ANATOMY AND DEVELOPMENT OF GYNOECIUM IN *TAPISCIA SINENSIS* OLIV. AND ITS IMPLICATIONS FOR THE ORIGIN OF CARPELS

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Abstract

Tapiscia sinensis Oliv. (Tapisciaceae) is a rare tree endemic in China. Characteristic of its androdioecy is the coexistence of male and hermaphroditic flowers. Its bisexual flower bears five stamens surrounding the gynoecium, which is composed of a terminal style and an ovary at the base. The style has a bifid stigmata, which is hollow and longer than stamens. The ovary is syncarpous, unilocular, formed by two fused carpels, with a basal or subbasal placenta. Ovule is bitegmic, anatropous, borne on the placenta and supplied by an amphicribral vascular bundle arising directly from receptacle. The carpel wall is supplied by a collateral vascular bundle. The ovule's position changes from initially inserted on the ovary base to later attached to the middle of the ovary wall due to unequal growth of the embryo sac. Based on the present observation and others, the implications of vascular system in *Tapiscia* for the evolution of carpel are discussed.

Introduction

Tapiscia Oliv. was published as a monotypic genus by Oliver in 1890, based on the specimens collected in Sichuan Province, China. *Tapiscia sinensis* (Tapisciaceae) (APG IV 2016), an endemic and rare tree under protection in China, is distributed south of the Yangtze River and in southwestern provinces of China as well as the north of Vietnam (Di and Yu 1989). *T. sinensis* is a relic plant surviving the tertiary glaciations and is a woody perennial androdioecious species with male and hermaphrodite individuals in the population. The male and hermaphroditic flowers blossom at the same time and both of them produce viable pollen. The mean pollen germination rate for males was higher than that of the hermaphrodites on the culture medium with 10% sucrose (Lü and Liu 2010, Ma 2013). The flower and fruit grow synchronously on the hermaphroditic individuals of *T. sinensis* (Liu *et al.* 2008, Lü and Liu 2010). Existence of males may be a balance for a trade-off between resource allocation to flower development and fruit growth at the same time on the hermaphroditic plant, producing excellent pollen for population, and the stamens of hermaphrodites may be a reproductive assurance and reward for pollinators (Zhou *et al.* 2016, Yang *et al.* 2017). Previously morphological and anatomical changes of flower and fruit in *T. sinensis* was investigated (Liu *et al.* 2008). The dormancy of zygote and fruit maturation until the second year in *T. sinensis* supports that *Tapiscia* should be raised to family level. *T. sinensis* has developed a set of unique strategies to survive from long winter with its naked ovary (undeveloped young fruit) (Teng and Liu 2009). Despite intensive effort, the unique reproductive feature of angiosperms, carpel, and its derivation from the counterparts in gymnosperms remains enigmatic. New researches showed that placenta and foliar part in female units of angiosperms are two parts supplied by two vascular bundles of different organizations, derived from two former primordia controlled by two different sets of genes (Zhang *et al.* 2017b). The carpel is a complex organ composed of branches and leaves, and ovules are borne on branches (Fu *et al.* 2018, Liu and Wang 2018). If a theory of carpel origin is practical, then it should also be applicable for *Tapiscia*,

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which is a member of angiosperms, and *Tapiscia* should also shed some light on this problem. In the present study, the development and anatomy of the carpel of *T. sinensis* in the hope of providing new insights on the origin of carpel were investigated.

Materials and Methods

Flower buds and fructifications at various developmental stages were collected from 5 hermaphrodites of *Tapiscia sinensis* cultivated on the campus of the Northwest University, Xi'an, Shanxi, China in 2011. The materials were fixed with FAA and then used in the preparation of 8 μm thick paraffin sections following the routine methods (Ruzin 1999). Part of the paraffin sections was stained with Safranin O and Fast Green, critically examined and photographed using a Nikon Eclipse 50i microscope with a Nikon DS-Fil digital camera. The other paraffin sections were stained with Aniline Blue, examined and photographed after excitation at 365 nm with a Leica DML epifluorescence microscope with a Leica DC300F camera. The longitudinal section of the fruits was observed and photographed using a Nikon D70 Digital Camera, and the flowers were observed and photographed using a Nikon Eclipse 50i microscope with a Nikon DS-Fil digital camera. The figures are organized for publication using Photoshop 7.0. All sections are deposited in the Northwest University, Xi'an, Shanxi, China.

The vascular systems in the ovaries or fruits of hermaphrodites were observed and documented at three levels to investigate the patterns of vascular bundles in carpels and ovules (Fig. 3). S1: cross-sections were made at the base of carpopodium, representing the total vascular system of fruit; S2: cross-sections were made at the joint between the fruits and carpopodium, representing the vascular system in transition before entering the fruits; S3: cross-sections were made at the fruit, representing the placenta vascular bundle and carpel vascular bundle, respectively. After Aniline Blue staining, cell walls of the vessel elements and callose in the sieve plates and sieve tubes appeared brighter than other tissues in the bundles.

Results and Discussion

The florescence of *Tapiscia sinensis* population under study was initiated in late May to early June. After fertilization, the ovary took nearly 17 months to develop into a mature fruit (Liu *et al*. 2008). Accordingly, flowers and fruits could be observed simultaneously existing on hermaphrodites from May to June (Fig. 1 A). The fruits of the preceding year and the young fruits of the current year grew synchronously on the same branch (Fig. 1B). The hermaphroditic inflorescences were 5 - 10 cm, very small (1 - 2 mm in diam.), yellowish, and fragrant floret, tightly clustered in contracted inflorescences (Fig. 1 A). Its bisexual flower born five stamens surrounding the gynoecium (Fig. 1 C). The gynoecium was composed of a terminal style and an ovary at the base. The style had a bifid stigmata, was hollow and longer than stamens (Fig. 1 D, E). Each of the hermaphroditic flowers has an ovary with one ovule. The ovary was syncarpous, unilocular, formed by two fused carpels, with a basal or sub-basal placenta (Fig. 1 F).

As seen in the longitudinal sections of ovaries collected from June to next March, ovule in hermaphroditic flowers was bitegmic, anatropous, borne on the placenta. The outer integument was shorter than inner integument, which forms the micropyle. Nucellus is composed of 4 - 6 cells under the integument (Fig. 1 D, 2 A). After fertilization, most of nucellar cells are disintegrated gradually to provide the nutrition for the embryo sac's development, but the nucellar cells next to chalaza end are split and reproduced (Fig. 2 B), resulting in an expanded apophysis that remains static until the seed becomes mature in the coming year (Fig. 2C, D). The ovule's position changes from initially inserted on the ovary base to later attached to the middle of the side ovary wall, due to unequal growth of the embryo sac. Therefore, the spatial relationship between expanded apophysis and micropyle are different from their original opposite position (Fig. 2 A, D).

Fig. 1. Flower and inflorescence of *T. sinensis*. (A) Flowers (arrow) and fruits (arrow head) co-exist on the same hermaphroditic plant; Bar = 1 cm. (B) Maturing fruit of the preceding year (arrow head) and young fruit of the current year (long arrow) exist on the same branch; Bar = 1 cm. (C) The hermaphroditic flower showing stamens; Bar = 1 mm. (D) The longitudinal section of ovary in a hermaphroditic flower; Bar = 20 μm. (E) Hermaphroditic flower has a hollow and longer style (long arrow); Bar = 20 μm. (F) Hermaphroditic flowers have an ovary with one ovule, with a basal placenta; Bar = 20 μm.

The total vascular system of fruits at S0 level was an amphicribral bundle, which had its vessel elements surrounded by several sieve elements (Fig. 4A). The transitional vascular system at S1 level was divided into one large central vascular bundle and seven independent vascular bundles at the periphery, separated by ground tissue in between (Fig. 4B, C). The seven peripheral

vascular bundles gave rise to the dorsal bundles in carpels. The vascular bundles in the carpel wall were typically collateral, namely, with adaxial xylem and abaxial phloem (Fig. 4D). The large central vascular bundle supplied the ovule, ascending to the chalazal end. The placental vascular bundle assumed an amphicribral organization. Taking an eccentric or central position, the vessel elements of the xylem in the placental bundle were surrounded by several bright dots of sieve elements in cross sections (Fig. 4E). In the meantime, several bright dots of sieve elements were observed at both sides of the vessel elements of the xylem in the longitudinal sections of placental bundle (Fig. 4F).
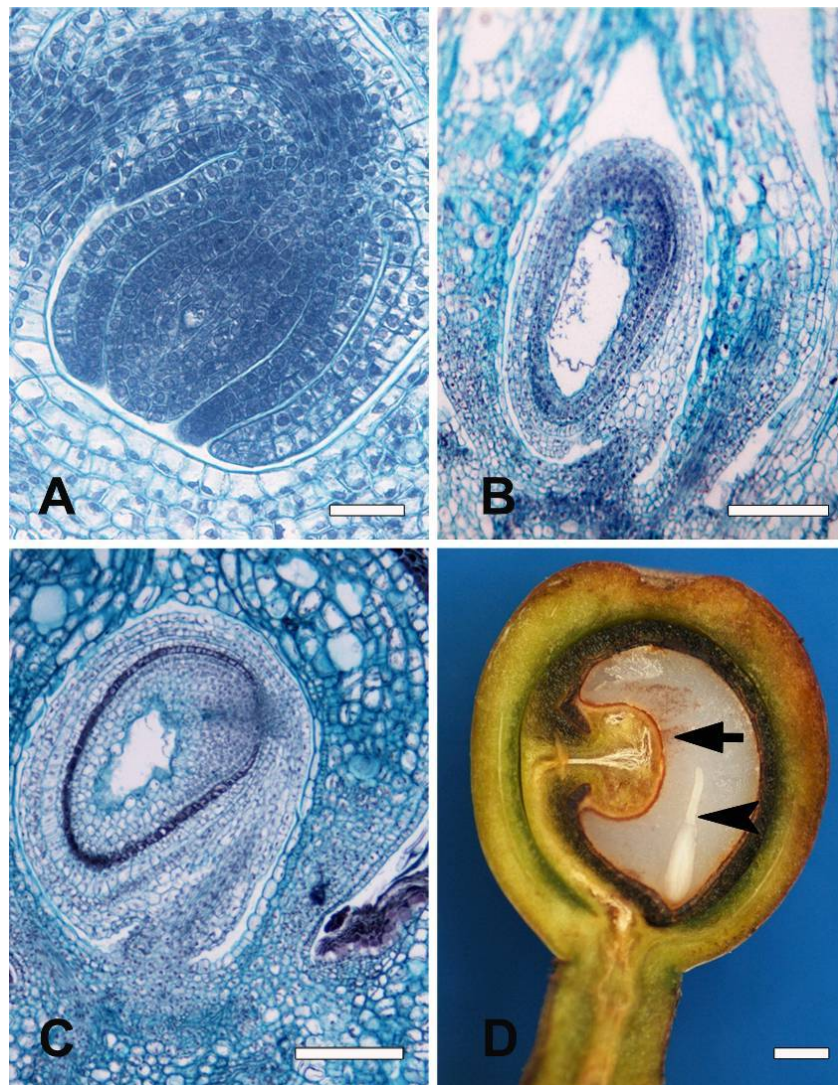

Fig. 2. Anatomy and development of ovule *T. sinensis*. (A) Anatropous ovule in blossoming, borne on a basal or subbasal placenta; Bar = 50 μm. (B) Anatropous ovule after fertilization; Bar = 50 μm. (C) Anatropous ovule during dormant period; Bar = 50 μm. (D) Anatropous ovule in mature fruit, attached to the middle of the ovary wall; Bar = 50 μm.

Tapiscia sinensis is a rare endemic tree, living in highly disturbed habitats. Once-contiguous population of *Tapiscia* has been broken into a few increasingly isolated populations. The extremely long reproductive cycle (lasting 17 months) of *T. sinensis* is an important trait of androdioecy. This phenomenon is very common in gymnosperms, but extremely rare in angiosperms (Chen and Lu 2001, Liu *et al.* 2008). After fertilization, the receptacle of hermaphrodite flower begins to expand and extend downwards; as a result, the receptacle gradually wraps the carpopodium. This structure is similar to fleshy seed collar of *Podocarpus* in gymnosperms (Florin 1958). These obvious changes of the dormant young fruit and other characteristics are adapted to the survival of dormant zygophase in winter (Teng and Liu 2009). During the process of fruit development, nucellus cells at the chalaza form a semicircle globular structure. It is possible that unequal growth of the embryo sac plus fusion of eccentric funiculus with ovary wall together contribute to the changes. The expanded apophysis moves aside continuously, therefore, the expanded apophysis and micropyle are finally vertical to each other, differing from their original opposite position. Vascular bundles are dispersed in hypostase, which helps to enlarge the contact area between expanded apophysis and embryo sac and enhance the transporting capacity of the materials to the embryo sac (Zhang *et al.* 2004, Zhang *et al.* 2017a).
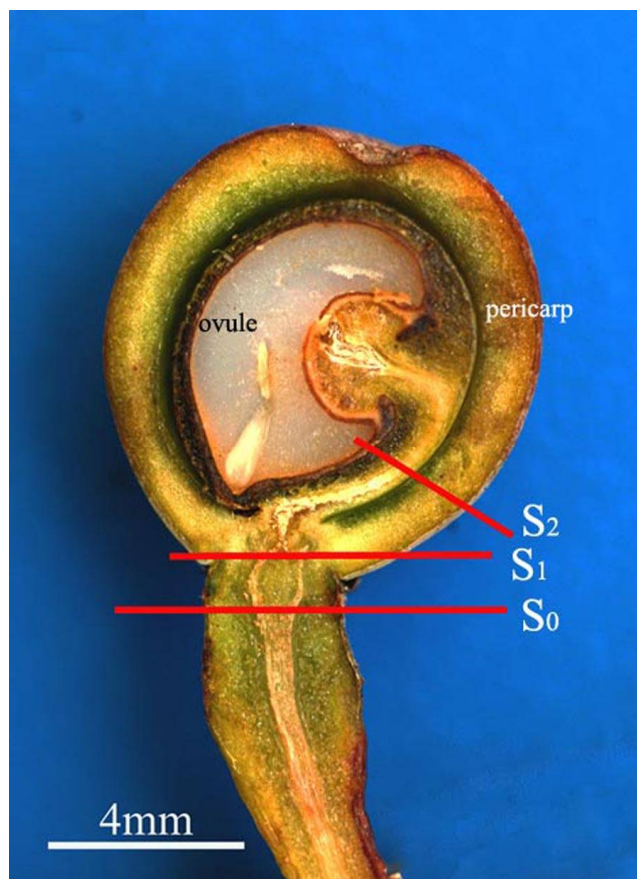

Fig. 3. The longitudinal section of mature fruits, showing the three levels at which the patterns of vascular bundle in carpels and ovules are shown in Fig. 4; Bar = 1 mm.
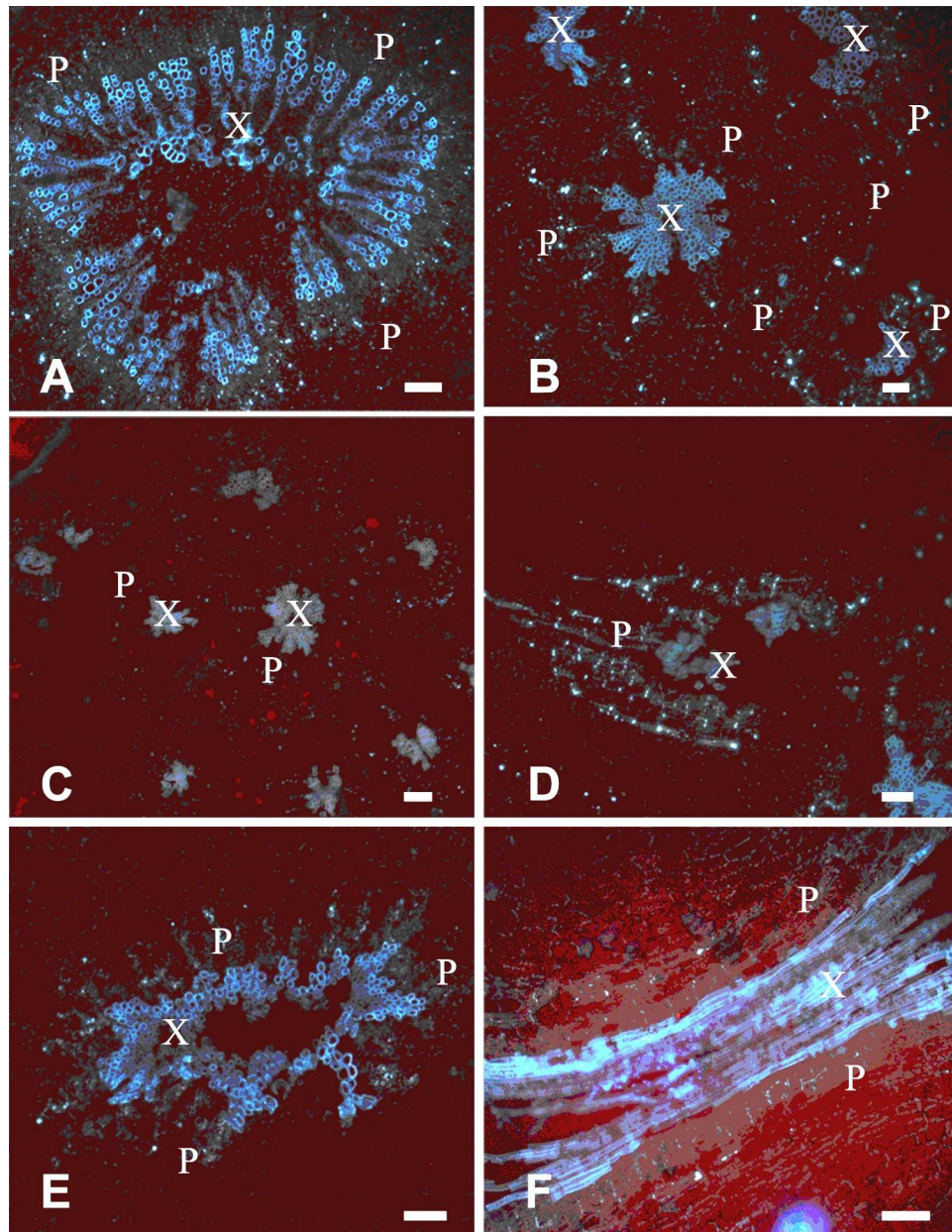
Fig. 4. Anatomy of vascular bundles in gynoecium. (A) The total vascular system of the fruit is an amphicribral bundle, cut at S0 level in Fig. 3; Bar = 10 μm. (B and C) Vascular system at the joint between the fruits and carpopodium, cut at S1 level in Fig. 3. Showing one large central vascular bundle in B and seven peripheral vascular bundles in C; Bar = 10 μm. (D - F) Sections were made at the fruit showing the carpel bundles and placental bundles, respectively, cut at S2 level in Fig. 3. (D) A collateral vascular bundle in carpel. (E) Cross-section of an amphicribral vascular bundle in placenta. (F) Longitudinal section of amphicribral vascular bundle in placenta; Bar = 10 μm. Abbreviations: P for phloem, X for xylem and V for vessel.

Homology of carpels in angiosperms has been a challenge in botany for long time (Tucker 1975). Many research works have been carried out to investigate whether there are two independent vascular systems in the carpels in the past years (Takhtajan 1969, Cronquist 1988, Thorne 1996). Wang (2010) raised the Unifying Theory, stating that angiosperm carpel is a composite organ derived from an ovule-bearing branch and its subtending bract. In favor of this theory, amphicribral bundles have been documented in placenta of many angiosperms (Dave *et al.* 1981, Endress and Armstrong 2011). However, comparable information on *T. sinensis*, crucial for this question, is still lacking. Trying to fill this gap in knowledge, here we investigate the gynoecium development and anatomy of *T. sinensis*.

A carpel as a leaf bearing ovule along its margins as assumed by the traditional interpretation (Cronquist 1988, Sokoloff *et al.* 2017) is not corroborated by the present research. Our observation indicates that the placenta vascular bundle in *T. sinensis* is distinct from that of the carpel wall's (Fig. 4a). This observation is fully understandable under the light of developmental genetics of model angiosperms, including *Arabidopsis*, *Petunia*, and *Oryza*, in which the developments of carpel wall and placenta are controlled by two exclusive gene sets, implying that placenta is equivalent to a secondary shoot independent of ovary wall (Roe *et al.* 1997, Skinner *et al.* 2004, Alvarez-Buylla *et al.* 2011). This conclusion is further confirmed by the results of gene manipulation on *Arabidopsis*, in which knocking out the genes controlling carpel wall development has resulted in naked ovules in *Arabidopsis*, not only turning this model angiosperm into a "gymnosperm", but also re-confirming the independence of ovules and placenta from ovary wall (Rounsley *et al.* 1995, Roe *et al.* 1997).

The amphicribral placental bundle of carpopodium and ovule in *T. sinensis* is anatomically distinct from the collateral one: an amphicribral bundle is featured by xylem surrounded by phloem, while a collateral bundle has adaxial xylem and abaxial phloem. This suggests a possible provenance of placental bundle from a former branch. Among angiosperms, amphicribral bundles are restricted to fruits and flowers, closely correlated to the gynoecium of angiosperms (Hu *et al.* 2010). However, the value of this intimate spatial correlation between amphicribral bundles and gynoecium is rarely explored. An incomplete survey of literature indicates that amphicribral bundles have been documented in the placenta in several families of angiosperms, including Papaveraceae (Kapoor 1973), Leguminosae (Lersten and Don 1966), Solanaceae (Dave *et al.* 1981), Gesneriaceae (Wang and Pan 1998), Buxaceae (Balthazar and Endress 2002), Annonaceae (Endress *et al.* 2011), Actinidiaceae (Habart 1974), Myrtaceae and Lythraceae (Schmid 1980). The present new observation (Fig. 4A-F) confirms the amphicribral organization of placental bundle in *Tapiscia*. The extensive existence of amphicribral bundle in the placenta of the above-listed distantly related clades in angiosperms implies that there is a common feature underlying the placentae in most angiosperms. It is thus logical to hypothesize that placenta in angiosperms is homologous to an ovule-bearing branch.

Acknowledgements

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