A review of current progress in our research on sex determination in *Diospyros*

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Abstract

This review summarizes current progress in our research on sex determination in *Diospyros* species. We used next generation sequencing technologies and *Diospyros lotus*, a close relative of the cultivated species *Diospyros kaki*, as our model. We compared floral initiation and developmental patterns of male and female flowers between *D. kaki* and *D. lotus* because the sexual systems of *D. kaki* differ from those of most other *Diospyros* species. The sexual system of *D. lotus* and most *Diospyros* species is dioecious while *D. kaki* is polygamous. After confirming that floral initiation patterns were similar in *D. lotus* and *D. kaki*, we used *D. lotus* as a model to study sexuality in *Diospyros*. We crossed *D. lotus* 'Kunsenshi Female' with *D. lotus* 'Kunsenshi Male' to produce an F_1 population (the KK F_1 population) that segregated distinct male and female phenotypes. Using 63 F_1 offspring, a bulked segregant analysis with amplified fragment length polymorphisms showed that the sexuality of *D. lotus* is controlled by a single locus or haploblock in a heterogametic male (XY) system. The genomes of *D. kaki* bearing male flowers contained the maleness DNA marker developed from *D. lotus*, suggesting that the genetic control of sexuality is similar in *D. lotus* and *D. kaki*. We conducted Illumina HiSeq paired-end sequencing of the genomes of 57 individuals from the KK F_1 population. We cataloged and assembled male-specific subsequences (male-specific k-mers) from the Illumina HiSeq genomic reads data from male and female pools. These analyses enabled us to isolate the male-determining regions on the Y-chromosome in *D. lotus*. Integrating transcriptome and evolutionary analyses led to the identification of one Y-specific sex-determinant candidate gene, *OGI*. *OGI* encodes a small RNA that in turn triggers transitive RNA interference on a feminizing gene, *MeGI*, located on the autosomal or pseudoautosomal region of the sex chromosome to produce male flowers. These data indicate that the genetic control of maleness expression in *Diospyros*, including *D. kaki*, is based on the *OGI*/*MeGI* system.

Keywords: dioecious, persimmon, sexuality, small RNA, Y chromosome

INTRODUCTION

Sex expression in fruit tree species affects cultivation and breeding systems as well as yields. Understanding sex expression and its controlling mechanism would be useful to develop stable and efficient production systems. Sexuality in plants can be determined at three different levels: the flower level, the individual level, and the population level (Dellaporta and Calderon-Urrea, 1993). In terms of flower sexuality, distinct unisexual types (male and female) and a hermaphrodite type are defined. The combinations of flower sexualities in an individual define sexuality at the individual level. The combinations of individual sexualities in a population, often at the plant species level, define sexuality at the population level, which is referred to as the "sexual system". Although sex expression is controlled by both environmental and genetic factors, the environmental and molecular controls of sexuality are yet to be fully clarified for most plants.

Most *Diospyros* species, including *Diospyros lotus* (known as date plum or Caucasian

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persimmon), form unisexual flowers, although there are a few exceptional cases of hermaphrodite flower formation in some species. At the population level, most *Diospyros* species are classified as dioecious, while some species are classified as monoecious (Duangjai et al., 2006). The sexuality of cultivated persimmon (*Diospyros kaki* Thunb.) is complicated, and this species is classified as polygamous. In *D. kaki*, some individuals have only female or male flowers and some have both female and male flowers (monoecious) (Kitagawa and Glucina, 1984). Some monoecious individuals bear hermaphrodite flowers as well as male and female flowers. Hermaphrodite flowers of most *D. kaki* cultivars or genotypes do not fully function as female flowers because they develop seedless fruit. However, in some individuals, normal fruit containing seeds develop from hermaphrodite flowers. Furthermore, individuals that usually bear only female flowers occasionally form male flowers (Yakushiji et al., 1995). Thus, the sexuality of *D. kaki* can be described as polygamous and the sex expression of *D. kaki* is apparently unstable.

Most commercial *D. kaki* cultivars, such as 'Fuyu', 'Jiro', and 'Hiratanenashi' bear only female flowers, while some cultivars such as 'Taishu' and 'Hanagosho' bear male flowers as well. Commercial orchards are inter-planted with pollinizer trees with male flowers to ensure good fruit production. In monoecious cultivars, the ratio of male-to-female flowers in a tree varies not only with the genotype or cultivar, but also with the environmental conditions and tree age (Hume, 1913; Kajiura and Blumenfeld, 1989). For example, old or weakened trees of 'Taishu' produce excessive male flowers, leading to an insufficient crop load. Thus, some growers are reluctant to plant 'Taishu', even though this cultivar produces excellent fruit. The fact that only limited numbers of cultivars bear male flowers limits crossing combinations in breeding programs.

Elucidation of the genetic and molecular basis of sex expression in *D. kaki* will allow for the development of methods to artificially control sex expression in this important fruit species. The complicated sex expression and polyploidy of *D. kaki* make it difficult to identify genes involved in sex determination. We used *D. lotus*, a diploid dioecious species closely related to *D. kaki*, as a model to uncover the genetic and molecular basis of sexuality in *Diospyros* species including *D. kaki*. This review summarizes our recent research on the sexuality of *Diospyros.* Although *D. kaki* shows polygamous sexuality, unlike most other *Diospyros* species, the genetic control of maleness expression in this species appears to be the same as that in *D. lotus*.

Flowering habit and floral induction

The sexual system of *D. lotus* differs from that of *D. kaki*; the former is dioecious and the latter is polygamous. However, the male and female flowers are quite similar in their morphology at blooming. Male flowers comprise a simple cyme with three florets and female flowers show a solitary form in both species. The flowering habit differs slightly between the two species (Figure 1) (Kajita et al., 2014). In *D. lotus*, male and female flowers form on almost all shoots from lateral buds at the base of the apical part of the previous year's growth. In contrast, flowers of different sexes show different flowering habits in *D. kaki*. Female flowers form on shoots from most apical buds and several adjacent lateral buds on the previous year's growth, while male flowers form on shoots from lateral buds at the basal part as well. The simplicity of the flowering habit of *D. lotus* made it an ideal candidate for research on sexuality determination.

We also observed seasonal flower bud development using *D. kaki* 'Hiratanenashi', which bears only female flowers, and *D. kaki* 'Zenjimaru', which bears both female and male flowers, as well as *D. lotus* 'Kunsenshi Female' and 'Kunsenshi Male'. Buds were collected monthly and internal flower development was observed by scanning electron microscopy. The initiation of the floral meristem had commenced by mid-June in *D. lotus* and by late June in *D. kaki* (Figure 2). After the initiation of the floral meristem, calyx differentiation progressed during autumn and winter in *D. lotus* and *D. kaki*. However, the formation of petals and stamens did not progress substantially until after endodormancy break in both species. These results demonstrated that the phenological control of flower bud development is similar between these species, suggesting that *D. lotus* can be used as a

model plant for studying floral development in *D. kaki*, and possibly other *Diospyros* species.

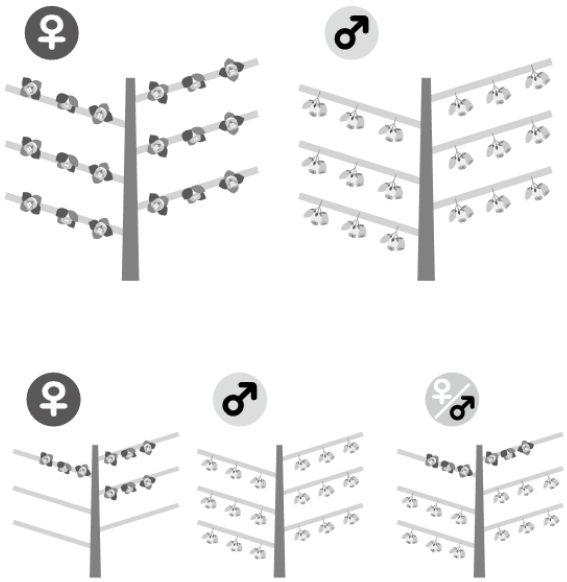

Figure 1. Schematic diagram of flowering habit of *Diospyros lotus* (top) and *Diospyros kaki* (bottom). Current year's growth (shoots: light grey bars) appears from previous year's growth (1-year-old branches: dark grey bars). Female flowers are solitary form and male flowers are a simple cyme.

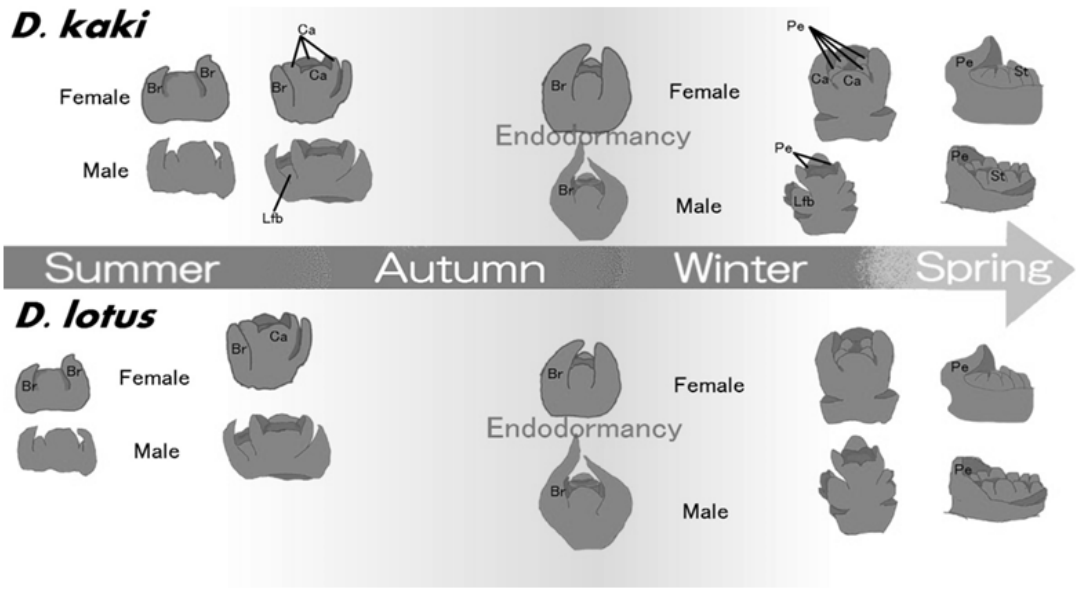

Figure 2. Schematic illustration of female and male flower development in *Diospyros kaki* and *Diospyros lotus*.

Development of DNA markers

Next, we developed DNA markers for sexuality (Akagi et al., 2014b; Kajita et al., 2015). We crossed *D. lotus* 'Kunsenshi Female' and *D. lotus* 'Kunsenshi Male' to produce an F_1 population, designated as the KK F_1 population, which segregated distinct male and female

phenotypes. Using 63 F_1 offspring, we found two amplified fragment-length polymorphism markers, DlSx-AF4 and DlSx-AF7, which cosegregated with maleness. This strongly suggested that the sexuality of *D. lotus* is controlled by a single gene or haploblock, and that male is dominant over female. Thus, *D. lotus*'s sexuality can be classified as heterogametic male, the XY-type, as reported for most other dioecious plant species. For unknown reasons, segregation of the phenotype of a sequence-characterized amplified region marker developed from DlSx-AF4 (DlSx-AF4S) and the male/female phenotype in this cross in *D. lotus* showed an apparent bias towards femaleness. The marker and male/female phenotypes better fitted 1:2 than 1:1, which is the theoretical segregation for a single genetic locus or haploblock in diploid *D. lotus*. DlSx-AF4S partially distinguished *D. kaki* individuals with female and male flowers from cultivars with only female flowers (Table 1). The DlSx-AF4S marker was always present in individuals with male flowers, although its presence did not necessary correspond to male flower formation. These results suggested that the same genetic system controls sexuality in *D. kaki* and *D. lotus*, and that DlSx-AF4S could be used as a genetic marker for sexuality in *D. kaki* breeding programs to some extent.

Table 1. Relationship between presence of *DLSx-AF4S* marker sequence and sex expression in *Diospyros kaki* cultivars (reproduced from Kajita et al., 2015).

Type of cultivar	No. tested	Cultivars with DLSx-AF4S (%)
Monoecious	39	36/39 (92%)
Female	135	23/135 (17%)

Identification of sex determinant

We conducted de novo whole-genome sequencing and transcriptome analysis to characterize the sex determination system in *D. lotus* (Akagi et al., 2014a). To identify male-specific sequences, we constructed genomic sequencing libraries from 32 female and 25 male trees of *D. lotus* from the KK F_1 population. Libraries pooled according to sex were sequenced to an estimated 45 to 50× coverage. Every 35-bp subsequence (35-mer or k-mer) present in these sequencing reads was catalogued, and reads including significant male-pool-specific k-mers (MSKs) were used to assemble 5100 contigs putatively located on the Y chromosome. Approximately 800 contigs were located on putative male-specific regions of the Y chromosome (MSYs) and amounted to a total length of about 1 Mb.

To identify genes expressed during floral initiation and development, we conducted high-throughput mRNA sequencing using tissues from mixed buds, including floral organ primordia. These tissues were obtained from the nine males and nine females from the KK F_1 population that were used for the genomic analysis, and their parents. We used several approaches to interpret the expression data. Eventually, we focused on a pair of class I homeodomain transcription factors. The first gene, which we named *OGI* (Japanese for "male tree"), showed male-specific expression in developing buds, and was identified as a Y-specific sex determinant candidate with no homologous sequence in the female genomic reads. Its coding sequence was found to contain multiple disruptive stop codons. The second gene, which we named *MeGI* (Japanese for "female tree") showed female-biased bud and flower-specific expression, but was not MSY-linked and was found to be located in an autosomal region. A sequence analysis of *OGI* predicted the presence of a hairpin structure with high similarity to the homologous region of the *MeGI* gene, indicating that *OGI* functions to repress *MeGI* through RNA interference. Despite multiple disruptive mutations, the *OGI* gene sequence and male specificity are highly conserved in the *Diospyros* genus. Phylogenetic analyses suggested that the establishment of *OGI* predated *Diospyros* radiation and that suppressed recombination has retained *OGI* on the Y chromosome for tens of millions of years.

There is no proven method for transformation of *D. lotus*. Therefore, we functionally characterized *OGI* and *MeGI* in tobacco (*Nicotiana* spp.) instead. In transient co-expression assays in *Nicotiana benthamiana*, overexpression of *OGI* suppressed the expression of *MeGI*,

suggesting that *OGI* can repress *MeGI* in plants. Furthermore, three out of 11 *Arabidopsis thaliana* plants independently transformed with *MeGI* driven by the constitutive CaMV35S promoter showed stunted growth and formed sterile androecia that occasionally produced nonfunctional pollen-like grains, whereas the carpels produced fertile seeds upon cross-pollination. Similarly, in *Nicotiana tabacum*, five out of 12 plants transformed with *MeGI* driven by its native promoter showed low pollen germination and formed shorter androecia, but normal carpels. Taken together, the phenotypes observed in transgenic *A. thaliana* and *N. tabacum* were consistent with the morphology of female flowers in *D. lotus*. The *OGI* RNA hairpin structure suggested that RNA interference plays a role in repressing *MeGI*. Consistent with this inference, *MeGI*-specific small RNAs were found to accumulate in the buds and flowers of males only.

Our data suggest a model for sex determination in *Diospyros* in which *OGI*, or *Oppressor of MeGI*, represses the expression of the feminizing *Male Growth Inhibitor* gene, *MeGI*, in male flowers. There is no evidence that *MeGI* promotes gynoecia formation (Figure 3). Instead, observations of *MeGI* expression in hermaphroditic *Arabidopsis* suggest that it plays a feminizing role through androecia sterilization. The mechanisms affecting gynoecia formation in *Diospyros* are still missing from this model, and an additional Y-linked locus may be required, as postulated by the two-mutation model for the evolution of dioecy. Alternatively, a single master regulator of sex determination, such as SRY in humans, may be sufficient.

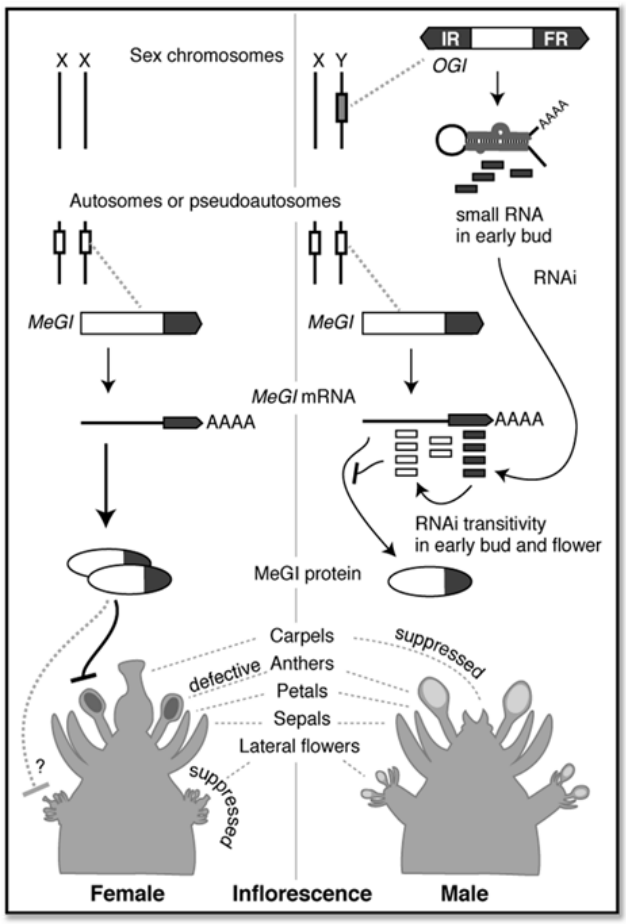

Figure 3. Chromosomal map of *OGI* and *MeGI*, with model of their interaction and potential function in female (left) and male (right) flower development in *Diospyros lotus* (reproduced from Akagi et al., 2014a).

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