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ANI1: A Sex Pheromone–Induced Gene in Ceratopteris Gametophytes and Its Possible Role in Sex Determination

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Antheridiogen (A_{CE}) is a pheromone that is required for the development of male gametophytes in the homosporous fern *Ceratopteris richardii*. Subtractive hybridization of cDNAs isolated from A_{CE}-treated and non-A_{CE}-treated gametophytes was used to isolate genes that are induced by the pheromone. The expression of one gene, *ANI1* (for antheridiogen induced), was induced within 3 hr of A_{CE} treatment, but its expression was transient. Patterns of *ANI1* expression in wild-type and mutant gametophytes show that *ANI1* expression inversely correlates with the predicted activity of one of the sex-determining genes, *TRANSFORMER5* (*TRA5*). These data suggest that *ANI1* transcription or transcript accumulation is directly or indirectly prevented by TRA5 in the absence of A_{CE} and that A_{CE} inactivates the *TRA5* gene or its product, leading to the upregulation of *ANI1*. Cycloheximide (no A_{CE}) induced the expression of *ANI1*, also indicating that *ANI1* expression is subject to negative regulation in the absence of A_{CE}. The sequence and inferred protein structure of *ANI1* suggest that it is a novel, extracellular protein. The secreted portion of the ANI1 protein potentially forms a β barrel with superficial similarities to lipocalins, which bind small hydrophobic molecules such as pheromones, steroids, and odorants. ANI1 may be an extracellular carrier of A_{CE} that is required to initiate the male program of development as the sexual fate of the young gametophyte is determined.

INTRODUCTION

The haploid gametophytes of many homosporous ferns, including *Ceratopteris richardii*, develop as either males or hermaphrodites (Figures 1A and 1B). The sex of the gametophyte is determined by the pheromone antheridiogen, or A_{CE} in Ceratopteris (for antheridiogen Ceratopteris) (Schedlbauer and Klekowski, 1972; Banks et al., 1993). Ceratopteris spores develop as male gametophytes with numerous sperm-forming antheridia if they are continuously exposed to exogenous A_{CE} from the time of spore germination. If A_{CE} is removed from the medium surrounding an older male gametophyte, undifferentiated cells of the male prothallus will divide and differentiate a hermaphroditic prothallus, indicating that A_{CE} is required to both initiate and maintain the male program of expression. In the absence of A_{CE}, single spores develop as hermaphroditic gametophytes. These begin to produce and secrete A_{CE} into the surrounding medium after they lose the ability to respond to the pheromone. The loss of sensitivity to A_{CE} coincides with the development of a lateral, multicellular meristem, which is unique to the hermaphrodite (Figure 1A) (Banks et al., 1993). Exogenous antheridiogen thus performs several functions in the young, sexually undetermined gametophyte: it promotes the differentiation of sperm-forming antheridia and simultaneously suppresses the development of the meristem, the egg-forming archegonia, and the synthesis of antheridiogen (Banks, 1997a).

Although the structure of A_{CE} is unknown, several lines of evidence suggest that A_{CE} is a gibberellin (GA). GA biosynthetic inhibitors block the male-inducing response, indicating that GA and A_{CE} share a common biosynthetic pathway in Ceratopteris (Warne and Hickok, 1989). Abscisic acid, a known antagonist of GA responses, also antagonizes the male-inducing A_{CE} response (Hickok, 1983; Warne and Hickok, 1991), further implicating a GA-like molecule in sex determination in Ceratopteris. All known antheridiogens of other homosporous ferns have been identified as GAs and include GA_{109} (Wynne et al., 1998), GA_{73} methyl ester (Takeno et al., 1989; Yamauchi et al., 1996), antheridic acid (Yamauchi et al., 1991), 3-epi-GA_{63}, and 3α-hydroxy-9,15-cycle-GA_9 (Yamauchi et al., 1995). Finally, partial characterization of an antheridiogen of Ceratopteris indicates that it is a small hydrophobic molecule with a molecular mass of $\sim$300 D (Koitabashi, 1996), similar to other GAs.

Ceratopteris is a particularly useful system for studying antheridiogen and how it regulates sex determination in ferns because it is very easy to isolate and genetically characterize mutations that affect sex expression in this species. Although a genetic approach has been useful for identifying

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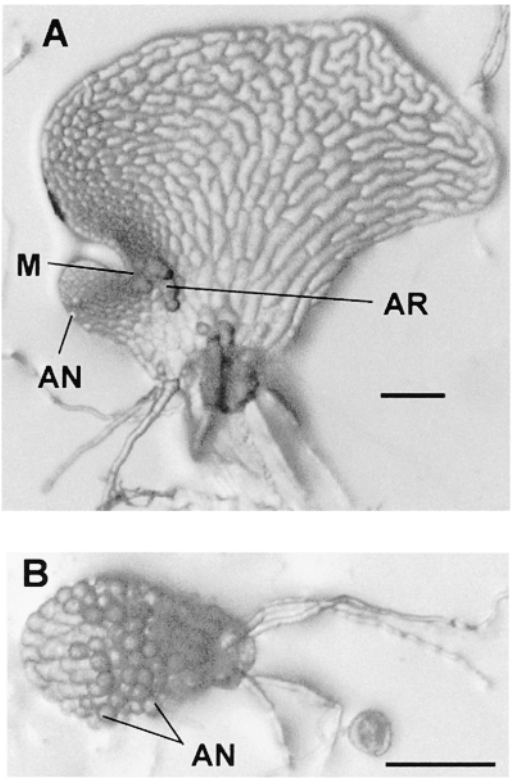

Figure 1. The Ceratopteris Gametophytes.

(A) The hermaphrodite, which develops in the absence of A_{CE}, forms a meristem (M), several egg-forming archegonia (AR), and sperm-forming antheridia (AN).

(B) The male, which develops only in the presence of A_{CE}, has no meristem or archegonia. Almost all cells of the male prothallus differentiate as antheridia.

Both gametophytes are 14 days old. Bars = 250 μm.

sex-determining genes and dissecting the sex determination pathway in Ceratopteris (Banks, 1994, 1997b; Eberle and Banks, 1996), cloning these genes will be difficult due to the large genome size of Ceratopteris ($\sim 8 \times 10^9$ bp per haploid genome) and the lack of a transformation system. To understand how antheridiogen controls the sex of the gametophyte, we used a polymerase chain reaction–coupled subtractive hybridization technique (Wang and Brown, 1991) to clone genes whose expression is induced by A_{CE} during the brief period of time that the sex of the gametophyte is determined by the presence of A_{CE}. One such gene, termed *ANI1* (for antheridiogen-induced), has been cloned, and its expression in wild-type and mutant gametophytes has been assessed. The predicted structure of the protein encoded by *ANI1* indicates that it may be an extracellular carrier of small hydrophobic molecules. Its possible role in sex determination is discussed.

RESULTS

Timing of A_{CE} Secretion and the Competence to Respond to A_{CE} in Developing Gametophytes

Because this study was intended to identify early A_{CE}-inducible genes, it was first necessary to define the period of time during which a population of developing gametophytes is competent to respond to exogenous A_{CE}. This was determined by periodically transferring developing gametophytes grown in medium lacking A_{CE} (fern medium [FM]) to medium containing exogenous A_{CE} (conditioned fern medium [CFM]). If a gametophyte is competent to respond to A_{CE} at the time of transfer, it will develop as a male gametophyte in the presence of exogenous A_{CE}. If a gametophyte is not competent to respond to A_{CE} at the time of transfer, it will develop as a hermaphrodite. Gametophytes that were transferred to CFM 80 hr after spore inoculation developed exclusively as males. Of those transferred to CFM 99 hr after spore inoculation, only 33% developed as males, whereas 16% of the spores transferred to CFM 104 hr after spore inoculation developed as males. These results indicate that if gametophytes have not perceived A_{CE} after 104 hr, most of them will have lost the competence to respond to A_{CE} and will therefore develop as hermaphrodites. Accordingly, adding A_{CE} (CFM) to gametophytes $<$80 hr old should be sufficient to induce A_{CE}-responsive genes in all members of a population.

When grown in the absence of exogenous A_{CE}, some members of a population of gametophytes develop as males in response to endogenous A_{CE} secreted by the faster growing, hermaphroditic members of the population. The presence of endogenous A_{CE} in the culture medium makes it difficult to generate the population of gametophytes not exposed to A_{CE} and thus not expressing A_{CE}-induced genes required for this study. Therefore, it was necessary to establish when biologically effective amounts of A_{CE} are first detectable in the medium supporting growing gametophytes. Medium lacking exogenous A_{CE} that had supported the growth of gametophytes for 72, 82, 96, and 102 hr was added to wild-type spores. These spores developed exclusively as hermaphrodites, indicating that biologically effective amounts of A_{CE} were absent in the medium. Medium that had supported the growth of gametophytes for 112 hr resulted in the development of 100% male gametophytes.

Based on these results, biologically detectable amounts of endogenous A_{CE} are not present in the medium supporting the growth of gametophytes until between 102 and 112 hr after spore inoculation. Because the assay for A_{CE} is biological, trace amounts of endogenous A_{CE} may be present in media that have supported the growth of gametophytes for $<$102 hr, but the concentration is below that required to initiate male development. Thus, RNA isolated from spores germinated for up to 100 hr in FM should not contain messages from any A_{CE}-inducible genes required for the initiation of male development.

Cloning of A_{CE}-Inducible Genes by Subtractive Hybridization

Genes induced by exogenous A_{CE} in gametophytes that were competent to respond to A_{CE} were cloned using a polymerase chain reaction–coupled subtraction and hybridization procedure. This procedure removes sequences that are common between two populations of cDNAs generated from two populations of gametophytes. To generate the two populations of gametophytes, we initially cultured spores in medium lacking A_{CE} (FM) for 72 hr. One population was then transferred to A_{CE}-containing medium (CFM) and cultured for an additional 28 hr, and the other population was cultured for the same period of time but in the absence of exogenous A_{CE}. cDNAs generated from CFM- or A_{CE}-treated gametophytes are referred to as tracer cDNA; cDNAs generated from FM- or non-A_{CE}-treated gametophytes are referred to as driver cDNA. After each round of hybridization/subtraction to remove driver cDNAs from the population of tracer cDNAs, the efficiency of subtraction was tested by probing subtracted tracer cDNA populations with Ceratopteris malate dehydrogenase (MDH) or elongation factor-1α (EF-1α) probes that had been cloned previously (C.K. Wen and C. Juarez, unpublished results). Virtually no MDH cDNA remained after one round of hybridization and subtraction, whereas virtually all EF-1α cDNA was removed after two rounds of hybridization and subtraction (data not shown). After two rounds of hybridization and subtraction, the remaining tracer cDNAs were cloned. Six independent cDNA inserts were used to probe RNA gel blots; their corresponding messages were shown to accumulate in young gametophytes whether or not they had been treated with exogenous A_{CE} (data not shown). The majority of the cloned cDNAs thus appeared to represent germination-related genes and not the desired A_{CE}-induced genes.

To further eliminate germination-related genes from the population of cloned tracer cDNAs, we probed 20,000 *Escherichia coli* colonies, each containing a tracer cDNA insert, with labeled cDNAs originating from 78-hr-old gametophytes grown in FM (no A_{CE}). The same colonies also were probed with a cDNA population that had been enriched for cDNAs present only in non-A_{CE}-treated gametophytes by subtractive hybridization. After primary and secondary screenings using these two probes, 51 non-cross-hybridizing colonies were obtained. The inserts of each of the 51 clones were partially sequenced and then sorted into 15 unique groups based on their sequence. A representative cDNA insert of each group was then used to probe gel blots carrying RNA isolated from A_{CE}-treated and non-A_{CE}-treated gametophytes. Based on these results (data not shown), 12 of the 15 unique cDNA inserts appeared to represent A_{CE}-inducible genes.

Any cDNA insert identified using this protocol is likely to represent only part of a full-length gene because the cDNAs were initially digested with AluI early in the hybridization and subtraction protocol. To determine how many genes were represented by the collection of enriched cDNA fragments, we used the inserts individually as probes to screen a cDNA library that was constructed from mRNA isolated from 100-hr-old gametophytes that had been grown in the presence of exogenous A_{CE} for 28 hr before harvesting. Each cDNA insert hybridized with one of two unique recombinant plaques. Based on these results, the enriched cDNAs represent parts of two distinct genes or gene families, known as *ANI1* and *ANI2*. The expression and characterization of *ANI1* are described here.

Expression of *ANI1* in Wild-Type and Mutant Gametophytes

RNA gel blots show that the ~1.3-kb *ANI1* transcript is induced in young gametophytes between 30 min and 3 hr after treatment with exogenous A_{CE} (Figure 2). A low basal level of *ANI1* expression can be detected in 87- and 100-hr-old gametophytes that have not been exposed to exogenous A_{CE} (Figure 2). In gametophytes that have been continuously exposed to exogenous A_{CE}, the expression of *ANI1* rapidly declines between 110 and 130 hr (Figure 2). Even after prolonged exposure of RNA gel blots, virtually no expression of *ANI1* could be detected in a mixed population of mature wild-type male and hermaphroditic gametophytes (data not shown) or in sporophylls of the diploid sporophyte plant (Figure 2). The temporal pattern of *ANI1* expression in wild-type gametophytes indicates that *ANI1* is induced by A_{CE} and that its expression is transient in the developing gametophyte. The transient expression of *ANI1* correlates with the initiation, but not the maintenance, of the A_{CE}-induced male program of development in wild-type gametophytes.

The control of sexual identity in Ceratopteris gametophytes is ultimately controlled by two classes of key regulatory genes, the *FEMINIZATION* (*FEM*) and the *TRANSFORMER*

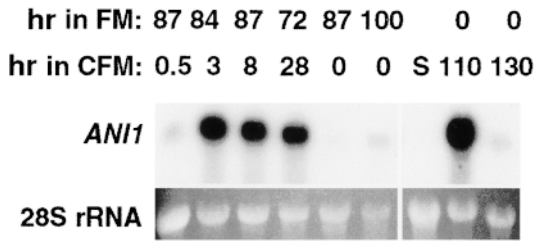

Figure 2. *ANI1* Is Upregulated by A_{CE} in Young Gametophytes.

Total RNAs were prepared from variously treated wild-type gametophyte populations and sporophylls of the sporophyte plant (lane S). The period of time that gametophytes were grown in FM (lacking A_{CE}) is indicated at top. Gametophytes were then transferred to CFM (containing A_{CE}) for the period of time indicated below. Ethidium bromide–stained 28S rRNA bands are shown as loading controls.

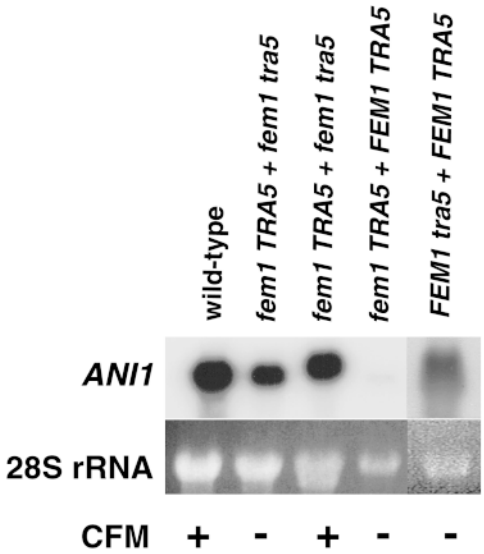

Figure 3. *ANI1* Expression in Various Mutant Lines.

Total RNAs were prepared from gametophyte populations grown in FM lacking A_{CE} for 90 hr (lanes − CFM) or grown for 72 hr in FM and then transferred to CFM and cultured for an additional 28 hr (lanes + CFM). Each population contained wild-type gametophytes or equal numbers of gametophytes of the given genotypes. Ethidium bromide–stained 28S rRNA bands are shown as loading controls.

(*TRA*) genes. Mutant *fem* gametophytes always develop as females, even in the presence of A_{CE}, whereas mutant *tra* gametophytes always develop as males, indicating that the male program of expression is activated independently of A_{CE} in the *tra* male. If *ANI1* is involved in sex determination, its expression or accumulation is predicted to be affected by mutations that alter the sex of the gametophyte. To test the involvement of *ANI1* in sex determination, we assessed the expression of *ANI1* in *fem1* and *tra5* mutant gametophytes. Because both *fem* and *tra* mutant gametophytes cannot be self-fertilized (they make only one kind of gamete), it is impossible to produce a pure population of *fem* or *tra* gametophytes. Therefore, mixed populations of gametophytes of known genotypes were used to assess *ANI1* expression in mutant backgrounds.

In a mixed population of gametophytes containing equal numbers of mutant *fem1 TRA5* and *fem1 tra5* double mutant gametophytes, *ANI1* RNA accumulates to high levels when grown in the absence or presence of A_{CE}, as shown in Figure 3. Therefore, a mutation in either the *tra5* or *fem1* gene leads to an accumulation of *ANI1* in the absence of exogenous A_{CE}. To determine which of these mutations results in A_{CE}-independent expression of *ANI1*, we assessed the expression of *ANI1* in mixed populations of either *FEM1* and *fem1* gametophytes or *TRA5* and *tra5* gametophytes by using RNA gel blot analysis. The population containing both *FEM1* and *fem1* gametophytes did not accumulate *ANI1* when grown in the absence of A_{CE}, whereas the population of *TRA5* and *tra5* gametophytes did accumulate *ANI1* transcripts in the absence of exogenous A_{CE} (Figure 3). These results indicate that a mutation of *TRA5*, but not *FEM1*, results in the accumulation of *ANI1* in the absence of A_{CE}. The expression of *ANI1* in the *FEM1 fem1* and *TRA5 tra5* populations treated with A_{CE} is uninformative because *ANI1* is expressed in the wild-type members of each population in response to the added A_{CE}.

Cycloheximide Treatment Results in A_{CE}-Independent Expression of *ANI1*

Young gametophytes were pretreated with cycloheximide (CHX) before adding A_{CE} to determine whether A_{CE}-induced expression of *ANI1* in wild-type gametophytes requires de novo protein synthesis. Preliminary studies, summarized in Tables 1 and 2, demonstrated that 20 μM CHX was sufficient to inhibit de novo protein synthesis by 85% in young gametophytes after 30 min of CHX treatment. Gametophytes that were treated with 20 μM CHX for 30 min before adding A_{CE} accumulated ANI1 at high levels, as determined by RNA gel blot hybridization (Figure 4). Thus, A_{CE}-induced expression of *ANI1* does not require the synthesis of new proteins. However, a 30-min treatment of gametophytes with 20 μM CHX alone (no A_{CE}) also results in an accumulation of *ANI1* RNA (Figure 4). This suggests that *ANI1* can be induced independently of A_{CE} by preventing the synthesis of a protein that acts as a repressor of *ANI1*, a destabilizer of the *ANI1* transcript, or both.

Structure of the *ANI1* Gene and Inferred Protein

The nucleotide sequence of *ANI1*, based on the sequence of the longest cDNA clone obtained, is shown in Figure 5. The length of the *ANI1* transcript (1.3 kb) determined by RNA gel

Table 1. Effects of CHX Concentration on Protein Synthesis in Gametophytes

CHX (μM)[a]	CPM[b]	Incorporation (%)[c]
0	10,166	100
5	5,700	56
10	3,408	34
20	2,951	29

[a] CHX and ^{35}S-L-methionine were added to gametophytes at 3 hr and 20 min and at 3 hr, respectively, before harvesting.
[b] Total protein was extracted from each gametophyte population and trichloroacetic acid precipitated, and the tricholoracetic acid precipitate was counted.
[c] Percentage of ^{35}S-L-methionine incorporated into total protein relative to no CHX treatment control.

Table 2. Effects of Varying the Time of CHX Treatment on Protein Synthesis in Gametophytes

Time (hr) in CHX[a]	CPM[b]	Incorporation (%)[c]
0	87,002	100
0.5	12,840	15
1.0	7,585	9
2.0	6,425	7
3.0	7,635	9
4.0	6,555	8

[a] Gametophytes were incubated for various periods of time in 20 μM CHX before adding ^{35}S-L-methionine; gametophytes were then cultured for 3 hr before harvesting.

[b] Total protein was extracted from each gametophyte population and trichloroacetic acid precipitated, and the tricholoracetic acid precipitate was counted.

[c] Percentage of ^{35}S-L-methionine incorporated into total protein relative to no CHX treatment control.

blot hybridization is somewhat longer than is the longest cDNA clone (939 bp). Based on DNA gel blot analysis, shown in Figure 6, *ANI1* is a member of a multigene family in Ceratopteris. The entire *ANI1* gene hybridizes with two major and several minor genomic fragments when used to probe DNA gel blots containing genomic DNA digested with two different restriction enzymes and washed under stringent conditions. Hybridization patterns using a probe corresponding to the 5′ end of the cDNA or the 3′ untranslated region of the gene were similar in that both probes hybridized strongly to two DNA fragments and more weakly to other DNA fragments (Figure 6), indicating that *ANI1* is a member of a gene family. Three independent *ANI1* cDNA clones obtained from the same cDNA library were sequenced and shown to be >99% identical to one another (data not shown).

The deduced protein encoded by the largest open reading frame of the *ANI1* cDNA (Figure 5) is 182 amino acids with a predicted pI of 9.4 and molecular mass of 22 kD. The nucleotide and predicted amino acid sequences of *ANI1* share no significant similarity with other expressed sequence tags or proteins present in the databases, and thus *ANI1* encodes a novel protein. The putative ANI1 protein contains two different repetitive motifs: two tandem copies of the 18–amino acid sequence PSTTVY(G/E)(K/E)EEKP(D/E)F(D/Y)(Y/R)YK and five tandem copies of the eight–amino acid consensus sequence VVIYKPKP. A sixth repeat (VVVYKEKE), which is less similar to the consensus sequence than it is to the others, precedes and is separated from the five tandem repeats. These repetitive sequences account for more than one-half of the total amino acid residues of ANI1.

The N-terminal residues (7 to 29) are hydrophobic and are predicted to form an inside–outside type II transmembrane domain, indicating that ANI1 is targeted to the outside of the plasma membrane. The sequence NASA, located at positions 25 to 28, corresponds to the consensus of N-glycosylation sites, indicating that ANI1 may be glycosylated at position 25. A potential signal sequence cleavage site (Nielsen et al., 1997) occurs at residues 28 to 29. If cleaved, ANI1 is likely to be an extracellular protein that is not anchored to the membrane. A hydrophilic region (positions 31 to 81) containing the two 18–amino acid repeats follows the transmembrane domain of the protein. Following the hydrophilic region, the protein is predicted to form a series of short, hydrophobic β strands that alternate with hydrophilic loops/turns. The number of predicted β strands varies, depending on the algorithm used, and ranges from five to eight. However, five of the six repeats are always predicted to form β strands, regardless of the algorithm used. Making up these β strands are the first four residues (usually VVIY) of the eight–amino acid repeat (Figure 5). One or usually two proline residues separate the β strands and may cause a turn where they occur. Taken together, this region of the polypeptide could form a repeated anti-parallel structure with a +1 topology that folds into a β barrel.

DISCUSSION

The antheridiogens of homosporous ferns are small, hydrophobic, GA-like pheromones that are required to induce male and repress female development of the gametophyte

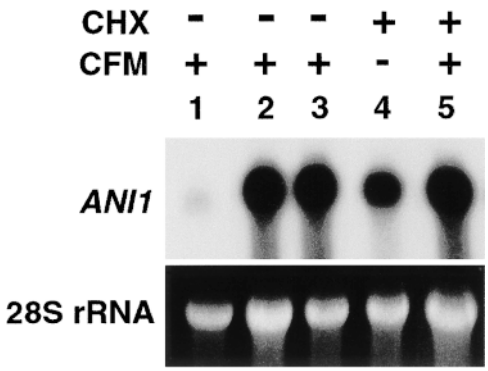

Figure 4. Effects of CHX on *ANI1* Expression.

Total RNAs were prepared from wild-type gametophyte populations grown for 84 hr before adding CFM (lanes + CFM) and/or CHX (lanes + CHX). Gametophytes grown only in FM (no A_{CE}) or without CHX are indicated by − CFM and − CHX, respectively.

In lane 1, gametophytes were cultured in CFM for 30 min before harvesting; in lane 2, gametophytes were cultured in CFM for 3 hr before harvesting; in lane 3, gametophytes were cultured in CFM for 8 hr before harvesting; in lane 4, gametophytes were cultured in 20 μM CHX (no A_{CE}) for 3 hr before harvesting; and in lane 5, gametophytes were cultured in 20 μM CHX (no A_{CE}) for 30 min, then transferred to CFM and cultured for 3 hr before harvesting. Ethidium bromide–stained 28S rRNA bands are shown as loading controls.

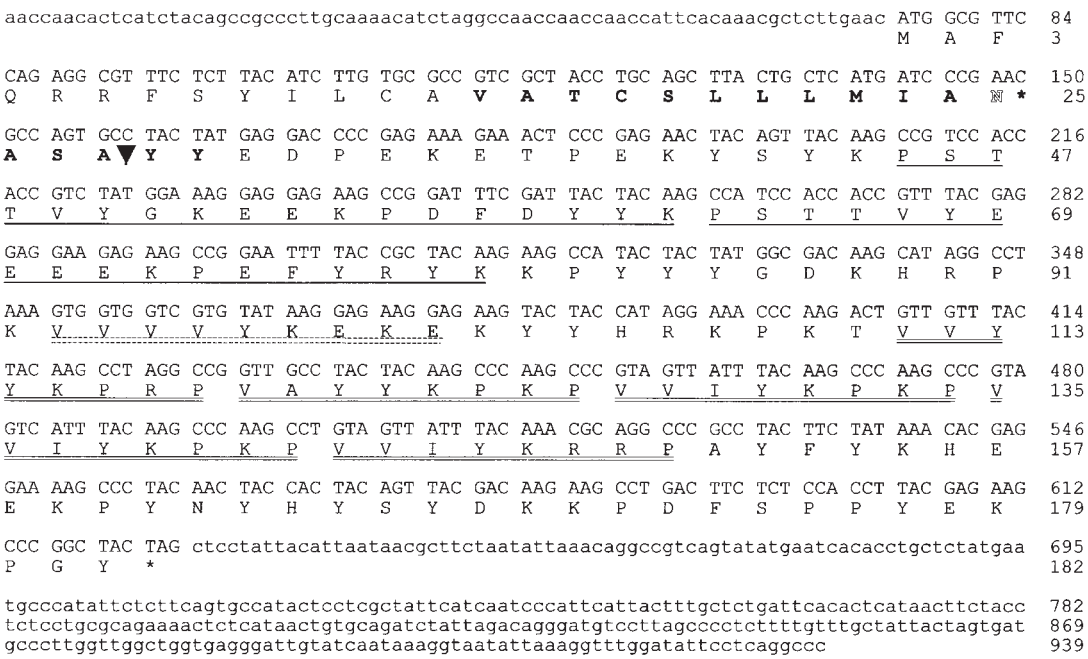

Figure 5. *ANI1* cDNA and Deduced Amino Acid Sequence.

The DNA sequence of the longest *ANI1* cDNA (GenBank accession number AF113324) is shown, with the predicted amino acid sequence indicated underneath. Nucleotides in lowercase represent 5′ and 3′ untranslated sequences. The residues predicted to form a transmembrane region are shown in boldface. A potential cleavage site is indicated by a solid triangle, and a potential N-glycosylation site is indicated by a boldface asterisk adjacent to a shadowed N. The two 18–amino acid repeats are underlined, and the five highly conserved six–amino acid repeats are double underlined, with a sixth, less highly conserved repeat indicated by dashed underlines.

at concentrations as low as 10^{-14} M (Yamane, 1998). To understand how the antheridiogen of Ceratopteris functions at a molecular level, we isolated and characterized the gene *ANI1*, which is induced by A_{CE} in young Ceratopteris gametophytes at a time when they are competent to respond to the sex-determining signal. The rapid induction of *ANI1* by A_{CE} and the A_{CE}-independent expression of *ANI1* in *tra5* mutant gametophytes that constitutively express the male program of expression in the absence of A_{CE} provide evidence that *ANI1* functions in determining the sex of the developing gametophyte.

The A_{CE}-independent expression of *ANI1* in CHX-treated gametophytes indicates that A_{CE} induces the expression of *ANI1* by downregulating a repressor of *ANI1* transcription or by downregulating a factor that destabilizes the *ANI1* transcript. By preventing the synthesis of this factor or repressor, CHX treatment results in the accumulation of *ANI1* in gametophytes grown in the absence of A_{CE}. Accordingly, when A_{CE} and CHX are not present in the medium, *ANI1* transcription is repressed, or if transcribed, its transcripts are rapidly degraded in the developing gametophyte.

The proposed regulatory interactions between the major sex-determining genes in Ceratopteris that have been identified by mutation are shown in Figure 7A. By analyzing the expression of *ANI1* in *fem1* and *tra5* mutant backgrounds, we have shown that the expression of *ANI1* inversely correlates with the predicted activity of the *TRA5* gene and shows no correlation with other sex-determining genes (Figure 7B). Based on this inverse correlation, it is likely that the repressor of *ANI1* expression, or the factor that destabilizes its transcript, is either encoded by or regulated by the *TRA5* gene. Therefore, we propose that A_{CE} initiates male sex determination in the gametophyte by repressing or inactivating *TRA5*. Because *TRA5* encodes the factor that directly or indirectly affects *ANI1* expression, the repression or inactivation of *TRA5* by A_{CE} results in the accumulation of *ANI1* transcripts.

The induction of *ANI1* by A_{CE} in Ceratopteris is typical of other hormone-induced genes of flowering plants, including the auxin (*Aux*)/*IAA* family of genes, which are induced by indoleacetic acid (IAA) (reviewed in Abel and Theologis, 1996), and the *IBC6* and *IBC7* genes, which are induced by cytokinin (Brandstatter and Kieber, 1998). Their induction is relatively rapid (within 10 to 30 min after hormone treatment) and CHX insensitive. Each is also induced by CHX alone. Although the lag between A_{CE} treatment and *ANI1* induction is longer (between 0.5 and 3 hr of A_{CE} treatment), *ANI1* is induced by CHX, whereas its induction by A_{CE} is CHX insensi-

tive. The CHX insensitivity of these pheromone/hormone–induced genes suggests that all may be primary response genes in that their induction does not require the synthesis of new proteins. However, that all are also induced by CHX alone suggests that these genes are subject to negative regulation and that the hormone or pheromone acts to derepress their transcription or to prevent transcript degradation (Koshiba et al., 1995; Brandstatter and Kieber, 1998). Because CHX alone induces *ANI1* in Ceratopteris, it is not clear if the induction of *ANI1* by A_{CE} is insensitive to CHX or not.

Many of the auxin-, cytokinin-, and ethylene-induced genes in plants encode proteins that are components of their own signal transduction pathways. The *Aux*/*IAA* genes encode short-lived proteins that form homodimers or interact with other members of the Aux/IAA family of proteins to activate the transcription of auxin-responsive genes (Kim et al., 1997). One member, *IAA24*, is similar to ARF1, a transcription factor that binds to an early auxin-responsive element (Ulmasov et al., 1997). The recent demonstration that *AXR3*, a gene defined by mutations that result in increased auxin responses, is *IAA17* provides direct evidence that the *Aux*/*IAA* genes are involved in auxin signaling (Rouse et al., 1998). The cytokinin-induced *IBC6* and *IBC7* genes encode proteins that are similar to the receiver domain of bacterial two-component regulators, indicating that these proteins are involved in cytokinin signaling. Although neither IBC6 nor IBC7 has been shown to bind cytokinin, further evidence that bacterial two-component regulator-like proteins are involved in cytokinin signaling comes from the recent cloning of *CKI1* (Kakimoto, 1996). The *cki1* mutant of Arabidopsis is insensitive to cytokinin, and *CKI1* encodes a protein homologous to histidine kinase and receiver domains of two-component regulators. Finally, three of the five members of the ethylene receptor gene family in Arabidopsis (*ERS1*, *ETR2*, and *ERS2*) are upregulated by ethylene (Hua et al., 1998). These genes also encode proteins homologous to bacterial two-component regulators. A fourth member of this family (*ETR1*) has been shown to bind ethylene in vitro (Schaller and Bleecker, 1995).

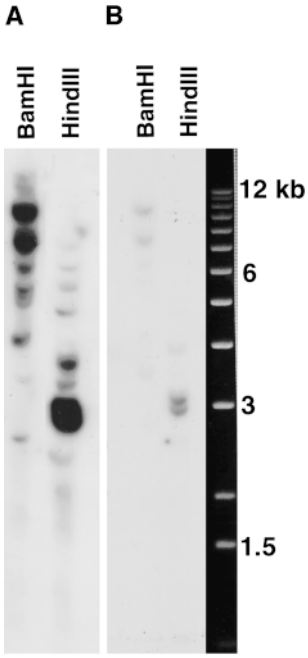

Figure 6. DNA Gel Blot Analysis of *ANI1*.

Ceratopteris genomic was DNA digested with BamHI or HindIII.
(A) The 5′ end of *ANI1* (nucleotides 1 to 351) was used as a probe.
(B) The 3′ untranslated region of ANI1 (nucleotides 723 to 939) was used as a probe. An ethidium bromide–stained 12-kb ladder is shown at right.

Although the putative protein encoded by *ANI1* shows no homology to other proteins, its predicted tertiary structure superficially resembles the lipocalin superfamily of proteins. Lipocalins are small (151 to 188 amino acids) extracellular proteins with eight antiparallel β strands that fold into a β barrel. Although lipocalin family members may share $<20\%$ overall sequence identity, they are unified by their similar tertiary structures (reviewed in Flower, 1996). In animals, lipocalins bind small hydrophobic molecules, among them pheromones, retinoids, odorants, steroids, and prostaglandin. They function in olfaction, sterol, retinol, and pheromone transport, prostaglandin synthesis, cell regulation, immune modulation, and invertebrate coloration (Flower, 1996). Most are synthesized by secretory tissues and transported through the plasma to target tissues. Some lipocalins, such as retinol binding protein (RBP), bind to cell surface receptors, at which either the retinol–RBP complex or retinol alone is transferred into the interior of the cell (Bavik et al., 1992; Smeland et al., 1995). Other lipocalins, such as the pheromone and odorant binding proteins (OBPs), are thought to concentrate specific odorants or pheromones at the site of the receptor. Although some OBPs have been shown to bind specific ligands (Lobel et al., 1998), proof that binding of ligand–OBP complex to specific receptors or evidence that OBPs concentrate odorants remains elusive.

Although the crystal structure of ANI1 is unknown at this time, its superficial resemblance to lipocalins suggests that ANI1 might also bind small hydrophobic molecules. Given that A_{CE} is a small hydrophobic molecule (Koitabshi, 1996), we speculate that ANI1 is an extracellular protein that binds to A_{CE} to facilitate the transport of antheridiogen from an aqueous medium to its receptor, to concentrate A_{CE} at the site of the receptor, or to bind to the receptor itself. All of these possibilities place ANI1 early in the A_{CE} signal transduction pathway that leads to the initiation of the male program of development in the gametophyte by A_{CE}. Because *ANI1* is only transiently expressed, it is unlikely that it functions in the maintenance of the male program of development, which requires continued exposure to A_{CE}. Studies to determine the crystal structure of ANI1, its cellular localization, and its ability to bind A_{CE} are currently in progress.

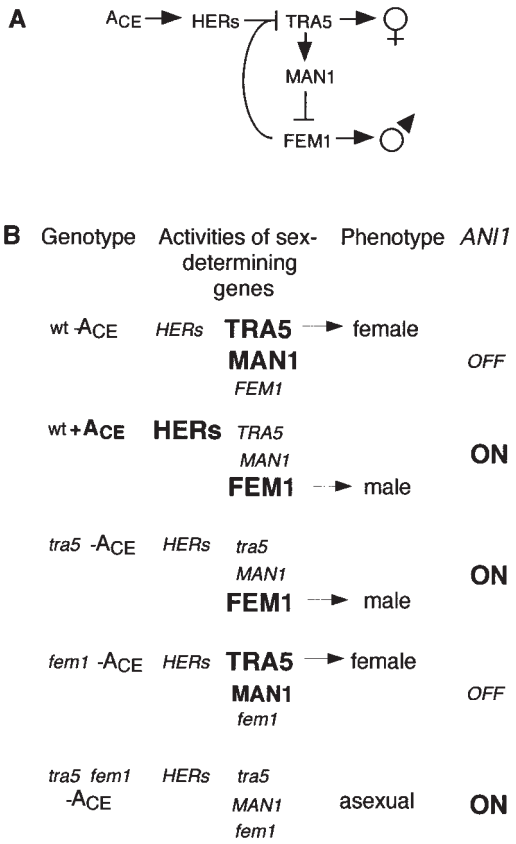

Figure 7. A Model of the Sex-Determining Pathway in Ceratopteris.

(A) The predicted regulatory interactions among the major classes of sex-determining genes identified by mutation are shown (described in detail by Banks [1997b]). Arrows indicate positive (activating) interactions; t-bars indicate negative (repressing) interactions. The *her* (*hermaphroditic*) mutants are hermaphroditic in the presence or absence of A_{CE}; the *tra* (*transformer*) mutants are always male; the *man1* (*many antheridia1*) mutant is male in the presence of A_{CE} and hermaphroditic in the absence of A_{CE}, but each hermaphrodite produces ~10 times more antheridia than does the wild-type hermaphrodite; and the *fem1* (*feminization1*) mutant is similar to the *her* mutants except that the *fem* gametophyte produces no antheridia.
(B) The predicted activities of the sex-determining genes shown in **(A)**, depending on the absence or presence of A_{CE} and the genotype of the gametophyte. Genes in boldface indicate that the genes are active, whereas genes in italics indicate that the genes are not active. Also indicated is the expression of *ANI1* in the same gametophytes (results of this study). ON indicates that *ANI1* is expressed, and *OFF* indicates that it is not expressed. wt, wild type.
Because the model in **(A)** and predicted activities of the sex-determining genes in **(B)** are derived from genetic data only, the molecular mechanisms underlying these gene interactions or gene activities are unknown.

Why plant hormones induce genes that encode proteins involved in their own signal transduction pathways is unknown. It has been suggested that the induction of some ethylene receptor genes by ethylene may be a mechanism for adapting to ethylene. If the different receptors have different affinities for ethylene, the induction of receptors with a low affinity for ethylene could allow the plant to become desensitized to ethylene such that higher and higher ethylene concentrations are needed to maintain a response (Hua and Meyerowitz, 1998). In the case of cytokinin-induced genes that also display a basal level of expression in the absence of cytokinin, their upregulation by cytokinin may be necessary to amplify the signal and its response (Brandstatter and Kieber, 1998). Given that *ANI1* is expressed at low levels in non-A_{CE}-treated gametophytes, it is possible that the induction of *ANI1* is required to amplify A_{CE} signaling or its response. Such amplification may be necessary to initiate male development in the gametophyte when antheridiogens are present at extremely low concentrations.

Given that there are at least two *TRA* loci (Banks, 1997b), two different active antheridiogens in CFM that are separable by thin-layer chromatography and HPLC (E. Strain, C. Chapple, and J.A. Banks, unpublished data), and multiple *ANI1*-like genes in Ceratopteris, the regulation of sex determination by A_{CE} is likely to be complex. Continued studies to identify and integrate these components in the sex determination process in Ceratopteris should be useful in helping us to understand how pheromones regulate complex developmental processes in plants.

METHODS

Strains and Growth Conditions

The origins of Hnn, the wild-type stain of *Ceratopteris richardii*, and the *feminization1* (*fem1*) and *transformer5* (*tra5*) sex-determining mutants as well as the conditions for gametophyte and sporophyte culture are described by Banks (1994, 1997b). Gametophytes were cultured at a density of 1 g L^{-1} liquid medium unless noted otherwise. Spores were cultured either in fern medium (FM) lacking Ceratopteris antheridiogen (A_{CE}) or in conditioned fern medium (CFM) containing exogenous A_{CE}, prepared according to Banks (1994). CFM is a crude aqueous extract of medium that has supported the growth of wild-type gametophytes for 14 days.

Physiology of the A_{CE} Response

The period of time that developing gametophytes are sensitive to exogenous A_{CE} was determined by adding wild-type spores to FM in a single flask. Spores or developing gametophytes were periodically removed from the flask and placed individually into microtiter wells containing CFM. The sex of each gametophyte was scored 14 days after spore inoculation. The timing of A_{CE} secretion was determined by adding wild-type spores to FM in a single flask. At varying periods of time, an aliquot of medium was removed, the spores or developing

gametophytes removed by filtration, and the medium placed in individual microtiter wells. Fresh wild-type spores were then added individually to each well. The sex of each gametophyte was then scored 14 days after spore inoculation. In all experiments, 300 gametophytes were scored per time point or treatment.

DNA and RNA Isolation and Preparation of cDNA

Nucleic acids were isolated from gametophytes by mixing ground gametophytes in a buffer containing 4 M guanidine thiocyanide, 1 M ammonium thiocyanide, 0.3 M sodium acetate, pH 5.2, 1% sarcosine, 1% polyvinylpolypyrrlidone, and 0.5% β-mercaptoethanol. After three chloroform–isoamylalcohol (24:1) extractions, nucleic acids were precipitated with 2.5 volumes of ethanol. RNA was further purified by hexadecyltrimethylammonium bromide (CTAB) precipitation (Murray and Thompson, 1980), except that 2% CTAB and 0.84 M NaCl were used. DNA was isolated in the same way, except that 0.3 M sodium acetate in the initial extraction buffer was replaced with 0.1 M Tris-HCl, pH 7.5.

cDNAs were synthesized with an oligo(dT) primer, using the SuperScriptII system (Life Technologies, Gaithersburg, MD). cDNAs were either used in the gene expression screen or cloned into the λ ZipLox vector (Life Technologies).

Gene Expression Screen

Two populations of cDNAs, one derived from 80-hr-old A_{CE}-treated gametophytes (the tracer cDNA) and the other from 80-hr-old non-A_{CE}-treated gametophytes (the driver cDNA), were digested with AluI. The remaining subtraction/hybridization steps to remove driver cDNAs from tracer cDNA population were performed according to Wang and Brown (1991) with the following modifications. Two sets of adapters, rather than one, were ligated to the AluI-digested cDNA fragments. The sequences of the oligonucleotides used for linker preparation are as follows: J1, 5′-CTCTTGCTTGAATTCGGACTA-3′; J2, 5′-TAGTCCGAATTCAAGCAAGAGCACA-3′; CH1, 5′-ATCAGGCTTAAGTTCGTTCTC-3′; and CH2, 5′-GAGAACGAACTTAAGCCTGATCACA-3′. Biotin-labeled J1 or CH1 was used to generate driver cDNA, whereas nonlabeled J1 or CH1 was used to generate tracer cDNA. After three rounds of hybridization and subtraction, the remaining cDNA fragments were cloned into the pBluescript SK+ vector (Stratagene, La Jolla, CA).

Differential Screening by Colony Hybridization

Twenty thousand colonies containing single tracer cDNA inserts were transferred to nitrocellulose membranes and hybridized with ^{32}P-labeled cDNA isolated from 80-hr-old gametophytes not treated with A_{CE}. Replicate membranes were probed with labeled driver cDNA from the gene expression screen. The cloned inserts from colonies that did not hybridize to either probe were selected.

RNA and DNA Gel Blot Analyses

For RNA gel blots, 5 μg of total RNA prepared from gametophytes of various ages either treated or not treated with A_{CE} was size fractionated on formaldehyde gels and blotted onto nylon membranes according to the manufacturer's instructions (Hybond N^+; Amersham, Piscataway, NJ). For DNA gel blots, 10 μg of restriction-digested genomic DNA was size fractionated and blotted onto nylon membranes, according to the manufacturer's instructions (Hybond N^+; Amersham). RNA and DNA gel blots were hybridized to probes labeled according to Mertz and Rashtchian (1994) by using hybridization conditions described by Church and Gilbert (1984).

DNA Sequencing and Sequence Analysis

DNA sequencing was performed by the Iowa State University (Ames, IA) sequencing facilities, by the Purdue University sequencing facility, or by manual sequencing according to the manufacturer's instructions (Fidelity; Ventana Medical Systems, Tucson, AZ). Sequence similarities were sought using the BLAST system available through the National Center for Biotechnology Information (Bethesda, MD) (http://www.nim.nih.gov/cgi-bin/BLAST/). The TMpred program (Hofmann and Stoffel, 1993) was used to predict membrane-spanning regions and their orientation. Predictions of protein secondary structure were made using the following programs available on the World Wide Web: Predator (Frishman and Argos, 1996), Gibrat (Gibrat et al., 1987), Double Prediction (Deleage and Roux, 1987), SSP (Solovyev and Salamov, 1994), and GOR IV (Garnier et al., 1996).

Cycloheximide Treatment

The amount of cycloheximide (CHX) required to block in vivo protein synthesis was determined by culturing spores (400 mg per 400 mL of FM) for 72 hr and then adjusting the density of spores to 400 mg per 4 mL of FM. The spores were divided into four tubes, 100 μCi of ^{35}S-L-methionine was added to each tube, and each tube was incubated for 20 min. CHX (0, 5, 10, or 20 μM) was then added, and the gametophytes were cultured for an additional 3 hr before extracting total protein (Hames, 1990). To determine the minimum period of time that gametophytes must be exposed to CHX to block protein synthesis, similar experiments were performed, except that gametophytes were cultured in the presence of 20 μM CHX for varying periods of time before adding 100 μCi of ^{35}S-L-methionine; cultures were then incubated for 3 hr before extracting total protein. Total protein was fractionated by SDS-PAGE and stained with Coomassie Brilliant Blue R 250 (Life Technologies) to ensure that each sample contained approximately the same amount of protein. The incorporation of ^{35}S-L-methionine into proteins after various treatments was determined by autoradiography of gels and trichloroacetic acid precipitation according to Boefey (1990).

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ANI1: A Sex Pheromone–Induced Gene in Ceratopteris Gametophytes and Its Possible Role in Sex Determination

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