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Comparison of Stomatal Structure and Distribution between Ovules and Leaves in *Ginkgo biloba*

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Abstract: Stomata are plant epidermal structures that play essential roles in photosynthesis, respiration, and transpiration. Although stomata on plant leaves have been extensively studied, their structure and distribution on other organs remain poorly understood. The "living fossil ", *Ginkgo biloba*, has naked ovules that are thought to be primitive reproductive structures in ancient seed plants. Therefore, we hypothesized that there are some distinct stoma features in *G. biloba* ovules that have not been reported. In this study, we investigated the morphological development of stomata on ovules and leaves of *Ginkgo biloba* using scanning electron microscopy, then examined the anatomical characteristics of the general stalk and petiole using semi-thin sectioning. We found that stomata were distributed on the epidermis of the whole ovule, except near the micropyle; these stomata persisted until harvest, indicating that ovules perform gross photosynthesis to an extent similar to the photosynthesis observed in leaves, which is beneficial to ovule development. Ovule and leaf stomata share similar orientation, composition, and development; however, their distribution and subsidiary cell morphology significantly differ. The morphology of the general stalk was similar to the morphology of the petiole, but xylem cell development was minimal, and no sclerenchyma cells were present beneath the epidermis; these findings suggested that the general stalk is biomechanically weaker than the petiole. Overall, these results suggest that despite their differences, *G. biloba* ovules and leaves share many morphological and anatomical similarities in terms of stomatal architecture and stalk anatomy. These findings will help to elucidate the leaf origins of "flowers" in ancient plants.

Keywords: *Ginkgo biloba*; leaf; ovule; stalk; stomata; development

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1. Introduction

Plants exhibit various functional traits that facilitate resource acquisition and allow adaptation to constantly changing environments [1,2]. Stomatal differentiation has been linked to the success of terrestrial plants because the stomata allow plants to counterbalance the conflicting physiological requirements of increased carbon dioxide uptake and reduced water loss [3,4]. Thus, terrestrial plants achieve rapid diffusive conductance in favorable habitats and greater water use efficiency under dry conditions through the swelling and shrinking of stomatal guard cells [5,6]. The mechanism of stomatal movement has received considerable research attention [7–9]. Stomata are found on the leaves, stems, petioles, primary roots, and nectaries of various plant species [10,11]. Although stomata are not typically found on seeds, some species have stomata on their seed coats; these tend to differ in shape, size, and frequency from the stomata on leaves [12]. In these non-leaf organs, stomata presumably have many functions such as the optimization of carbon dioxide uptake, minimization of water evaporation, respiration, and perception of environmental changes; however, the distribution and structure of stomata on these non-leaf organs remain poorly understood.

Ginkgo biloba is a dioecious species that is among the oldest gymnosperm lineages [13]; its ovule has many distinct morphological and anatomical features [14–16]. For example, *G. biloba* ovules and leaves are arranged in a spiral manner at the apices of short shoots on morphologically similar stalks that are regarded as the general stalk and petiole, respectively [17]. The ovule epidermis bears stomata on its integument [14,18], and female gametophytes produce chlorophyll and engage in photosynthesis [19]. However, although several studies have revealed the presence of stomata on both leaves and ovules in fossil and modern *Ginkgo* specimens [14,18,20–22], few studies have compared stomatal architecture between these organs. Thus, a comprehensive understanding of ovular morphology in *Ginkgo* has remained elusive. The hypothesis is that some primitive features of ovule stomata in *G. biloba* may be similar to the leaves.

In this study, we investigated the functions of ovular stomata by examining their distribution and development, including the morphology of subsidiary cells, using scanning electron microscopy. We conducted semi-thin sectioning to compare stomatal architecture between the ovules and leaves, particularly between the general stalk and petiole, respectively. In this context, we also discussed the shortening or disappearance of the ovule stalk during the evolution of *Ginkgo*.

2. Materials and Methods

2.1. Materials Collection and Treatment

Ovules and leaves of *G. biloba* were collected at the Ginkgo Experimental Station at Yangzhou University, Yangzhou, China (32°20′ N, 119°30′ E) from March until May (2020). During the sampled stage, ovules developed from early-stage (before pollination) to late-stage (after pollination), and the leaves developed from the young (unexpanded) to mature stage (fully expanded). The integument, collar, stalk, and general stalk of the ovule, as well as the leaf lamina and petiole, were carefully removed using a razor blade; they were then fixed (as described below) and stored in a refrigerator at 4 °C until use.

2.2. Scanning Electron Microscopic Observations

The scanning electron microscopic observations were performed as previously described with minor modifications [23,24]. Samples were fixed in an improved formalin aceto–alcohol solution (70% alcohol:glacial acetic acid:formaldehyde, 90:5:5) for 1 week, then dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%; 15 min per step). After samples had been infiltrated twice in 100% acetone, they were immersed in a series of acetone–isoamyl acetate solutions (acetone:isoamyl acetate, 1:1, 1:2, and 0:1; 15 min per step). After critical point drying, the samples were coated with a layer of gold and observed under an S-4800 field emission scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 15 kV. The stomatal density was measured from six pictures, and the stomatal length and width were calculated from at least 25 stomata using ImageJ software (V1.51; National Institutes of Health, Bethesda, MD, USA). The experimental data between ovules and leaves were analyzed based on Student's *t*-test at $p \leq 0.05$ (SPSS 25.0) (SPSS, Chicago, IL, USA) [25].

2.3. Confocal Laser Scanning Microscopic

To detect stomatal morphology on the leaf lamina, samples were stained for 1 min with 50 μM propidium iodide in water as a molecular probe. Excess dye was removed with water, and the samples were observed under a Leica TCS SP5 confocal laser scanning microscope. Propidium iodide fluorescence emission was observed at emission and excitation wavelengths of 550–680 and 488 nm, respectively.

2.4. Semi-Thin Sectioning

Semi-thin sectioning experiment was conducted as our previous method with minor modifications [26]. Briefly, specimens were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for ≥1 day. Then, the samples were dehydrated in a graded ethanol series

(20%, 40%, 60%, 80%, 90%, 95%, and 100%; 10 min per step), infiltrated with propylene oxide on a gyrator, and embedded in Spurr resin at 70 °C for 12 h. Semi-thin sections (thickness, 1 µm) were cut with a glass knife, stained with 1% (*w*/*v*) toluidine blue, and observed under a microscope (Zeiss Axioskop 40, Carl Zeiss Shanghai Co., Ltd., Shanghai, China).

3. Results

3.1. Stomatal Distribution on Ovules

The *G. biloba* ovule consists of the integument, nucellus, collar, and stalk (Figure 1A). Stomata were distributed on the epidermis of the whole ovule, except the nucellus. Before the female bud scales opened, the stomata began to develop, such that the stomata on the collar developed earlier than the stomata on the integument (Figure 1B). After the female bud had opened, the number of stomata on the integument and collar rapidly increased (Figure 1C). Stomata on the integument were irregularly distributed and randomly oriented, except near the micropyle, where no stoma formed (Figure 1D,E). Significantly more stomata formed on the collar than on the integument, but the distribution and orientation patterns were similar (Figure 1F, Table 1). The size, especially the width of the stomata on the collar, was also larger than that on the integument. Among our samples, some collars showed a vein-like epidermis with regularly distributed cells, among which no stomata formed (Figure 1G). Stomata on the ovule stalk were sparse and oriented in a manner parallel to the long axis of the ovule (Figure 1H). Most stomata were distributed on the upper part and base of the abaxial side of the stalk (Figure 1I); however, stomata rarely formed at the base of the adaxial side, where a few trichomes were observed (Figure 1J). In *G. biloba*, the ovule stalk usually degenerates or is absent, and the collar is directly attached to the general stalk. The stomata on this connecting area were similar in distribution to the stomata on the collar (Figure 1K). A few ovules developed distinct ovule stalks, with stomatal structures intermediate between structures of the collar and structures of the general stalk. Numerous stomata were distributed near the collar (Table 1), with an irregular arrangement and random orientation (Figure 1L); stomata near the general stalk were sparse, arranged in longitudinal lines, and oriented parallel to the long axis of the ovule (Figure 1M). Moreover, the density of stomata on the ovule stalk was higher than that on the integument, while the difference in stomatal size was not significant (Table 1).

3.2. Stomatal Development and Ovular Morphology

Stomatal guard mother cells originated from ovule epidermal cells, which were smaller than all other epidermal cells (Figure 2A). Each guard mother cell divided into two semicircular cells known as guard cells, with substantially thickened cell walls between the two cells (Figure 2B). Subsequently, the surrounding cells were modified to form subsidiary cells. Stomatal guard cells on the integument were slightly sunken, whereas subsidiary cells were mainly unspecialized and similar in size to the other epidermal cells (Figure 2C). Stomata were typically separated from each other by one to five epidermal cells, or they were occasionally contiguous (Figure 2D); stomata rarely shared subsidiary cells. Mature stomata on the collar were deeply sunken and irregularly distributed (Figure 2E); they were sometimes compactly arranged in groups of two or three, separated by a common subsidiary cell (Figure 2F). The subsidiary cells had a dome-like appearance and were larger than the other epidermal cells (Figure 2E,F). Some subsidiary cells developed faint papillae that would bend toward the stomatal pit (Figure 2G). Stomata on the ovule stalk and general stalk had a regular appearance, with rare sunken guard cells but slightly bulging subsidiary cells (Figure 2H,K). Among our samples, the stomata sometimes showed enlarged subsidiary cells that extended over deeply sunken guard cells (Figure 2I). We also observed double stomata on the ovule stalk; these shared two common subsidiary cells (Figure 2J). Stomata on the general stalk were usually longitudinally or slightly obliquely oriented; occasionally, stomata were oriented perpendicular to the long axis of the ovule (Figure 2K). As ovule development progressed, the epidermal cells increased in size, such that the stomatal guard cells appeared deeply sunken, whereas subsidiary cells appeared

similar to the surrounding cells (Figure 2). We observed both closed and open stomata distributed over the ovule (Figure 2).

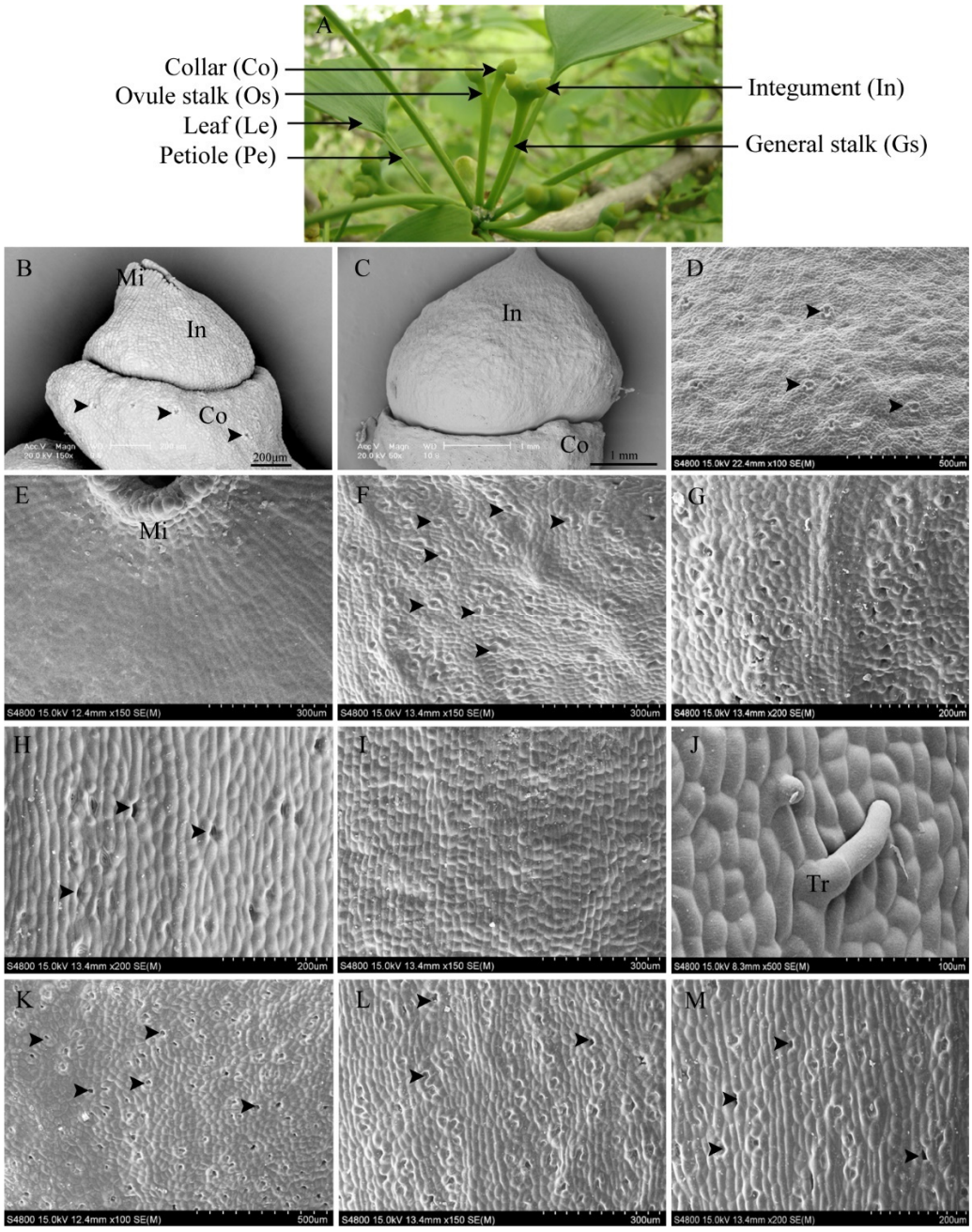

Figure 1. Stomatal distribution on *G. biloba* ovules. (**A**) Ovules and leaves clustered on short branches. (**B**) Stomatal development began earlier on the collar than on the integument. (**C**) Stomata increased in number on the integument and collar. (**D**) Stomatal distribution on the integument. (**E**) Very few stomata formed around the ovular micropyle. (**F**) Irregular stomatal distribution on the collar. (**G**) Regularly arranged cells on the collar, without stoma. (**H**) Stomatal distribution on the abaxial side of the ovule stalk. (**I**) Very few stomata formed on the adaxial side of the general stalk. (**J**) Trichomes on the base of the adaxial side of the general stalk. (**K**) Stomata in the area connecting the collar and general stalk of the ovule. (**L**) Irregular stomatal distribution on the ovule stalk near the collar. (**M**) Stomata on the ovule stalk near the general stalk were parallel to the long axis of the ovule. Black arrows indicate stoma. Co, collar; Gs, general stalk; In, integument; Le, leaf; Mi, micropyle; Os, ovule stalk; Pe, petiole.

Table 1. Density and size of stomata on different parts of ovules and leaves.

	Integument	Collar	Ovule Stalk (Abaxial Side)	Leaf Lamina (Abaxial Side)	Petiole (Abaxial Side)
Density (mm^2)	32.00 ± 3.27 c	218.00 ± 51.41 a	149.67 ± 13.47 b	112.00 ± 11.78 b	28.00 ± 3.27 c
Length (μm)	18.88 ± 1.50 b	23.37 ± 1.15 b	18.19 ± 4.29 b	22.07 ± 1.22 b	31.97 ± 3.54 a
Width (μm)	9.30 ± 0.85 bc	11.32 ± 0.65 a	8.16 ± 0.71 c	10.07 ± 0.67 ab	10.69 ± 0.67 ab

Lowercase letters indicate the significant difference at 0.05 level.

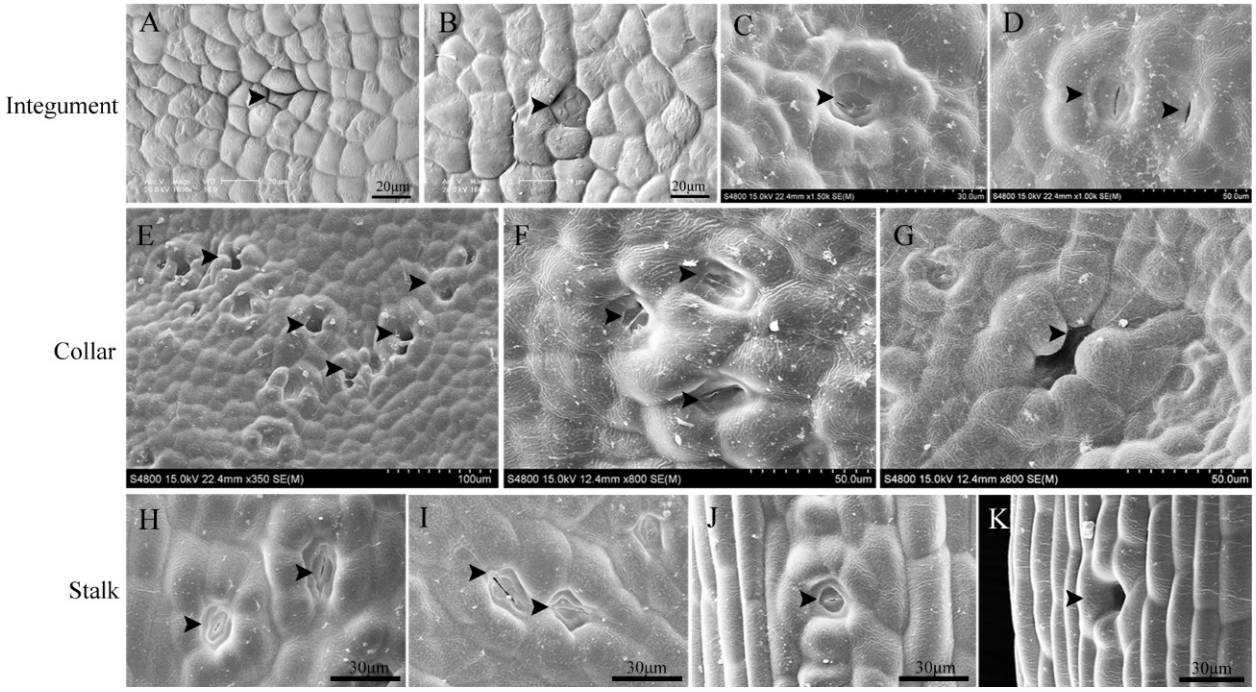

Figure 2. Stomatal development and morphology on the *G. biloba* ovule. (**A**) Guard mother cell. (**B**) Developed guard cells. (**C**) Stomata on the integument. (**D**) Two stomata on the integument, with shared subsidiary cells. (**E**) Stomata on the collar. (**F**) Three stomata on the collar, with shared subsidiary cells. (**G**) Weakly developed subsidiary cell papillae on the collar. (**H**) Stomata on the ovule stalk, with a pair of deeply immersed guard cells. (**I**) Double stomata on the ovule stalk. (**J**) Stomata on the general stalk. (**K**) Stomata on the general stalk with deeply immersed guard cells and obscure subsidiary cells. Black arrows indicate stoma. Bars: 20 μm (**A**,**B**); 30 μm (**C**,**H**–**K**); 50 μm (**D**,**F**,**G**); 100 μm (**E**).

3.3. Stomatal Distribution on Leaves

Stomata mainly formed on the abaxial side of the leaf prior to unfolding (Figure 3A), with distinct stomatal and vein regions. After the leaf unfolded, the number of stomata rapidly increased, with random orientation (Figure 3B). Stomata were rarely observed at the center of the adaxial side of the leaf (Figure 3C); a few stomata were observed at the base or along the margin of the adaxial side (Figure 3D,E). However, the morphology of the subsidiary cells of these stomata was similar to the morphology of the surrounding epidermal cells, though without papillae (Figure 3D,E). Stomata on the petiole mainly appeared on the abaxial side, with a banded distribution that was parallel to the long axis of the petiole (Figure 3F). The stomata density on the abaxial petiole was lower than that on the abaxial leaf lamina, while the stomata length on the abaxial petiole was the longest (Table 1). Stomata were rarely observed on the adaxial side of the petiole (Figure 3G,H), and trichomes were found on the base (Figure 3I).

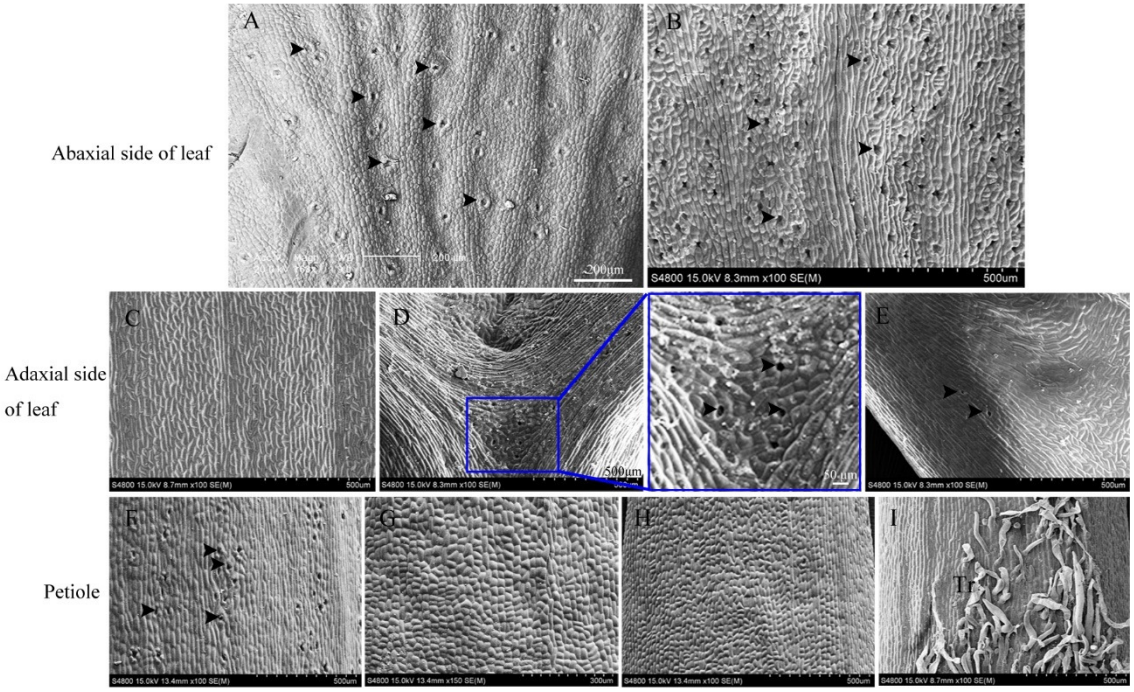

Figure 3. Stomatal distribution and morphology on *G. biloba* leaves. (**A**) Stomata occurred on the abaxial side of the young leaf lamina. (**B**) Abaxial side of the mature leaf lamina, showing stomatal and vein regions. (**C**) Stomata were absent from the adaxial side of the mature leaf lamina. (**D**) Stomata on the base of the adaxial side of the mature leaf lamina. (**E**) Stomata on the lateral part of the adaxial side of the leaf lamina. (**F**) Stomata on the abaxial side of petiole. (**G**,**H**) Very few stomata formed on the adaxial side of the petiole. (**I**) Trichomes on the base of the adaxial side of the petiole. Black arrows indicate stoma. Tr, trichome. Bars: 20 μm (**A**); 500 μm (**C**–**F**,**H**,**I**); 300 μm (**G**).

3.4. Stomatal Morphology on Leaves

Mature stomata on the abaxial side of the leaf consisted of pairs of guard cells with surrounding subsidiary cells (Figure 4A). Mature guard cells were deeply sunken, and the subsidiary cells developed papillae that extended over the stomatal pit (Figure 4B,C). Stomata on the petioles were typically composed of pairs of guard cells with subsidiary cells; however, the appearance of these stomata differed from the appearance of stomata on leaves (Figure 4D–F). Most stomata on petioles had sunken guard cells and distinct subsidiary cells (Figure 4D), and a few stomata on petioles had slightly sunken guard cells and obscure subsidiary cells (Figure 4E). The subsidiary cells occasionally increased in size to cover the deeply sunken guard cells (Figure 4F).

3.5. Anatomical Characteristics of the Ovule Stalk, General Stalk, and Petiole

The transverse section of the petiole was horseshoe-shaped (Figure 5A). The petiole had one layer of small epidermal cells that extended over one or two layers of compactly arranged sclerenchymatous cells (Figure 5B). The parenchymal tissue was composed of six to eight layers of parenchymal cells surrounding a secretory cavity (Figure 5B). There were two vascular bundles at the center of the petiole, which originated separately; the phloem and xylem were slightly concentrated toward the abaxial and adaxial sides, respectively (Figure 5C). The general stalk was ellipsoid in the transverse section, with a thicker cuticle (Figure 5D,E). The epidermal cells were small and arranged in a compact manner, whereas the parenchymal cells were large and loosely arranged (Figure 5E). The general stalk had two vascular bundles in opposite positions (Figure 5D). The phloem was located toward the center of the general stalk, with orderly arranged cells, whereas the xylem was located toward the epidermis and underdeveloped (Figure 5F). The ovule stalk was composed of cortical, parenchymal, and vascular tissue (Figure 5G). The cortical tissue consisted of a

single layer of orderly cells, with a few stomata among them (Figure 5H). The parenchymal tissue comprised numerous loosely arranged parenchymal cells; their size increased from the outer layer to the inner layer (Figure 5G). The ovule stalk had a single vascular bundle that was mainly composed of phloem cells without xylem cells (Figure 5I). A cylindrical protrusion on the cortex opposite the vascular bundle showed a thicker cuticle (Figure 5H).

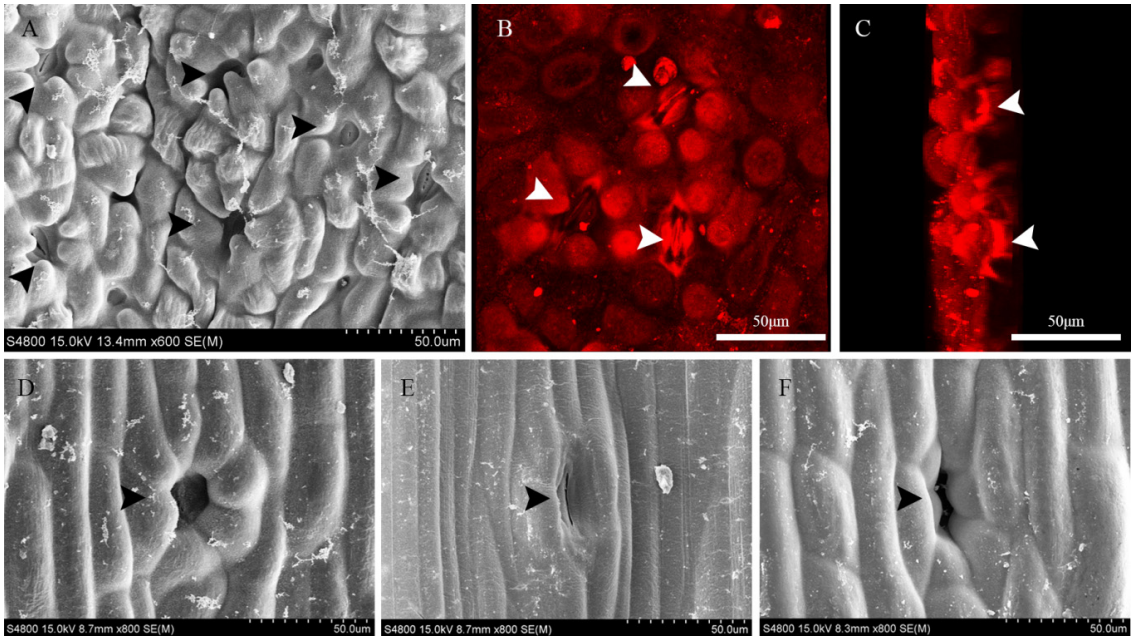

Figure 4. Stomatal morphology on the leaf lamina and petiole in *G. biloba*. (**A**) Mature stomata on the abaxial side of the leaf lamina. (**B**) Stomata stained with propidium iodide on the abaxial side of the leaf. (**C**) Lateral view of (**B**), showing deeply sunken guard cells. (**D**–**F**) Differential stomatal morphology on the abaxial sides of different petioles. Black and white arrows indicate stoma. Bars: 50 μm.

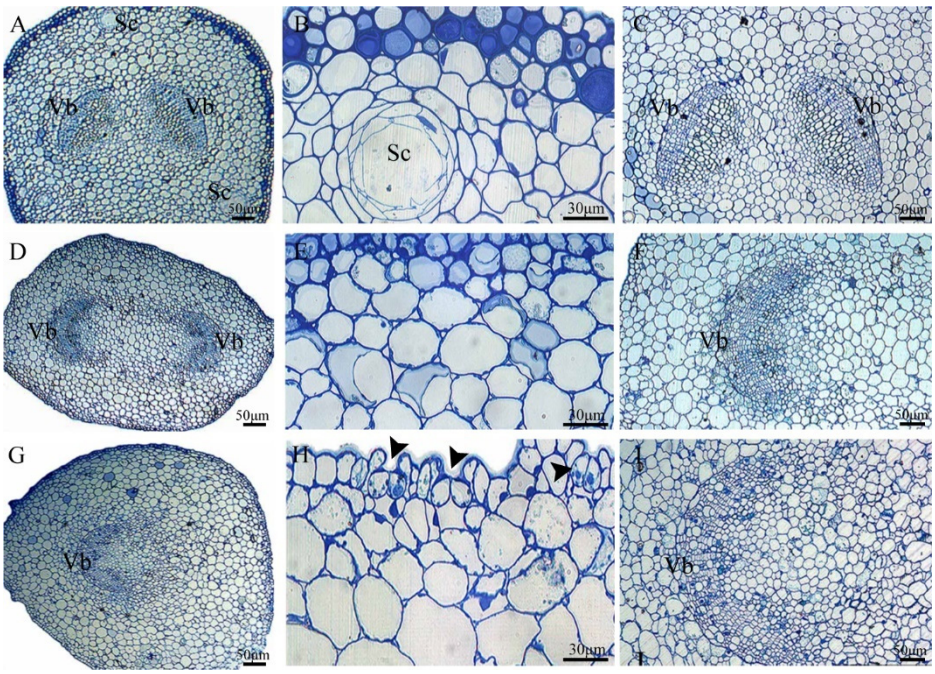

Figure 5. Anatomical structure of three stalk types in G. biloba. (**A**) Transverse section of the ovule stalk. (**B**) Epidermis, sclerenchyma, and secretory cavity of the petiole. (**C**) Vascular bundles of the

petiole. (**D**) Transverse section of the general stalk. (**E**) Epidermis and parenchyma of the general stalk. (**F**) Vascular bundle of the general stalk. (**G**) Transverse section of the ovule stalk. (**H**) Stomata and cylindrical projection of the ovule stalk. (**I**) Vascular bundle of the ovule stalk. Black arrows indicate stoma. Sc, secretory cavity; Vb, vascular bundle. Bars: 50 μm (**A**,**C**,**D**,**F**,**G**,**I**); 30 μm (**B**,**E**,**H**).

4. Discussion

Stomata play important roles in plant development; they control the entry of carbon dioxide assimilated in photosynthesis and optimize water use efficiency by regulating the turgor pressure of the guard cells [27,28]. Stomata are generally distributed on the epidermis of plant leaves [29], but they have also been found on other organs, including young stems [30], hypocotyls [31], corollas [32], nectaries [33], fruit [34], and seeds [35]. However, few species have stomata on their ovules. *G. biloba* is an ancient gymnosperm that has stomata on its ovules [14]. In this study, we observed that stomata were distributed on the epidermis of the whole ovule in *G. biloba*, except near the micropyle; the stomata were well-developed on the integument, collar, and stalk. Stomata found on the seed coats of some species remain perpetually open, which suggests that they are involved in respiration and absorption but not transpiration or photosynthesis [36]. Unlike these seed coat stomata, we found both open and closed stomata on *G. biloba* ovules at proportions similar to the proportions observed on leaves. Thus, we conclude that the ovular stomata of *G. biloba* contribute to nutrition transport through effects on transpiration demand. Considering that the ovules observed in this study were green and naked, we propose that the ovular epidermis of *G. biloba* performs photosynthesis aided by stomata.

Previous studies showed that *G. biloba* exhibited differential stomatal density on the abaxial and adaxial sides of the leaf; stomata were mainly distributed on the abaxial side of the leaf [22,37]. Stomatal morphological traits (stomatal density and the length of guard cells) are also plastic and linked to environmental changes such as long-term aridity and air temperature [38,39]. In *G. biloba*, we observed that stomatal density and size were different between ovules (integument, collar, and ovule stalk) and leaves (leaf lamina and petiole). These differences in stomatal morphological traits between ovules and leaves may be due to their different responses to the environment.

In addition to the differences in stomatal density and size, there are also differences in stomatal distribution. First, stomata were mainly distributed on the abaxial side of the leaf lamina; on the adaxial side, stomata were confined to a small area at the base near the petiole, with broad variation among individuals. Unlike stomata on leaves, stomata on the integument and collar were randomly distributed. The ovule stalk and general stalk showed similar stomatal distribution and orientation, generally on the abaxial side and parallel to the long axis. Second, both perigenous and mesoperigenous patterns of stomatal development have been observed on leaves [22]. However, ovular stomata are considered exclusively perigenous because subsidiary cells form from specialized surrounding cells after guard cell formation. Third, the composition of the stomatal apparatus was similar between leaf and ovule; this apparatus consisted of a pair of sunken guard cells and several subsidiary cells, with differential subsidiary cell morphology. On the abaxial side of the leaf lamina, subsidiary cells had distinct papillae that extended over the guard cells; on the adaxial side of the leaf lamina and on the ovules, subsidiary cells of the stomata usually exhibited a slight bulge and did not cover the guard cells. Based on the above analysis, we conclude that despite their differences, the ovular stomata of *G. biloba* exhibited consistent similarities with their counterparts on leaves, including their distribution and orientation patterns, developmental processes, and composition. These similarities suggest that *G. biloba* ovules and leaves may have similar functions in terms of photosynthesis and nutrient synthesis. In fact, transcriptome data also revealed a degree of gene co-expression in ovules and leaves, particularly in genes associated with stomata development [24]. As a bold hypothesis, we propose that *G. biloba* ovules exhibit signs of leaf origin, such that the ovular stomata may be remnants of leaf structural transformation. This hypothesis would

explain the great similarity between ovules and leaves in terms of stomatal distribution and morphology.

Another indication of similar structures for the ovules and leaves of *G. biloba* is the stalk that connects the leaf and ovule. Because petioles show less anatomical diversification within species than among species [40,41], the anatomical characteristics of the petiole are essential for dividing plant taxa and identifying their phylogenetic relationships. Petioles mainly function to support leaves and transport nutrition; leaf unfolding toward the most convenient orientation for photosynthesis is favored through adjustments of length and angle [42,43], as well as facilitation of nutrient transport between the stem and leaf [44,45]. In this study, we found that the petiole and general stalk of the ovule had similar architectures, each of which comprised compactly arranged epidermal cells, several layers of loose parenchymal cells, and two separate vascular bundles. However, the general stalk showed less xylem development and lacked sclerenchyma cells beneath the epidermis; both of these components are involved in mechanical support. Thus, the general stalk may have less biomechanical strength compared with the petiole; accordingly, the general stalk may exhibit greater flexibility, which prevents breakage as the increasingly heavy ovule pulls the stalk from an upright to a pendulous position. The *Ginkgo* ovule has increased in size during long-term evolution, in a manner counterbalanced by decreasing ovule numbers and the disappearance of the ovule stalk; therefore, the ovular collar is usually directly attached to the general stalk in modern *G. biloba* [4]. However, a shortened ovule stalk has occasionally been reported [17]. In this study, we investigated the anatomy of the ovule stalk using semi-thin sectioning; we found that the ovule stalk contained only one vascular bundle with extremely underdeveloped xylem, which suggested minimal support for the transport of mineral nutrients and water. We suggest that the presence of a less developed vascular bundle may be an important reason for the shortening or disappearance of the ovule stalk during the evolution of *Ginkgo,* as indicated by Zhou et al. [46]. Overall, these results suggest that stalk architecture in the modern *Ginkgo* ovule (i.e., a well-developed general stalk and rare ovule stalk) supports ovule/seed development.

5. Conclusions

In this study, we investigated the roles of stomata on *G. biloba* ovules and explored the relationship between ovules and leaves on the basis of stomatal architecture. Our results suggest that green, naked ovules execute gross photosynthesis resembling the photosynthesis in leaves because the universal distribution of stomata on the ovular epidermis contributes to ovule development. Despite some differences, the similarities in stomatal structure and stalk anatomy indicate that *G. biloba* ovules and leaves may have similar functions in photosynthesis and nutrient synthesis. The underdeveloped xylem and sclerenchyma cells in the ovule stalk suggest minimal biomechanical support for the transport of mineral nutrients and water. These findings provide morphological and anatomical evidence for a leaf origin of "flowers" in ancient plants.

Author Contributions: Z.L. and L.W. conceived and designed the study. S.C., D.W., W.L. and N.X. collected these samples and performed the experiments. S.C., D.W. and Z.L. analyzed the anatomical results and wrote the manuscript. S.C., X.S., C.Z. and Z.L. performed measurements, data analysis, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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