

Sex Conversion in *Ginkgo biloba* (*Ginkgoaceae*)

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A male *Ginkgo* tree at Kami Yagisawa, Minobu-cho, Yamanashi Prefecture, Japan, is shown to possess a small, localized, branch that produces ovules that mature into viable seeds. This tree is recognized as an Ohatsuki Ichu because of the occasional production of pollen sacs on otherwise normal vegetative leaves, but most of the abundant male cones that it produces are of normal morphology. Localized sex conversion, such as that seen in the Kami Yagisawa tree, by which part of otherwise male *Ginkgo* tree switches to producing seeds, may be more common than has previously been noted. Dioecy in extant *Ginkgo biloba* most likely evolved from monoecious ancestors that had ovulate and pollen cones on different parts of the plant. This change from monoecy to dioecy has also been accompanied by the differentiation of a ZW system of sex chromosomes (heterogametic females ZW, homogametic males ZZ) in which the W chromosome is slightly larger. The molecular developmental basis for sex conversion in *Ginkgo* is unknown, but in certain angiosperms a specific transcription factor regulates the expression of the female phenotype. A microRNA, which seems to be male specific, negatively affects this transcriptional regulator, resulting in the suppression of female characters and expression of the male phenotype. Further studies are needed to determine whether a similar or different mechanism operates in *Ginkgo*, and whether localized disruption of the male specific microRNA is responsible for ovule production on a plant that is otherwise morphologically male.

Key words: Dioecy, *Ginkgo biloba*, male Ohatsuki Ichu, sex conversion.

Ginkgo biloba has been cultivated in China for its edible nuts for about one thousand years and almost from the beginning it was known that two different trees were required for seed production (Crane 2013). *Ginkgo biloba* was first introduced into the West sometime between

about 1720 to 1750 (Loudon 1854), about 30 to 60 years after it was encountered by Engelbert Kaempfer in southern Japan. One of these early introductions, which still survives at the Royal Botanic Gardens, Kew in the U.K., was the first specimen in Europe to be recorded as reaching



Fig. 1. Male *Ginkgo* trees showing sex conversion. A. Tree at The Royal Botanic Gardens, Kew, U.K. B–E. Tree at Kami Yagisawa, Minobu-cho, Yamanashi Prefecture, Japan. A. Kew tree showing ovules (photographed autumn 2006). B. Male Ohatsuki Icho *Ginkgo* tree (photographed on 12 May 2015). C. Mature seeds in the crown of the male Ohatsuki Icho *Ginkgo* (photographed on 14 September 2015). D. Short shoot from sex converted branch on male Ohatsuki Icho showing developing ovules (photographed on 12 May 2015). E. Leaf of male Ohatsuki Icho showing pollen sacs on the leaf (photographed on 12 May 2015).

sexual maturity. Like other early *Ginkgo* introductions the Kew tree was male, and pollen cones were first recorded in 1795 (Loudon 1854).

It was almost 20 years later that the first female tree was recognized in Europe, but in

1814 Augustin Pyramus de Candolle noted ovulate reproductive structures on a mature *Ginkgo* at the Bourdigny Estate near Geneva (Loudon 1854). However, in the absence of male trees the ovules failed to mature. Viable *Ginkgo* seeds were first obtained in Europe at the

Montpellier Botanical Garden in 1835 from a female branch grafted onto a male tree (Loudon 1854).

During the nineteenth century the practice of grafting a female branch onto male tree was common. Such a graft was made onto the original male tree at Kew (Bean 1973) and also onto the male *Ginkgo* at the Botanical Garden of University of Vienna. It was from this Vienna tree that Richard von Wettstein sent young ovules every two weeks to Strasburger at Bonn University. Strasburger used these samples to study the development of germinated pollen in *Ginkgo* ovules (Strasburger 1892), although he overlooked the formation of sperm at the last moment of pollen development. This was recognized just a few years later, on 9 September 1896, by Hirase working at the Koishikawa Botanical Garden of the University of Tokyo (Hirase 1896).

In 2006 surprisingly, the old male tree at Kew produced about 13 seeds (Fig. 1A) all of which occurred on the same small branch (Crane 2006). However, without more detailed study it was impossible to determine whether this localized production of seeds reflected the reemergence of an old graft, or the *de novo* production of seeds resulting from localized sex conversion. Here we report an unambiguous case of localized sex conversion in a male tree at Kami Yagisawa, Minobu-cho, Yamanashi Prefecture, Japan. In 2011, a small branch on this male tree produced ovules that matured into viable seeds. We briefly report our observations on this tree and discuss the evolutionary and developmental context in which such sex conversion in *Ginkgo* occurs.

Materials and Methods

Ohatsuki Ichō, literally “seed-bearing leaf *Ginkgo*”, is the name given to *Ginkgo* trees that produce reproductive structures that deviate from the morphology typical of the species, most typically in bearing seeds on otherwise normal vegetative leaves (Shirai 1891). The first Ohatsuki Ichō was discovered at the Jyotakuji

temple, Minobu-cho in Yamanashi Prefecture, Japan (Shirai 1891). This tree, which is female, is unusual not only in bearing seeds on some of the leaves, but also in having ovule-bearing structures that are often irregularly branched and frequently bear several seeds. Typically the seed-bearing stalks of *Ginkgo* are unbranched and bear just two sessile seeds.

The Jyotakuji temple belongs to the Nichiren Sect of Buddhism, and because the Ohatsuki Ichō was considered a religious object associated with Nichiren, the founder of Nichiren sect, further studies of this tree were not permitted. However, Fujii surveyed the region around the Jyotakuji temple and discovered another female Ohatsuki Ichō close by at Honkokuji (Fujii 1896).

In the course of his survey Fujii also discovered a male Ohatsuki Ichō, located not far from the Jyotakuji temple at Kami Yagisawa on the other side of the Fuji River (Fig. 1B) (Fujii 1896). In addition to producing normal pollen cones this tree also produces occasional pollen sacs on otherwise normal vegetative leaves (Fig. 1E). A total of nine Ohatsuki Ichō with unusual morphological features, seven females and two males, have now been identified in the area of Minobu-cho. Female Ohatsuki Ichō are now known from many places in Japan and in China (Nagata 2014) and also from elsewhere including in the West (e.g., Vienna; Stadt Wien 2000). However, the two male Ohatsuki Ichō at Minobu-cho are the only ones that are currently known.

In 2011 a brief report in the local Yamanashi Nichinichi newspaper noted that seeds were formed on the male Ohatsuki Ichō at Kami Yagisawa (Mochizuki 2015). This, in addition to the occurrence at Kew and other reports (see Discussion), was the stimulus to undertake a more detailed study of this phenomenon.

Since 1940 the male tree at Kami Yagisawa has been considered a Natural Monument of Japan. Therefore collecting of samples, or experiments on this tree, requires special

permission from the Cultural Agency of the Japanese Government. We applied for the necessary permissions on 9 February 2015 and received the official response on 4 April 2015 (dated March 13, 2015).

Results

We visited Minobu-cho on 12 May 2015. One of us (TN) revisited the tree again the following September. The tree is about 20 m tall (Fig. 1B) and grows not far from the Eastern bank of the Fuji River in the Shinto Shrine area of Yamanokami (latitude 35°24'40"N, longitude 138°27' 35"E, 223 m in altitude). The tree is isolated from other *Ginkgo* trees. Pollen cones had been formed and shed earlier in the season but their abundance on the ground made it clear that the tree produced normal pollen cones profusely. Among the shed pollen cones, there were also shed leaves bearing a few pollen sacs (Fig. 1E). These leaves were rare, both among the debris beneath the tree, and also among those leaves on tree that could be examined from the ground.

The branch exhibiting sex conversion was about 8 m above the ground. The ovules were still small and at an early stage of development and the branch could not be observed from beneath the tree. One of us (HT) climbed the tree with aid of climbing equipment, and was able to identify the sexually converted twig. The twig was hanging down in the interior of the crown close to the main trunk. It had numerous well-developed short shoots, many of which bore both ovulate reproductive structures and leaves (Fig. 2). Some of the ovules were borne in pairs on more or less unbranched ovule-bearing stalks, as is typical in *Ginkgo*. Others were borne singly at the tips of ovule-bearing structures that were more irregularly branched (Figs. 1D, 2). The tip of the ovule-bearing twig was harvested using horticultural scissors for further studies. In September the remaining ovule-bearing branch could be easily identified from the ground by its numerous yellow seeds (Fig. 1C).

Discussion

Other records of sex conversion in *Ginkgo*

In addition to the male Ohatsuki Ichō at Kami Yagisawa, and the male tree at Kew that produced a few viable seeds in 2006, we are aware of two other clear examples, both in the eastern USA, of the localized production of seeds on a *Ginkgo* tree that is otherwise male.

At Clay Hill cemetery in Louisville, Kentucky, a large male *Ginkgo*, which appears to date from the early nineteenth century, has developed a witches broom high in its crown. This tree produces abundant pollen cones in the spring, but within the witches broom there is localized abundant production of ovules on the modified branches. These ovules mature into seeds (Crane 2013).

At the Blandy Experimental Farm near Boyce, northern Virginia, there is a plantation of more than 600 *Ginkgo* trees, most of which were planted between 1929 and 1939. Successive surveys of the 638 surviving trees in 1978, 1979 and 1981 demonstrated a sex ratio not statistically significantly different from 1:1. However, the most intensive survey that used a lift to carefully inspect the crown of each tree, was carried out in May 1982. This careful examination, carried out during the time at which ovulate shoots and pollen cones are produced, showed that four of 144 male trees had been classified as female in previous surveys, based on the production of mature seeds (Baldwin 1972, Santamour et al. 1983). As half sibs that are the progeny of a single mother these four trees may be of particular value for further research on sex conversion in *Ginkgo*.

From the examples given above we suggest that localized sex conversion in *Ginkgo*, while rare, may occur more often than has previously been supposed. In all cases seeds are produced only on a small, localized portion of the crown. Because, typically, only a few seeds are produced, and because the seeds only become obvious in the autumn, long after the pollen cones have been shed, sex conversion on a



Fig. 2. Sex converted branch about 8 m from the ground on the male Ohatsuki Ichō *Ginkgo* tree at Kami Yagisawa, Minobu-cho, Yamanashi Prefecture, Japan. Note prominent short shoots bearing developing ovules (photographed on 12 May 2015).

small part of the tree could be easily overlooked. Observations of the same tree are needed at two different times of the year. Such observation is most likely to happen in botanical gardens, but in these settings, as in the case at Kew, it may be difficult to conclusively exclude the possibility

of the reemergence of a former graft.

Evolution and development

Based on our observations, sex conversion in *Ginkgo biloba* is uncommon. Also, in all cases that we are aware of, the production of ovules

on otherwise male trees is highly localized and confined to a single branch. This suggests that the responsible developmental perturbation occurs in the living cells resulting from a single apical meristem and is manifested only in the reproductive tissues derived from that meristem. Since irregularly branched ovule-bearing stalks also occur on other Ohatsuki Icho, and because some of the ovules produced on the Kew male tree were borne in normal pairs, we think it likely that the genetic changes that cause sex conversion are different from those that cause irregularly branched ovule-bearing stalks.

The evolution of dioecy in plants has been studied mainly in angiosperms, where it occurs in almost 1,000 genera and has clearly evolved many times (Ming et al. 2011). In angiosperms it is often straightforward to infer that dioecy has evolved from an ancestral hermaphrodite that had bisexual flowers containing both staminate and pistillate parts (Darwin 1877). Understanding the evolution of dioecy in *Ginkgo*, however, is less straightforward because living *Ginkgo biloba* is phylogenetically isolated and there is no evidence of hermaphrodite reproductive structures in fossil members of the *Ginkgo* lineage. However, analogy with conifers, where dioecy seems to have evolved from monoecy at least ten times (Leslie et al. 2013), may be instructive. As in conifers, dioecy in *Ginkgo biloba* most likely evolved from monoecious ancestors that had ovulate and pollen cones on different parts of the plant. Therefore, in an evolutionary sense, this sex conversion reflects a partial reversal to the ancestral condition. In conifers there is also a common association between dioecy and the presence of fleshy seeds with presumed animal dispersal (Givnish 1980, Leslie et al. 2013). This association is also manifested in *Ginkgo biloba*.

The evolution of dioecy is often accompanied by the differentiation of sex chromosomes (Ming et al. 2011). In *Ginkgo* there are 12 pairs of chromosomes: 22 autosomes and a pair of larger sex chromosomes that differ from

each other in the position of their centromeres. Early cytological observations suggested XY chromosome differentiation with heterogametic males (Tanaka et al. 1952, Newcomer 1954, Lee 1954), although interpretations were further complicated by differing considerations of the significance of satellite chromosomes (Hizume 1997). However, subsequent work suggested a ZW system (heterogametic females ZW, homogametic males ZZ) with a slightly larger W chromosome (Chen et al. 1987, Lan et al. 2008). Subsequently, manual separation of the presumed W chromosome, followed by PCR amplification and FISH analyses revealed a positive signal along its entire length, with only a partial corresponding signal on the Z chromosome, suggesting that the unique region of the W chromosome may be female specific (Lan et al. 2008). The expectation from theory is that a Z-linked loss of function mutation on the Z chromosome creates a female sterile allele that results in male plants (ZZ), while a W-linked dominant male sterile allele results in female plants (ZW) (Ming et al. 2011).

A better understanding of the molecular genetic developmental basis of localized sex conversion in *Ginkgo*, may give insights into the evolution of dioecy, not only in *Ginkgo*, but also in other conifers. Comparison with what is known about the molecular genetics of dioecy in angiosperms may also be instructive to develop hypotheses for a mechanism of sex conversion in *Ginkgo* that could be tested by future research.

In the Caucasian persimmon (*Diospyros lotus*), a dioecious tree, a gene in male trees encodes a microRNA that affects expression of the different sex organs (Akagi et al. 2014). The female phenotype is caused by a transcriptional factor that regulates the expression of female characters. A microRNA, that seems to be male specific negatively affects this transcriptional regulator, resulting in the suppression of female characters and expression of the male phenotype. A similar mechanism seems to function also in *Arabidopsis* and *Nicotiana*

(Akagi et al. 2014). In both genera experimental transformation with the DNA corresponding to this microRNA resulted in the anticipated change in the sex organs, suggesting that the mechanism discovered in persimmon could be widespread. Against this background, and with the forthcoming availability of a genome sequence for *Ginkgo* (Ray Ming, Department of Plant Biology, University of Illinois, USA, pers. comm.), the identification of the mechanism that causes the expression of female characters in the male Ohatsuki Ichō may be within reach.

An interesting aspect that may be helpful in the future is that seeds from the Ohatsuki Ichō at Kami Yagisawa are viable and six plants are cultivated at the Koishikawa Botanical Garden of the University of Tokyo. Given the strong possibility that the ovules were fertilized by pollen from the same tree it is likely that the resultant seedlings will be homozygous in mutant loci. It will be many years until these trees reach maturity, but trees with mutant phenotypes can be expected to result from these offspring. In the meantime further studies of sex conversion in the male tree at Kami Yagisawa, and in similar trees at the Blandy Plantation, will be worthwhile to further clarify the molecular developmental correlates of sex conversion in *Ginkgo*.

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 P. R. Crane^d: イチョウ (イチョウ科) における性転換

山梨県身延町上八木沢の雄のオハツキイチョウの一
 枝にギンナンがなり、そのギンナンから実生が得られ
 た。この木は多くは正常な花粉嚢をつけるが、葉の上に
 花粉嚢をつけることで、オハツキイチョウとして発見さ
 れている。上八木沢のオハツキイチョウで見られた局所
 的な性転換は、これまで考えられていたより、もっと多
 く起こっていると思われる。また、現生イチョウの雌雄
 異株性は、同一植物の別な位置に花粉と胚珠を付ける雌
 雄同株から進化してきたと思われる。このような雌雄同
 株から雌雄異株への転換は、性染色体のZW型の分化
 を伴って起こった可能性があるが、この場合Z型染色
 体の方が若干大きくなっている。なお、ZWの組み合わ
 せで雌性を発現し、ZZの組み合わせで雄性を発現する。
 イチョウにおける性転換の分子機構は、未だ分かって

いないが、ある種の被子植物においては雌性の発現は特
 定の転写因子により制御されていることが知られてい
 る。その転写因子が、雄性由来のマイクロRNAにより
 負に制御されて雌性を抑制し、雄性の発現をもたらすこ
 とが報告されている。同様なシステムがイチョウで起こ
 っているのかどうか、あるいはまったく別の機構である
 かどうかは今後の研究に待たねばならないが、雄性特異
 的マイクロRNAの局所的破壊によりこの雄のオハツキ
 イチョウにおいて胚珠の形成された可能性は検討に値
 する。

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