

Evidence for a Gibberellin Biosynthetic Origin of *Ceratopteris* Antheridiogen¹

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ABSTRACT

The species-specific chemical messenger, antheridiogen A_{ce} , mediates the differentiation of male gametophytes in the fern *Ceratopteris*. In order to investigate the biochemical origin of antheridiogen, the effect of the inhibitors, 2'-isopropyl-4'-(trimethylammoniumchloride)-5'-methylphenylpiperidine-1-carboxylate (AMO-1618), 2-chloroethyl trimethylammonium chloride (CCC), and α -cyclopropyl- α -(4-methoxyphenyl)-5-pyrimidine methyl alcohol (ancymidol) on gametophytic sex expression was determined in *C. richardii*. Both AMO-1618 and ancymidol blocked the production of male gametophytes in three genetically defined strains of *C. richardii* that exhibit different sensitivities to antheridiogen. Antheridiogen supplementation overcame inhibition by AMO-1618 and ancymidol, except in one strain (HaC18) that is insensitive to antheridiogen supplementation. These data suggest that the synthesis of *Ceratopteris* antheridiogen, a taxon that is insensitive to exogenously supplied gibberellins, occurs via a pathway that may include steps in common with gibberellin biosynthesis or involves similar reactions.

In many ferns a chemical messenger, antheridiogen, controls sex expression in gametophytes either by promoting premature initiation of male sex organs (antheridia) or by determining morphologically distinct males (18). In *Ceratopteris*, antheridiogen accumulates within a population of developing gametophytes and affects sensitive gametophytes, presumably those which develop more slowly or germinate later (24, 26). At sexual maturity a dimorphic gametophytic population consists of large cordate and small spatulate individuals. Cordates are either female or hermaphroditic and possess a defined meristematic region, whereas males are ameristic and bear only antheridia. Characteristic frequencies of sexual types depend upon population dependent antheridiogen production and accumulation, the sensitivity of gametophytes, and asynchrony in population development (17, 24, 29, 31, 32). A genetic basis for this trait, suggested by extensive phenotypic variability, has recently been identified and described (15, 29, 33).

Antheridiogen-like responses occur in a wide variety of ferns; however, most information on antheridiogen comes from four taxa: *Pteridium* (Polypodiaceae), *Anemia*, *Lygodium* (Schizaceae), and *Ceratopteris* (Parkeriaceae) (18). Variability among taxa in biological cross-sensitivity to antheri-

diogens and responsiveness of gametophytes suggest extensive diversity among the chemical structures of antheridiogens and acceptor sites or receptors down to the species level (18, 20, 22). However, all antheridiogens apparently share some basic chemical properties, *i.e.* they are readily dialyzable and of low mol wt (<2000), possess a pKa of about 5.0, and are relatively stable at pH and temperature extremes (25).

Chemical structures are known for several antheridiogens from Schizeaceous taxa. An antheridiogen from *Anemia phyllitidis* (L.) Sw. (A_{An}), identified as a rearranged gibbane (19) has been synthesized and its structural formula revised (5, 6). Zanno *et al.* (37) demonstrated identity for antheridiogens from *A. phyllitidis* and *Anemia hirsuta* (L.) Sw. An antheridiogen of *Lygodium japonicum* (Thumb.) Sw. (A_{Ly}) has been identified as GA₉ methyl ester (36). Recently, Nester *et al.* (22) reported a partial characterization of an *Anemia mexicana* Klotzsch antheridiogen which is distinct from the antheridiogens of *A. phyllitidis*, *A. hirsuta*, and *L. japonicum*. Structural similarity of these antheridiogens to GAs is reflected in sensitivity of these and other Schizeaceous taxa to a variety of exogenously supplied GAs and conjugated GAs (27, 30). In addition, Weinberg and Voeller (35) demonstrated a block in germination and early gametophyte development by the GA biosynthetic inhibitor, AMO-1618²; however, Nester and Coolbaugh (20) were unable to confirm this.

Exogenously supplied GAs are ineffective in non-Schizeaceous taxa (30), such as *Ceratopteris* (23; TR Warne, LG Hickok, unpublished data) *Dryopteris filix-mas* (7), and *Polypodium crassifolium* (27). The absence of GA sensitivity and the limited studies on the chemical nature of non-Schizeaceous antheridiogen preclude biochemical, developmental and evolutionary comparisons to the GA and antheridiogen responses of *Anemia* and *Lygodium* and to the GA responses of higher plants (28).

In this paper, we demonstrate a positive effect of inhibitors of GA biosynthesis on sex expression in three strains of *Ceratopteris* that exhibit different sensitivities to antheridiogen.

MATERIALS AND METHODS

Plant Material

Spores of the diploid ($n = 39$) homosporous fern, *Ceratopteris richardii* from three strains were used in this study, the

² Abbreviations: AMO-1618, 2'-isopropyl-4'-(trimethylammoniumchloride)-5'-methylphenylpiperidine-1-carboxylate; CCC, 2-chloroethyl trimethylammonium chloride; ancymidol, α -cyclopropyl- α -(4-methoxyphenyl)-5-pyrimidine methyl alcohol.

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wild type (Hn-n) (12), and two derived mutants, HaA48, (13, 14), and HaC18 (33). HaA48 is an abscisic acid tolerant strain that exhibits enhanced sensitivity to antheridiogen, whereas HaC18 was selected for reduced sensitivity to antheridiogen. Based upon the responses of gametophytic populations (maximum percent males) to antheridiogen supplementation in the medium, these strains form a series of decreasing sensitivity to antheridiogen, *i.e.* HaA48 > Hn-n > HaC18.

Gametophyte Culture

Gametophytic cultures were axenically established according to standard procedures in 24-well tissue culture dishes (14, 34). About 100 spores/well were sown onto 4.2 mL of 1% agar solidified medium containing Parker's macronutrients and Thompson's micronutrients (16) pH 5.4. Cultures were maintained at a constant thermophotoperiod of $27 \pm 2^\circ\text{C}$ and $23 \pm 5 \text{ W m}^{-2}$.

Antheridiogen

Antheridiogen (A_{cc}) for supplemented medium was obtained as a crude aqueous filtrate (CAF-II) from cultures that previously supported gametophytic growth (33). Medium was directly filtered following liquid shaker culture (50 rpm) of 20 mg wild-type (Hn-n) spores per 1000 mL of basal medium without agar at a constant thermophotoperiod of $28 \pm 1^\circ\text{C}$ and 20 W m^{-2} for 20 d. CAF-II was incorporated (v/v %) into medium prior to pH titration and autoclaving.

Inhibitors

Inhibitors (ancymidol, AMO-1618, CCC) or solvents alone (controls) were filter sterilized ($0.2 \mu\text{m}$) into autoclaved and cooled medium. For AMO-1618 and CCC, 0.5% DMSO was used as a solvent (final concentration 0.05% for 1 mM treatment and controls). For ancymidol, a mixture of 0.5% DMSO and 5% ethanol (95%) were used as solvents (final concentrations; 0.05% DMSO and 0.5% ethanol (95%) for the 1 mM treatments and controls). Severe effects on gametophyte development were evident with 0.5% DMSO and greater (TR Warne, LG Hickok, unpublished data). CCC and AMO-1618 were purchased from Sigma Chemical Co. and Calbiochem Co., respectively.

Analysis

At 14 d following sowing, all gametophytes were sampled and mounted in Hoyer's medium (2) mixed with 0.5% aceto-carmin on slides. Sexual gametophytes were categorized as male (ameristic, antheridiate) or nonmale (meristemetic, archegoniate). Nonsexual or neuter gametophytes comprised <0.1% of all gametophytes and were omitted from all calculations.

RESULTS AND DISCUSSION

AMO-1618 and ancymidol inhibited the production of male gametophytes in all strains tested, whereas CCC was ineffective (Table I). Relative sensitivity of strains to inhibitors (HaC18 > Hn-n > HaA48) corresponded to relative insensi-

Table I. Effect of Inhibitors (AMO-1618, CCC, Ancymidol) on Sex Expression in Three Strains of *Ceratopteris richardii*

Stock	Inhibitor			
	mm	AMO-1618	CCC	Ancymidol
		<i>mean \pm SD % male gametophytes^a</i>		
HaC18	0 ^b	27 \pm 9.2	35 \pm 9.2	17 \pm 7.0
	0.01	25 \pm 7.0	30 \pm 7.9	9 \pm 3.4
	0.1	17 \pm 2.4	36 \pm 6.6	1 \pm 1.5
	1.0	7 \pm 3.5	30 \pm 2.6	— ^c
Wild type (Hn-n)	0	70 \pm 5.7	65 \pm 2.9	56 \pm 4.1
	0.01	66 \pm 5.6	60 \pm 8.4	42 \pm 6.3
	0.1	58 \pm 6.1	62 \pm 7.4	36 \pm 4.6
	1.0	36 \pm 2.1	69 \pm 6.1	— ^c
HaA48	0	88 \pm 1.6	90 \pm 4.0	81 \pm 7.9
	0.01	82 \pm 9.8	89 \pm 5.2	84 \pm 16.0
	0.1	80 \pm 6.2	85 \pm 2.5	59 \pm 3.9
	1.0	60 \pm 4.2	85 \pm 5.1	— ^c

^a $n = 3$. ^b Differences among 0 mM control treatments of a given strain reflect different solvents necessary to solubilize inhibitor; see "Methods and Materials." ^c The 1 mM treatment of ancymidol was completely lethal.

Table II. Effect of Inhibitors (AMO-1618, CCC, Ancymidol) in the Presence of 10% (v/v) *Ceratopteris* Antheridiogen Supplementation in Three Strains of *C. richardii*

Stock	Inhibitor + Supplemented Antheridiogen			
	mm	AMO-1618	CCC	Ancymidol
		<i>mean \pm SD % male gametophytes^a</i>		
HaC18	0 ^b	62 \pm 3.1	56 \pm 8.1	36 \pm 8.1
	0.01	46 \pm 3.2	53 \pm 5.5	17 \pm 7.2
	0.1	51 \pm 4.4	54 \pm 4.2	6 \pm 0.5
	1.0	64 \pm 4.2	63 \pm 4.4	— ^c
Wild type (Hn-n)	0	91 \pm 2.3	93 \pm 1.3	79 \pm 2.7
	0.01	87 \pm 3.5	92 \pm 2.1	74 \pm 1.3
	0.1	92 \pm 2.4	91 \pm 3.0	75 \pm 2.7
	1.0	97 \pm 1.3	84 \pm 16.8	— ^c
HaA48	0	100 \pm 0	100 \pm 0	99 \pm 0.1
	0.01	100 \pm 0.5	100 \pm 0	100 \pm 0
	0.1	100 \pm 0	100 \pm 0	91 \pm 6.1
	1.0	100 \pm 0	100 \pm 0	— ^c

^a $n = 3$. ^b Differences among 0 mM control treatments of a given strain reflect different solvents necessary to solubilize inhibitor; see "Methods and Materials." ^c The 1 mM treatment of ancymidol was completely lethal.

tivity to antheridiogen, *i.e.* inhibition was greatest in HaC18 which exhibits the lowest sensitivity to antheridiogen. AMO-1618, ancymidol, and CCC had no effect on the initiation, rate, and maximum of spore germination but resulted in decreased growth of gametophytes of all strains (data not shown). Ancymidol was lethal to all strains at 1 mM.

Since the major sites of activity for ancymidol, AMO-1618, and CCC are in the early stages of GA biosynthesis, inhibition of male gametophytes by such inhibitors suggests the involvement of this pathway or another comparable pathway in the synthesis of *Ceratopteris* antheridiogen (3). Both AMO-1618 and CCC primarily inhibit the initial cyclization of geranylgeranyl pyrophosphate, *e.g.* the A activity of the multienzyme

complex ent-kaurene synthase; however, CCC is typically much less effective than AMO-1618 (3, 10). The major sites of activity for ancymidol are the three oxidation steps from ent-kaurene to ent-kaurenoic acid (4, 11). At higher endogenous concentrations these inhibitors may affect other less sensitive biosynthetic pathways, e.g. sterol biosynthesis (3). For example, AMO-1618 and other similar quaternary ammonium compounds may inhibit acetate incorporation into mevalonic acid and the cyclization of squalene-2,3-epoxide (8, 9).

In addition to these known mechanisms of action, these inhibitors may be affecting an undefined biosynthetic pathway specific for antheridiogen synthesis. Such a pathway may include enzymes in the GA biosynthetic pathway that are acting on non-GA substrates, e.g. antheridiogen specific substrates, or enzymes distinct from GA biosynthesis that catalyze similar cyclization and hydroxylation steps (1).

The relative specificity of inhibitor action was examined by supplementing media with CAF-II antheridiogen. Antheridiogen reversed the inhibition of sex expression by AMO-1618 in all strains and by ancymidol in the wild type and HaA48 (Table II). Antheridiogen supplementation failed to overcome ancymidol inhibition in HaC18. However, the absence of reversal in HaC18 is consistent with the relative insensitivity of HaC18 to supplemented antheridiogen (33). Supplemented antheridiogen did not alleviate growth reduction by inhibitors nor lethality of 1 mM ancymidol. This suggests that the inhibition of maleness was independent from effects on gametophytic growth and lethality.

Successful inhibition of antheridiogen mediated sex expression by AMO-1618 and ancymidol, the enhanced inhibition of antheridiogen insensitive strains relative to antheridiogen sensitive strains and alleviation of inhibition by supplemented antheridiogen indicate the involvement of the GA biosynthetic pathway or other similar pathway in the synthesis of the *Ceratopteris* antheridiogen. Thus, *Ceratopteris* (Parkeriaceae) and the Schizaceae may possess similar biosynthetic pathways for antheridiogen production that share features in common with GA biosynthesis. In *Ceratopteris*, the absence of sensitivity to Schizaceous antheridiogen and various GAs may indicate species specific pathway for antheridiogen synthesis as well as high specificity of receptor or acceptor sites. Even in the Schizaceae extensive variability occurs in antheridiogen cross-sensitivity, response to GA moieties and antheridiogen structure at the genus and species level (20–22).

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