Inducing sex change and organogenesis from tissue culture in the endangered African cycad *Encephalartos woodii* (Cycadales, Zamiaceae)

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IF INCIPIENT SEX CHROMOSOME DIFFERENTIATION is caused by differential methylation between females and males, then methylating or demethylating cytosine nucleotides may induce sex change. Methylation may also stimulate regeneration of roots and shoots from tissue culture callus and increase genetic variation via greater mutation. We propose using these methods for conserving *Encephalartos woodii*, for which only a single male clone exists, sex change has never been induced, and regeneration from callus tissue has not been accomplished.

The conservation status of the African cycad *Encephalartos woodii* Sander is bleak. All extant plants are derived from a single male clone.[1] Although vegetative propagation is readily accomplished from offsets and more recently from leaf cuttings, genetic diversity is zero.

Osborne[2] proposed two conservation strategies for *E. woodii*: (1) repeated backcrossing of the existing male with females of a closely related species, and (2) chemically or environmentally inducing sex change in tissue-cultured plantlets from the existing male. The first approach resulted in an F_1 generation of *Encephalartos natalensis* × *E. woodii* in the 1980s and recently F_2 seedlings of (*E. natalensis* × *E. woodii*) × *E. woodii* (pers. obs.). We focus on sex change because it is the least well understood of the two approaches and has potentially the greater conservation benefits.

There are potentially six benefits to inducing sex change in *Encephalartos woodii*. First, our proposed method of sex change promotes mutation, thereby increasing genetic variation. Second, there would be increased epigenetic variation due to meiotic recombination.[3] Third, female plants produce megagametophytes and zygotes providing the cells of choice for cycad tissue culture.[4] Fourth, if backcrossing is the only realistic approach to conservation, then it is preferable to use *E. woodii* as the female parent because of maternal inheritance of chloroplast genomes. Fifth, induced sex change may assist in the conservation of other dioecious plants. Sixth, sex change provides fundamental insight into the biology of sex determination. We propose a method for inducing sex change in cycads and regeneration of roots and shoots from callus, including discussion of the pitfalls.

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Theory of sex determination

This article is predicated on the hypothesis that, in both females and males, incipient sex chromosomes arose as a different methylation pattern on one of two homologous autosomes and the altered methylation was of regulatory genetic elements that control sex hormone production. Sexual differentiation in plants is regulated by sex hormones.[5] Cytosine nucleotides are methylated via replacement of the hydrogen at the C-5 position by a methyl group and are highly heritable.[6,7] Methylation causes sex by blocking binding sites for enzymes that mediate transcription of sex hormones.[8–11]

Methylation suppresses transcription in several ways. Heterochromatic proteins bind to methylated cytosines, occupying protein binding sites.[12,13] Methylation alters interactions of histones with promoter regions by stimulating histone deacetylation.[14–16] Bound heterochromatic proteins and histones are called heterochromatin. Transcription is also suppressed because methyl groups are bulky and hydrophobic, thereby changing DNA conformation and blocking binding sites,[17] sometimes converting the normally right-handed DNA helix to left-handed.[18,19]

Dioecious seed plants generally have putative sex chromosomes that are indistinguishable under a light microscope.[20] Close inspection, however, sometimes reveals slight heterochromatin differences,[21,22] which is itself due to differential methylation.[23,24] Therefore, it is distinctly possible that methylation controls sex determination.

Methylation and accompanying heterochromatin can be removed by various factors — such as temperature,[25,26] light,[27] osmotic stress,[28] or hormones[29–31] — resulting in sex change.[32,33] Sex change occurs only in organisms that have (virtually) indistinguishable sex chromosomes, indicating that incipient sex chromosomes are formed by slight differences in methylation. Differential methylation is evolutionarily the first difference between females and males[34] and is the likely cause of reported sex changes in cycads.

Application of theory to sex change in cycads

Although sex-specific differential methylation or heterochromatin has not been examined in cycads, lack of identifiable sex chromosomes[1] and occasional induction of sex change via environmental shocks[35] suggest that sex determination in cycads is due to differential methylation. Definitive evidence for differential demethylation determining sex should be sought in cycads, especially in populations in which some individuals have undergone sex changes, using techniques such as chromomycin staining,[21] high-performance liquid chromatography (HPLC),[36,37] or bisulphide sequencing of promoters of genes that regulate hormone levels.[38–41]

We propose applying demethylating compounds to cycad cells in tissue culture, an approach that has resulted in sex change in at least one angiosperm species.[7] 5-azacytidine, and 5-aza-2′-deoxycytidine demethylate CpG dinucleotides in most eukaryotes,[42–46] while L-ethionine and dihydroxypropyladenine demethylate cytosines in CpNpG trinucleotides of plants (N can be any nucleotide base).[47]

Although demethylating agents have not been used to alter the sex of cycads, we propose that altering methylation of tissue-cultured cells of *Encephalartos woodii* could yield female plants. We suggest trying this procedure first on relatively common species of *Encephalartos*, such as *E. natalensis*. Tissue-cultured angiosperms have survived exposure to 5-azacytidine, although with lower viability and higher mutation rates.[44,48]

Measuring methylation levels using HPLC or bisulphide sequencing before and after application of demethylating compounds would provide a means for

determining appropriate amounts of these chemicals to use in subsequent experiments. The quantity of demethylating compounds could be adjusted so that the resulting diminution of methylation matches that found in cycads of the requisite sex, although we recognize that the 'wrong' methyl groups might get stripped away.[45]

If sex determination in *Encephalartos woodii* is controlled by differential methylation, then there is an *a priori* 50% chance that a male-to-female change can be induced via demethylation. The odds may be lower, however, because nearly all sex changes reported in *Encephalartos* have been from female-to-male (reviewed in ref. 35). If females of *E. woodii* have more heavily methylated promoters of sex determining genes than males, sex change of the extant male clone can be attempted via methylation. Addition of methyl groups to cytosine nucleotides may be possible by treating tissue cultures with 5-methyl deoxycytidine. This approach has been attempted with mammalian cells, where the dose of 5-methyl deoxycytidine was adjusted so that half the cell line survived.[49–51] However, much less experimental work has been done with methylation than with demethylation.

Ideally, the specific nucleotides responsible for regulation of sex hormones and sex should be identified, an assessment made of whether their regulation is controlled by methylation, and then only those specific nucleotides should be selectively methylated and/or demethylated in an attempt to change sex and regenerate roots and shoots from callus. Selective methylation can be attempted using methylated single-stranded oligonucleotides,[52,53] a technique that is not yet fully developed. Until then, attempts should be made by randomly methylating and demethylating the genome, although most such attempts are likely to be unsuccessful.

Regeneration of roots and shoots from tissue culture callus

Even if the sex of *E. woodii* can be changed with demethylating or methylating chemicals, other problems need to be addressed before any protocol can be considered effective, such as growing sex-changed tissue cultures up to full-sized cone-bearing plants, difficulties relating to small effective population size, and evolutionary loss of organelle genomes.

Early attempts to propagate cycads from tissue culture, including *Encephalartos* species, met with limited success (reviewed in ref. 54). Tissue culture of stem, root, and leaf material from several species of *Encephalartos* has given rise to callus.[54,55] Cell cultures from zygotes of two species of *Encephalartos* have been partially successful: shoots were formed, roots were not, and no plants matured.[56] Recent work using tissue from megagametophytes and zygotes of the Mexican cycads *Ceratozamia hildae, C. mexicana, Zamia fischeri, Z. furfuracea, Z. pumila* and particularly *Dioon edule,* has been encouraging, producing plants that can be grown in soil.[4,57,58] Tissue culture of megagametophyte and zygote explants probably leads to more successful regeneration of entire plants because global demethylation and *de novo* methylation may be a prerequisite for proper development, as with most eukaryote embryos.[59–61] An important but as yet unattained objective is to take leaf, stem, or root explants of *Encephalartos* and induce shoot and root formation from calli. This is necessary for E. *woodii,* in which neither megagametophytes nor zygotes presently exist. Another incentive for inducing sex change of the extant male *E. woodii* clone is that megagametophytic and zygotic cells can then be obtained, cultured, and treated with demethylating or methylating agents.

Although regeneration of tissue cultured *E. woodii* has not yet been accomplished, our theoretical framework of sex determination via methylation may provide the method by which differentiation of callus tissue can be induced.[62] Ontogenetic regulation of all plant and animal tissues is largely controlled by demethylation, although many of the methylated genes controlling regulation have not yet been identified. Until now, regeneration of tissue-cultured roots and shoots relied on the application of plant hormones, which work largely by demethylating chromosomal cytosines.[29,31,63] Until we know which genes to demethylate and how to demethylate them selectively, we propose skipping the intermediary of plant hormones and instead applying 5-azacytidine to *E. woodii* callus to induce root and shoot regeneration.[64] We also suggest first applying 5-methyl deoxycytidine to the callus to simulate early embryonic *de novo* methylation.

As with sex change, we expect that regeneration from callus will usually be unsuccessful because of the randomness of methylation and demethylation when applying compounds like 5-azacytidine. Although regulation is largely controlled by methylation of promoters, methylation of downstream regions of genes may also affect regulation.[65] Our best hope is for selective methylation and demethylation or, less elegantly, for many random attempts in the hope that one explant will be successfully regenerated.

The proposed applications of methylating and demethylating agents serve a dual purpose: sex change and regeneration of roots and shoots. The optimal amounts of these chemicals may, however, be different for each function. The targeted loci for methylation may also be different.

Problems following sex change and regeneration

Even if male-to-female sex change can be induced and viable sexual offspring formed in *E. woodii,* there will still be no genetic variability in the species. There will be only epigenetic variation due to different methylation patterns. It is noteworthy that F_1 and F_2 backcrosses of *E. natalensis* × *E. woodii* have been raised.

Methylation-induced mutations, although often deleterious, could be of conservation benefit for *E. woodii* because the surviving mutants increase genetic variability of the population.[61] Methylation creates a tension between increased genetic variation and decreased viability.[66,67] The balance between these two will determine the conservation implications of the proposed methylation/demethylation induced sex change of *E. woodii.* The best possible outcome is that viable sex-changed females will emerge from this protocol carrying a large number of mutations. It is not necessary that all these mutations appear on a single female; the genetic variation of the entire population is critical. A successful protocol for tissue culture of *E. woodii* will produce many sex-changed mutants to be grown into explants with (collectively) as much genetic variation as possible, thereby reducing the probability of extinction.

Cycads have maternally inherited chloroplasts[68] (and mitochondria?). The main disadvantage of backcrossing the existing male *E. woodii* with females of other *Encephalartos* species is that these non-Mendelian organelle genomes from the male parent are irrevocably lost from all backcrosses.[68] A preferred option would be to backcross an induced female *E. woodii* with males of a closely related species so that the *E. woodii* organelle genomes are preserved, providing additional impetus for inducing sex change in *E. woodii.*

Conclusion

If sex is determined by methylation, then attempts should be made to induce sex change of tissue-cultured cycad cells using specific demethylating or methylating agents. A prerequisite is to verify whether differential methylation of specific genes, primarily those producing and regulating hormones, is correlated with sex in cycads. It is desirable also to quantify degree of methylation in cycad clones for which sex change has been reported. Or, better yet, to identify which nucleotide loci contain regulation-controlling methylation.

Establishment of a successful protocol for *in vitro* culture of diploid vegetative or embryonic tissue of *Encephalartos*, particularly *E. woodii*, including organogenesis and the ultimate re-establishment of plantlets in soil, remains a *sine qua non* for this project. As with sex change, the formation of roots and shoots may also be possible by applying methylating and demethylating agents to the callus tissue. This seems especially plausible because plant hormones are known to cause the following: (i) heritable changes in methylation patterns, (ii) sexual differentiation in plants, and (iii) regeneration of roots and shoots from callus.

If sex change is successful with *Encephalartos woodii* and the resulting female plants are sexually viable, then economics will certainly lead to programmes for breeding nascent female clones to the extant male clone. Backcrossing the female clones to males of other *Encephalartos* species should also be encouraged to help overcome the extreme genetic bottleneck for this species unless the methylation or demethylation treatments add substantial genetic variation to the nascent females.

Putting these ideas into practice will require an enormous amount of work and good fortune. Even if attempts to change the sex and regenerate roots and shoots of the male clone of *Encephalartos woodii* are unsuccessful, however, the proposed testing may still provide fundamental insights into the biology of sex determination in seed plants.

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