an abnormal sexual phenotype. Prior genetic screens for X-ray induced, single gene mutations effecting the sex of the gametophyte were successful in identifying ACE and ABA-insensitive mutations. Antheridiogen-insensitive mutations do not respond to ACE and develop as hermaphrodites in its presence (Warne and Hickok, 1991; Warne et al., 1988). Mutant gametophytes that are ABA insensitive do not respond normally to ABA and develop as males in the presence of ACE and ABA, conditions that normally promote hermaphroditic development (Hickok, 1983; Warne and Hickok, 1991). In addition to these mutants, we have identified and characterized an additional 17 mutant lines generated by EMS mutagenesis. Phenotypically, all of the sex-determining mutants fall into four different categories: the hermaphroditic (her) mutants; the transformer (tra) mutants; the feminization (fem) mutants and the ABA-insensitive (abi) mutants. Together, these mutations define at least some of the major regulatory, sex-determining genes in Ceratopteris.

MATERIALS AND METHODS

Plants and growth conditions
The origin of the diploid (n=39) wild-type inbred strain of Ceratopteris richardii (Hmn) is described in Scott and Hickok (1987). The antheridiogen-insensitive mutation HtC23-12, designated here as her1, was selected from X-irradiated Hmn spores of C. richardii (Warne et al., 1988). Procedures for sterilization of spores, gametophyte culture and obtaining antheridiogen are described in Banks et al. (1993). Sporophyte plants were transferred to soil (3:1 mix of Promix:perlite) three weeks after fertilization, covered with plastic sheeting to maintain humidity and allowed to grow for three additional weeks under 40-W cool white fluorescent tubes (23 ±5 W.m⁻²). Plants were then transferred to a greenhouse and placed under a misting system delivering deionized water and fertilized each week. Spores were collected by harvesting mature leaves in glassine bags. Spores release inside the bag as the leaf dries out and germinate at frequencies sufficiently high for genetic analysis one month after leaves are harvested.

Isolation of mutants and genetic analysis
Wild-type or her1 spores were mutagenized with EMS at concentrations that resulted in approximately 50% spore germination and further gametophyte growth. EMS (Sigma Chemical Company) was dissolved in 0.2 M phosphate buffer (pH 6.5) to final concentrations of 0.0 M, 0.85 M, 1.70 M and 2.55 M and then added individually to 10 mg of wild-type or her1 spores. Spores were incubated for 24, 30 and 36 hours at 28°C, washed several times in distilled water, sterilized, then plated on appropriate medium. Between 84 and 96% of spores germinated and grew if not treated with EMS. Incubation in 0.85 M EMS for 30 hours resulted in 59 and 50% germination and growth of wild-type and her1 spores, respectively. These conditions were used to mutagenize 10⁶ wild-type and 8x10⁵ her1 spores. Spores were plated at ~3x10⁴ spores per 150 mm Petri dish.

In selecting her mutations, gametophytes were selected from a mutagenized population of wild-type spores cultured on ACE and placed in microtiter wells with culture medium lacking ACE. Several drops of water were added to each well to promote self-fertilization of the gametophyte. If no embryo formed after two weeks, wild-type sperm were added to each gametophyte. In some cases, gametophytes may have been fertilized by other members of the mutagenized population. The tra1 mutant gametophyte was selected from EMS-treated her1 spores. Males from this population were isolated and grown in microtiter wells on media lacking ACE. Sperm from gametophytes that continued to produce antheridia and no meristem were added to her1 hermaphrodites to produce a sporophyte heterozygous for tra1 and homozygous for her1.

In performing crosses, sperm were obtained by adding room temperature water to a plate of 10-day-old wild-type male gametophytes cultured at 28°C with ACE. The water and the drop in temperature cause the sperm to release. Sperm were then added dropwise to each female in a microtiter well. Immediate swarming of sperm around the archegonia and swelling of the egg 48 hours after adding sperm were indications that the desired fertilization event had occurred. Sperm swarm to the archegonia only if all eggs of the gametophyte are unfertilized.

RESULTS

Wild-type morphology
The wild-type male and hermaphroditic gametophytes can be morphologically distinguished by size and shape as well as type of sex organ produced. Hermaphroditic gametophytes (Fig. 1) are much larger than males due to the development of a lateral meristem that generates the two-dimensional sheet of cells that makes up most of the hermaphrodite thallus. Egg-bearing archegonia develop only adjacent to the meristem notch (Fig. 1). In hermaphrodites, antheridia develop distal to the meristem notch and archegonia. Male gametophytes have no organized meristem (are americist) and are smaller than hermaphrodites (Fig. 1). Most cells of the male thallus differentiate as antheridia with distinctive ring cells, cap cells and helical sperm cells (Fig. 1). The antheridia and archegonia are the only structures of the gametophyte that result from cell divisions and growth in three dimensions. A description of Ceratopteris richardii sporophyte morphology is provided elsewhere (Lloyd, 1974).

The completely ACE-insensitive her mutations
Hermaphroditic (her) mutant gametophytes are insensitive to ACE and are hermaphroditic in the presence of the male-inducing pheromone. These mutations were screened by plating mutagenized wild-type spores on medium containing ACE. Potential her mutants are meristic hermaphrodites on this medium and are easily distinguished from the smaller americist wild-type males that develop in the presence of ACE. From the mutagenized population, 531 hermaphrodites were selected and either selfed or backcrossed by wild-type sperm. Among these, 15 M₁ gametophytes produced F₁ progeny that formed higher frequencies (>20%) of hermaphrodites than wild-type gametophytes when grown on medium containing ACE. Further genetic analysis of the mutant lines showed that, in 10 of the 15 lines (her5, 7, 9, 10, 11, 13, 14, 15, 17 and 19), the her phenotype resulted from a single gene mutation (Table 1). Pairwise crosses to determine allelism by complementation is not possible since the gametophyte is haploid. However, linkage analysis to determine whether two different her mutants are genetically unlinked and non-allelic is currently in progress.

Phenotypically, all ten of the completely ACE-insensitive her mutant lines appear identical to each other at the gametophyte
Sex-determining genes in fern *Ceratopteris*

**Fig. 1.** Phenotypes of wild-type and mutant gametophytes. (A) A 15-day-old wild-type meristic hermaphrodite. (B) A 15-day-old wild-type ameristic male. (C) A 20-day-old *her8* gametophyte that began development as an ameristic male then switched to the meristic hermaphroditic form of development. (D) A 15-day-old *fem1* gametophyte. (E) A magnified view of the archegonia that form adjacent to the meristem notch of wild-type, *her* and *fem* mutant gametophytes. The neck of one archegonium has spread open and attracted several sperm which appear as squiggly lines at the entry of the archegonium. (F) A magnification of the tip of a male gametophyte showing multiple antheridia, each consisting of a cap cell and a ring cell. Abbreviations: an, antheridia; ar, archegonia; mn, meristem notch; rc, ring cell; cc, cap cell; s, sperm. Bar A-D, 200 µm; E and F, 50 µm.
level, i.e. are exclusively hermaphrodites with a normal-sized and -shaped meristem and normal numbers of antheridia and archegonia. Andriatheriogen is secreted from populations of each of these mutant lines in quantities sufficient to induce >90% male development of wild-type gametophytes grown in isolation (data not shown). Sporophytes homozygous or heterozygous for each of these ten mutations are phenotypically indistinguishable from wild type and are fully fertile.

The partially ACE-insensitive her mutations

In the remaining five her lines, the her phenotype did not segregate as a single Mendelian trait but was heritable through at least two generations of backcrossing by wild-type sperm. Hermaphrodites from each of these mutant lines, when crossed by wild-type sperm or self-fertilized, produced greater numbers of hermaphroditic F2 progeny when grown on medium containing ACE compared to wild type but did not produce hermaphrodites to males in a 1:1 or 1:0 ratio, respectively (Table 1). Among these partially ACE-insensitive her lines, considerable phenotypic variation was observed, particularly in the sporophyte. Normal sporophytic development was observed in only two lines. Plant homozygous for her6 and her16 were indistinguishable from wild-type sporophytes. The remaining mutant lines (her8, her12 and her20) each resulted in a unique phenotype in the gametophyte and/or sporophyte generation. These phenotypes were evident in all homozygous sporophytes analyzed after two generations of backcrossing ACE-insensitive hermaphrodites by wild-type sperm. This indicates that the her gametophytic and associated sporophytic phenotypes are due to either a mutation of a single gene or mutations of two different but closely linked genes.

The progeny gametophytes of a heterozygous her8 HER8 sporophyte parent were of three types when grown on medium containing ACE: meristic hermaphrodites (genotypically her8); americist males (genotypically HER8 and/or her8); and gametophytes that began development as americist males which then initiated development of the meristic, hermaphroditic form from undifferentiated cells of the male gametophyte (genotypically her8; Fig. 1). Although undifferentiated cells of the wild-type americist male gametophytes can also initiate the hermaphroditic program of development, the switch requires that the male gametophyte be removed from medium containing ACE for a period of at least one week (data not shown). The her8 gametophytes are unusual in that the switch from male to hermaphrodite can occur in the presence of ACE. Hermaphroditic ‘switching’ her8 gametophytes, when self-fertilized, produce homozygous sporophytes that are abnormal in many ways. They are short-lived, weak and small, producing up to six deformed leaves before dying (Fig. 2). After self-fertilization of her8 gametes, gametophyte growth does not cease as it does in wild type (Fig. 2). Polyembryony is rarely observed in wild type yet is common in self-fertilized her8 plants. Sporangia, if formed in these plants, do not contain viable spores. Heterozygous her8 HER8 sporophytes are phenotypically indistinguishable from wild-type sporophytes, indicating that in the sporophyte, her8 is a recessive lethal mutation.

The her12 mutation also results in partial insensitivity to ACE as less than one-half of the progeny of a her12 HER12 sporophyte parent are hermaphroditic after two generations of backcrossing her12 ACE-insensitive hermaphrodites by wild-type sperm (Table 1). Mutant her12 males and hermaphrodites that develop in the presence of ACE are indistinguishable from wild-type males and hermaphrodites. In the sporophyte, the her12 mutation effects both plant size and structure. Homozygous her12 plants generated by self-fertilizing ACE-insensitive hermaphrodites are of two distinct types, either dwarf and bushy or diminutive in size relative to wild type (Fig. 3). The leaf length of dwarf plants does not exceed 6 cm, averaging only 4.1 cm (Table 2). The mature leaves of the diminutive form of the her12 homozygote average 11.1 cm in length, larger than the dwarf form yet smaller than wild-type leaves of comparable developmental ages (Table 2). The extent of frond dissection is also reduced in all her12 sporophytes relative to wild-type plants (Table 2).

The her12 mutation also affects sporangial development in the sporophyte. In wild-type sporophytes, spore-bearing sporangia develop on the abaxial surface of the leaf (Fig. 4). Marginal leaf tissue curls over the developing sporangia giving the dissected leaf blade a horned appearance and the plant its name (Ceratoic horn). The sporangia of the dwarf plants are frequently replaced by adventitious bud initials. These bud initials develop as plantlets that appear to line each margin of the sporophyte.
Sex-determining genes in fern *Ceratopteris* 1953

**Table 2. Phenotypes of homozygous her sporophytes.**

<table>
<thead>
<tr>
<th>Leaf length</th>
<th>Sporangial development</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>8-40 cm (<em>x</em>=25, <em>n</em>=20)</td>
</tr>
<tr>
<td><em>her12</em> dwarf</td>
<td>2.0-5.4 cm (<em>x</em>=4.1, <em>n</em>=17)</td>
</tr>
<tr>
<td><em>her12</em> diminutive</td>
<td>8.4-15.3 cm (<em>n</em>=11.1, <em>n</em>=19)</td>
</tr>
</tbody>
</table>

**Blade/total leaf length**

<table>
<thead>
<tr>
<th>wild type</th>
<th><em>her18</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>.51:1-.78:1 (<em>x</em>=0.68:1, <em>n</em>=14)</td>
<td>.27:1-.43:1 (<em>x</em>=0.36:1, <em>n</em>=14) spores aborted</td>
</tr>
</tbody>
</table>

**Frond dissection**

<table>
<thead>
<tr>
<th>wild type</th>
<th><em>her18</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>simple, pinnate, bipinnate or tripinate</td>
<td>simple or rarely pinnate</td>
</tr>
<tr>
<td><em>her12</em></td>
<td>simple or rarely pinnate</td>
</tr>
<tr>
<td><em>her18</em></td>
<td>simple or pinnate, rarely bipinnate</td>
</tr>
<tr>
<td><em>her20</em></td>
<td>rarely simple, pinnate, bipinnate or tripinate.</td>
</tr>
</tbody>
</table>

*her20* sporophytes are also only partially insensitive to AC_{E} (Table 1). Hermaphroditic *her20* gametophytes, when selfed, produce sporophytes with an exaggerated horned appearance. In wild-type plants, the transition from sterile to fertile, sporangia-producing leaves is gradual such that the leaves closest to the base of the plant are broad, less dissected and are sterile (Fig. 5), while younger, more distal leaves are more dissected and furled (horned) to support the development of sporangia. In homozygous *her20* sporophytes, the transition from vegetative to reproductive development occurs earlier than in wild type such that almost all leaves have a horned appearance (Fig. 5). Heterozygous plants are phenotypically indistinguishable from homozygous plants, indicating that in the sporophyte, the *her20* mutation is dominant.

**The transformer mutation.**

The *transformer* (*tra*) mutation causes the transformation of hermaphrodites into males under conditions that normally promote hermaphroditic development. Phenotypically, these mutants develop as males in both the absence and presence of AC_{E}. *Transformer* mutations are difficult to select from a population of wild-type gametophytes since in the absence of exogenous AC_{E}, between 40% and 70% of the gametophytes develop as males as a result of the endogenous AC_{E} secreted from hermaphroditic members of the population. The approach used to identify *tra* mutations was to mutagenize a population of AC_{E}-insensitive *HtrC23-12* spores (referred to here as *her1*, see methods) and select amsamic male gametophytes (suppressors of *her1*) on media containing AC_{E}. In a single mutagenesis of *her1* spores, ten male gametophytes were found and transferred to medium lacking AC_{E}. Five produced a meristem after three weeks of culture on media lacking AC_{E}. These resembled wild type and were not analyzed further. The other five continued to develop antheridia and failed to develop a meristem or archegonia after eight weeks on medium lacking AC_{E}. Two of these, when backcrossed to a *her1* hermaphrodite, produced sporophytes. One sporophyte produced no viable spores. Progeny spores of the second fertile sporophyte plant (segregants of the putative *her1 her1; tra1 TRA1* sporophyte) were plated on media with and without AC_{E} in isolation. If *tra1* is a true *transformer* mutation and *her1* and *tra1* are

---

**Fig. 2.** Phenotypes of young wild-type and homozygous *her8* sporophytes. (A) A wild-type sporophyte 10 days after fertilization showing the first three embryonic leaves that are completely round; the remnant gametophyte remains attached to the growing sporophyte. (B) Homozygous *her8* sporophyte 10 days after fertilization. The embryo produced from this gametophyte has one irregularly shaped embryonic leaf. The attached gametophyte continues to grow in the direction indicated by the arrow. Abbreviations: g, gametophyte; s, sporophyte. Bar, 1mm.

---

**Table 2. Phenotypes of homozygous her sporophytes.**

<table>
<thead>
<tr>
<th>Leaf length</th>
<th>Sporangial development</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>8-40 cm (<em>x</em>=25, <em>n</em>=20)</td>
</tr>
<tr>
<td><em>her12</em> dwarf</td>
<td>2.0-5.4 cm (<em>x</em>=4.1, <em>n</em>=17)</td>
</tr>
<tr>
<td><em>her12</em> diminutive</td>
<td>8.4-15.3 cm (<em>n</em>=11.1, <em>n</em>=19)</td>
</tr>
</tbody>
</table>

**Blade/total leaf length**

<table>
<thead>
<tr>
<th>wild type</th>
<th><em>her18</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>.51:1-.78:1 (<em>x</em>=0.68:1, <em>n</em>=14)</td>
<td>.27:1-.43:1 (<em>x</em>=0.36:1, <em>n</em>=14) spores aborted</td>
</tr>
</tbody>
</table>

**Frond dissection**

<table>
<thead>
<tr>
<th>wild type</th>
<th><em>her18</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>simple, pinnate, bipinnate or tripinate</td>
<td>simple or rarely pinnate</td>
</tr>
<tr>
<td><em>her12</em></td>
<td>simple or rarely pinnate</td>
</tr>
<tr>
<td><em>her18</em></td>
<td>simple or pinnate, rarely bipinnate</td>
</tr>
<tr>
<td><em>her20</em></td>
<td>rarely simple, pinnate, bipinnate or tripinate.</td>
</tr>
</tbody>
</table>

*her20* sporophytes are also only partially insensitive to AC_{E} (Table 1). Hermaphroditic *her20* gametophytes, when selfed, produce sporophytes with an exaggerated horned appearance. In wild-type plants, the transition from sterile to fertile, sporangia-producing leaves is gradual such that the leaves closest to the base of the plant are broad, less dissected and are sterile (Fig. 5), while younger, more distal leaves are more dissected and furled (horned) to support the development of sporangia. In homozygous *her20* sporophytes, the transition from vegetative to reproductive development occurs earlier than in wild type such that almost all leaves have a horned appearance (Fig. 5). Heterozygous plants are phenotypically indistinguishable from homozygous plants, indicating that in the sporophyte, the *her20* mutation is dominant.

**The transformer mutation.**

The *transformer* (*tra*) mutation causes the transformation of hermaphrodites into males under conditions that normally promote hermaphroditic development. Phenotypically, these mutants develop as males in both the absence and presence of AC_{E}. *Transformer* mutations are difficult to select from a population of wild-type gametophytes since in the absence of exogenous AC_{E}, between 40% and 70% of the gametophytes develop as males as a result of the endogenous AC_{E} secreted from hermaphroditic members of the population. The approach used to identify *tra* mutations was to mutagenize a population of AC_{E}-insensitive *HtrC23-12* spores (referred to here as *her1*, see methods) and select amsamic male gametophytes (suppressors of *her1*) on media containing AC_{E}. In a single mutagenesis of *her1* spores, ten male gametophytes were found and transferred to medium lacking AC_{E}. Five produced a meristem after three weeks of culture on media lacking AC_{E}. These resembled wild type and were not analyzed further. The other five continued to develop antheridia and failed to develop a meristem or archegonia after eight weeks on medium lacking AC_{E}. Two of these, when backcrossed to a *her1* hermaphrodite, produced sporophytes. One sporophyte produced no viable spores. Progeny spores of the second fertile sporophyte plant (segregants of the putative *her1 her1; tra1 TRA1* sporophyte) were plated on media with and without AC_{E} in isolation. If *tra1* is a true *transformer* mutation and *her1* and *tra1* are

---

**Fig. 2.** Phenotypes of young wild-type and homozygous *her8* sporophytes. (A) A wild-type sporophyte 10 days after fertilization showing the first three embryonic leaves that are completely round; the remnant gametophyte remains attached to the growing sporophyte. (B) Homozygous *her8* sporophyte 10 days after fertilization. The embryo produced from this gametophyte has one irregularly shaped embryonic leaf. The attached gametophyte continues to grow in the direction indicated by the arrow. Abbreviations: g, gametophyte; s, sporophyte. Bar, 1mm.
unlinked, the expected ratio of males to hermaphrodites on media with or without A CE would be 1:1; this was the ratio observed (Table 3). Sperm from males of this backcrossed population of gametophytes were crossed to wild-type hermaphrodites to determine the phenotype of tra1 HER1 gametophytes. In this cross (tra1 her1 x TRA1 HER1), a 3:1 ratio of males to hermaphrodites on media containing A CE is expected assuming that the tra1 HER1 gametophyte is male and tra1 and her1 segregate independently.

Three hundred progeny gametophytes from each of six independent crosses of this type were analyzed. An average of 71% of the gametophytes were male, indicating that the male phenotype was not quite segregating 3:1 (Table 3). The lack of 3:1 segregation is likely due to differences in the germination rates among spores of various genotypes. To test the possibility that either TRA1 HER1, tra1 HER1 or tra1 her1 males are underrepresented in the population of gametophytes, thus skewing the segregation ratios, males previously grown on medium containing A CE were transferred to medium lacking A CE. If TRA1 HER1, tra1 HER1 and tra1 her1 males are equally represented in the population, one-third of the males (i.e. TRA1 HER1 males) should switch to the hermaphrodite form of growth while two-thirds should remain male. Of the 24 male gametophytes transferred to media lacking A CE, 8 developed a meristem and 16 did not. Together, these results indicate that the tra1 gametophytic phenotype is male. The tra1 her1 double mutant gametophyte generated in these crosses is male, indicating that tra1 is epistatic to her1. Sporophytes heterozygous for tra1 are phenotypically indistinguishable from wild-type sporophytes.

The feminization mutations

Feminization (fem) mutations are female, i.e. produce archegonia and a meristem but no antheridia, in either the presence or absence of A CE (Fig. 1). One fem mutant (fem1) was isolated during the screen for her mutations previously described. While her mutations are A CE-insensitive and self-fertile, fem mutations are A CE-insensitive and self-infertile but are capable of being fertilized by wild-type sperm. The fem females, when crossed by wild-type sperm, produce heterozygous sporophytes which segregate female: male progeny gametophytes in a 1:1 ratio when grown on media containing A CE (Table 3), and a 1:1 ratio of female: hermaphrodite when grown on media lacking A CE (data not shown), indicating that fem1 segregates as a single Mendelian trait. Sporophytes heterozygous for fem1 are indistinguishable from wild type.

![Fig. 3. Phenotypes of mature wild-type and homozygous her12 sporophytes. (A). 8-week-old wild-type sporophytes. The tall, youngest leaves are furled and bear sporangia. (B). 10-week-old homozygous her12 sporophytes that are either diminutive (plant on left) or dwarfed (plant on right) in size. Bar, 5cm](image-url)
**Table 3. Segregation analysis of the fem1 and tra1 mutations.**

<table>
<thead>
<tr>
<th>Parent sporophyte genotype</th>
<th>Expected ratio of male: hermaphrodite (female) progeny</th>
<th>Observed ratio of male: hermaphrodite (female) progeny</th>
<th>n*</th>
<th>homogeneity chi square</th>
<th>summed data chi square</th>
<th>chi square</th>
</tr>
</thead>
<tbody>
<tr>
<td>fem1 FEM1</td>
<td>1:1†</td>
<td>1:1†</td>
<td>4</td>
<td>0.6; P&gt;0.5</td>
<td>0.6; P&gt;0.5</td>
<td>0.8; P&gt;0.3</td>
</tr>
<tr>
<td>tra1; her1</td>
<td>3:1†</td>
<td>1423:594</td>
<td>7</td>
<td>4.8; P&lt;0.05</td>
<td>23.1; P=0</td>
<td></td>
</tr>
<tr>
<td>tra1; her1 HER1</td>
<td>3:1†</td>
<td>1423:594</td>
<td>7</td>
<td>4.8; P&lt;0.05</td>
<td>23.1; P=0</td>
<td></td>
</tr>
</tbody>
</table>

*number of crosses analyzed.
†progeny grown on media with exogenous A CE.
‡progeny grown on media without exogenous A CE and in isolation.

**DISCUSSION**

**Major regulatory genes involved in sex determination in Ceratopteris and their function**

Several major genes necessary for appropriate sex expression, as determined by the absence or presence of the pheromone A CE, have been identified by EMS or X-ray mutagenesis of haploid, single-celled spores of *Ceratopteris*. Based on their mutant phenotypes and limited genetic analyses, these mutations fall into four different categories, including the hermaphroditic mutations, the transformer mutations, the feminization mutations, and the abscisic acid-insensitive mutations.

Mutations that completely block the ability of the developing gametophyte to sense or respond to A CE are always hermaphroditic. These her mutants have no effect on hermaphroditic development, indicating that wild-type HER activity is not required for the development of meristem, archegonia and antheridia in the meristic gametophyte. Wild-type HER gene activity is required for the suppression of meristic and archegonia development that is mediated by A CE. Given that all completely A CE-insensitive her lines produce and secrete A CE, these mutations do not appear to affect genes involved in the biosynthesis or secretion of A CE. Loss-of-function her mutations likely define genes whose wild-type gene products include the cellular receptor of A CE or components of the A CE signal transduction pathway. If the secreted form of A CE is converted or modified to an active form by the gametophyte in order to be effective in directing male development, her mutations may also affect this processing. Although many independent her mutations have been identified thus far, we do not know how many different gene loci are represented by these mutations. There are likely to be several her loci given that mutation of any of the genes involved in the A CE signal transduction pathway(s) will yield a her phenotype. Complementation analysis of two mutants is not possible in the gametophyte since it is haploid. Through a process referred to as apospory, it is possible to generate diploid gametophytes from diploid sporophytic tissue. By crossing two diploid gametes, tetraploid *Ceratopteris* sporophytes can be generated (Eberle, personal communication) which should produce diploid progeny spores. The ability of two mutations to complement each other in diploid gametophytes can be analysed by exploiting this process.

Sporophytes homozygous for all completely A CE-insensitive her mutations have wild-type phenotypes. This indicates that the antheridiogen response, or at least those aspects of the response affected by these mutations, is not essential nor involved in sporophyte growth and development. Since the A CE response is not essential for viability, it lends itself to the study of genes involved in hormone reception/perception in plants.

Several other her mutant lines identified in this study are partially insensitive to A CE. These mutations (herb, 8, 12, 16 and 20) alter, but do not abolish, the ability of the gametophyte to respond to A CE to develop as males. With the exception of herb and her16, these mutations also have a profound effect on sporophyte growth and development. The dwarf, bushy phenotype of some homozygous her12 sporophytes is typical of GA-sensitive and GA-insensitive dwarf mutants of *Arabidopsis* (Koornneef and van der Ven, 1980; Koornneef et al., 1985), maize (Phinney, 1956; Harberd and Freeling, 1989) and *Brassica* (Zanewich et al., 1991). If the phenotypes of her8, 12 and 20 homozygous sporophytes are due to defects in the ability to synthesize or respond to GA or some other hormone, it would suggest that hormones in addition to A CE are directly or indirectly involved in sex determination. Assuming that a single gene mutation is responsible for the gametophytic and sporophytic mutant phenotypes observed in her8, 12 or her20 plants, the pleiotropic effect on both gametophytic and sporophytic development could also be explained by the inappropriate expression of gametophytic genes in the sporophyte, which could alter sporophytic development. To illustrate, unlike wild-type gametophytes, the A CE-insensitive her8 gametophyte continues meristematic and sex organ growth after fertilization. This mutation thus appears to affect the repression of gametophytic genes, including sex-determining genes, that is mediated by the fertilization event. Expression of normally repressed gametophytic genes in the homozygous her8 sporophyte could hypothetically affect its development. Further genetic analysis will be required to determine whether the mutant gametophytic and sporophytic phenotypes are due to one or more closely linked mutations before a clear causal relationship between mutant gametophytic and sporophytic development can be made.

The tra1 mutant is also insensitive to A CE; however, unlike the her mutants, a meristem and archegonia fail to form under otherwise permissive conditions. Wild-type TRA1 activity is thus required for meristem and archegonia formation in the gametophyte. Not surprisingly, the stability of the male phenotype is also affected in tra1 mutants. Sexually undetermined or undifferentiated cells of the wild-type male gametophyte switch to the meristic, hermaphroditic form when A CE is removed from the supporting medium. The male program of expression in tra1 males, however, is stable and insensitive to fluctuations in A CE levels. This indicates that, in the wild-type male gametophyte, TRA1 activity can be activated or dere-
pressed if $A_{CE}$ is withdrawn from its environment. The ability to activate or derepress TRA1 activity in response to withdrawal of $A_{CE}$ is eliminated in the $tra1$ mutant gametophyte.

The phenotypes of $her$ and $tra$ mutants of Ceratopteris resemble those found in Caenorhabditis elegans. In C. elegans, the $tra$ gene is a major switch gene controlling its sexual phenotype (Hodgkin, 1987). Recessive loss-of-function $tra$ alleles cause XX animals (normally hermaphroditic) to develop into fertile males. Dominant gain-of-function $tra$ alleles cause XO animals (normally male) to develop into fertile females. By analogy, some gain-of-function $tra$ mutations in Ceratopteris are expected to have a $her$ phenotype and may be present among the collection of $her$ mutant lines. Epistatic suppression of $her1$ by the $tra1$ mutation in Ceratopteris is also similar to interactions between $tra$ and $her$ genes in Caenorhabditis (Hodgkin, 1980).

The $fem1$ mutant gametophyte produces a meristem, multiple archegonia and no antheridia in either the absence or presence of $A_{CE}$. This phenotype indicates that wild-type $FEM1$ activity is required for antheridial development in both meristic hermaphroditic and ameiotic male gametophytes. Genetic screens for mutations that prevent antheridial development in the meristic hermaphrodite but not in the ameiotic male are currently in progress. Such mutants, if they exist, would define genes that are required for antheridial development only in the hermaphrodite.

The fourth class of sex-determining genes in Ceratopteris is defined by mutations that render the gametophyte insensitive to $ABA$. Hickok (1983) first demonstrated that $ABA$ blocks the effects of $A_{CE}$ such that wild-type spores cultured with $A_{CE}$ and $ABA (>10^5 M)$ develop exclusively as small, meristic, archegoniate gametophytes with no or few antheridia. Hickok (1985) isolated two abscisic acid resistant ($abr$) mutant gametophytes by selecting large hermaphroditic gametophytes from a mutagenized population of spores grown on media containing $ABA$. Both $abr$ mutant gametophytes are insensitive to $ABA (>10^5 M)$ and develop as males in the presence of $A_{CE}$ and $ABA$ or as normal hermaphrodites in the presence of only $ABA$. The wild-type $ABR$ gene is thus required to block the effect of $A_{CE}$ in promoting male development when exogenous $ABA$ is present. However, there is no evidence or indication that $ABA$, like $A_{CE}$, is secreted to act exogenously as a pheromone. Endogenous levels of $ABA$ in populations of gametophytes are highest in ungerminated spores and drop as the spore walls crack and gametophyte growth begins (Warne and Hickok, 1989). High levels of endogenous $ABA$ correspond to the stage of development when the wild-type gametophyte is not competent to respond to $A_{CE}$ (stage 1; Banks et al., 1993). Mutant $abr$ gametophytes are more competent to respond to $A_{CE}$ during the first stage of development than wild-type gametophytes; i.e., produce more males when exposed to $A_{CE}$ during stage one than wild-type gametophytes (Eberle and Banks, unpublished data). These observations indicate that one possible role of endogenous $ABA$ in sexual development is to block the $A_{CE}$ response during this stage of development.

Fig. 4. Adventitious bud and sporangial development in wild-type and homozygous $her12$ sporophytes. (A) The adaxial surface of a wild-type sterile leaf showing the position of adventitious bud initiation and growth. (B) The abaxial surface of a wild-type fertile leaf showing the placement of sporangia along the margins of the leaf. Some of the lower leaf margin has been removed to reveal the sporangia. (C) The abaxial surface of a mature leaf of a homozygous $her12$ sporophyte (diminutive form) showing sporangia and adventitious bud initiation and growth where sporangia normally develop. (D) The abaxial leaf surface of a dwarf $her12$ sporophyte. Abbreviations: ab, adventitious bud; s, sporangium; sp, spores. Bar, 1 mm.
A simple model for the control of sex determination in Ceratopteris

A preliminary and simple model explaining how some of the key regulatory sex-determining genes interact to specify male or hermaphroditic development of the Ceratopteris gametophyte is illustrated in Fig. 6. This model is based on physiological studies of the antheridiogen response (Banks et al., 1993) and the phenotypes of her1, tra1 and fem1 single mutants and the her1 tra1 double mutant gametophytes. The nature of the regulatory steps in this model are unknown and cannot be predicted at this time.

The tra1 her1 double mutant gametophyte is phenotypically male, indicating that tra1 is epistatic to her1. One interpretation of this result is that HER1, when active, functions (directly or indirectly) to repress the TRA1 gene or its gene product. Because of this interaction, the sex-determining signal ACE is probably required only to activate HER1. This interpretation is most consistent with the observations that mutation of HER1 and/or absence of ACE leads to hermaphroditic development, while mutation of TRA1 and/or presence of ACE leads to ameristic male development. Therefore, when a wild-type gametophyte that is competent to respond to ACE is exposed to ACE, HER1 is activated. Because HER1 is a repressor of TRA1 activity, TRA1 is not active. Since TRA1 activity is required for archegonia and meristem formation, neither structure forms in the gametophyte. The FEM1 gene, which is active in the absence or presence of ACE, promotes the expression of genes required for antheridia development. The gametophyte is thus an ameristic male in the presence of ACE.

In the absence of ACE, HER1 is not active. TRA1 and FEM1 are both expressed, and the gametophyte develops archegonia, antheridia and a meristem (is hermaphroditic). The FEM1 gene, which is required for antheridia development, is thought to be constitutively active (in the sense that it is not regulated by ACE) because antheridia develop in both the hermaphroditic and male gametophyte and in the absence or presence of ACE. It is unknown at this time why antheridia do not form in the region of the hermaphroditic gametophyte occupied by the meristem and archegonia.

The fem1 gametophyte is meristic and produces archegonia even in the presence of ACE, indicating that TRA1 is expressed in the fem1 gametophyte even though its repressor, HER1, is active. This observation suggests that the active HER1 and FEM1 gene products are both necessary for the repression of TRA1. If FEM1 were not involved in repressing TRA1 activity, the fem1 gametophyte should develop as an ameristic, asexual gametophyte in the presence of ACE since under these conditions neither the TRA1 gene (required for meristem and archegonia development) nor the FEM1 gene (required for antheridia development) would be active. A genetic analysis of

Fig. 6. A model of proposed interactions between three major sex-determining genes when (A) ACE is present and the gametophyte is competent to respond to ACE, or (B) ACE is absent or ACE is present but the gametophyte is not competent to respond to it. Pointed arrows indicate activating interactions; barred arrows indicate repressing interactions. See text for further description.
double and triple mutant gametophytes will be used to test and develop this model further.

The general organization of the sex-determination gene network in *Caenorhabditis richardii* thus far appears similar in some aspects to that found in *Drosophila melanogaster* and, in particular, *Caenorhabditis elegans* (reviewed by Hodgkin, 1990). However, there are at least two major differences between *Ceratopteris* and these two animal species in the biology of sex determination that will likely be reflected in their underlying genetic and molecular mechanisms of sex determination. First, the primary sex-determining signal in *Ceratopteris* is a pheromone. In *Drosophila* and *Caenorhabditis*, the signal is the ratio of X chromosomes to autosomes. Second, *Drosophila* and *Caenorhabditis* have documented or proposed mechanisms to insure that once the sex is initially set by the X:A ratio during embryonic development, the organism maintains a single sexual fate throughout development without having to respond continuously to the primary sex-determining signal (reviewed by Hodgkin, 1990; Kuwabara and Kimble, 1992; Cline, 1993). While the same is true for the *Ceratopteris* hermaphroditic gametophyte, the male gametophyte adopts the meristic, hermaphroditic program of expression if the primary signal is removed. The underlying mechanism controlling male sex determination must, therefore, be able continually to monitor and respond to the sex-determining signal and regulate sex-determining genes appropriately. Given that sex determination is a vital developmental process in both plants and animals, it will be interesting to understand at a mechanistic and molecular level how such disparate organisms achieve the common goal of generating sexual dimorphism.

I wish to thank Jill Nemacheck and Corey Linkel for their assistance in scoring gametophytes and harvesting spores, James Eberle, Chi-Kuang Wen, Mitsuyasu Hasabe, George Rutherford and Les Hickok for helpful discussion and reading of the manuscript. This work is supported by a grant from the National Science Foundation. This is journal paper no. 14103 of the Purdue University Agricultural Experiment Station.

REFERENCES


(accepted 2 April 1994)