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Morphological Characterization of Metamorphosis in Stamens of *Anemone barbulata* Turcz. (Ranunculaceae)

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Abstract: The morphological characteristics of metamorphosis in stamens of *Anemone barbulata* Turcz. were investigated using morphological and histological analyses. The results showed that stamens were transformed into either white sepaloid organs or more frequently green leaflike structures with successive variations. The extreme metamorphic stamen was represented as a three-lobed leaflike structure with a long stalk, highly consistent with the morphological characters of the normal leaves of the plant. It was hypothesized that the connective and two pollen sacs of the anther were transformed into the three lobes of the metamorphosed stamen, respectively. The depression and circinate stages were identified as the important and necessary processes in the transformation of stamens from axial to foliar organs, suggesting probably the alternative evolutionary process of the formation of anthers derived from foliar organs. The morphological traces of leaf, sepal, and carpel observed in the metamorphosed stamens suggested the homeotic transformations among these organs. The foliar stage in the ancestral stamens of angiosperms was reflected ontogenically in the metamorphosed stamens of *A. barbulata*. Our findings of a series of metamorphic stamens probably represent the morphological evidence to support the hypothesis that the flowers of angiosperms were derived from metamorphic leaves with the progressive development mode in the evolution of floral organs.

Keywords: *Anemone barbulata*; Ranunculaceae; metamorphosis; stamen; homeosis; progressive development

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1. Introduction

The abnormal flowers in angiosperms substantially contribute to the evolutionary understanding of floral origins and have been extensively investigated since the golden ages in the 19th century [1–5]. Until 2000, reports on abnormal flowers were sparse, possibly because the abnormal flowers were generally not commonly found in the natural habitats [6–12]. The transformational changes among floral organs are commonly considered homeotic processes which are usually described by terms of metamorphosis, modification, teratology, and mutation. Homeosis is either the result of a gradual evolutionary process or a sudden mutation, considered an important developmental and evolutionary process [13]. Wolff [1] proposed a plant model based on homeotic variation to explain that the flower was derived from a gradual process of metamorphosis of foliage-leaves and the floral organs could be transformed to each other. Later, the most essential and remarkable investigations of metamorphic flowers were performed by von Goethe [2] to hypothesize that the floral parts (i.e., sepals, petals, stamens, and carpels) were structurally modified leaves, which were functionally specialized for reproduction or protection. To

date, this hypothesis has been widely recognized as the classical "foliar theory" of the floral origin [2,14–16].

During the ages of evolutionary and molecular genetics, i.e., since the 1990s, the extensive experimental studies have been performed to explore the functions of floral homeotic genes involved in the formation of floral organs in various model species, leading to an interpretative framework of the "ABC", "ABCDE", and "quartet" models of floral development, which requires the combined activities of genes of classes A, B, C, D, and E in the determination of floral patterns [10,17–20]. It is noted that the morphological mutants derived from the genes of various classes are generally generated in the laboratory settings. However, mutant flowers identified in nature have been rarely reported in the past ~20 years [16,21–27]. In particular, one floral homeotic mutant has been identified in nature within a wild type population of *Capsella bursa-pastoris* (Brassicaceae), which is the close relative of the molecular model plant *Arabidopsis thaliana* (L.) Heynh. and has been extensively investigated to explore the evolutionary significance of the metamorphosed floral organs and the floral homeotic mutants [25,26,28–33]. In this well-established floral model system of *C. bursa-pastoris*, the four petals are transformed into stamens, which is considered to be an example of non-gradualism evolution and an ideal model for the study of evolutionary significance of homeotic mutants and the speciation during the early developmental stages [29,31].

Notably, the above-mentioned studies focused on the transformation among the floral organs, whereas the conversions of floral organs into green leaflike organs in wild habitats are only reported in *Anemone barbulata* Turcz. [23,34,35]. It is noted that these metamorphosed floral organs into leaflike organs in wild are different from other transformed floral organs among each other [36–38]. The flowers of *A. barbulata* are excellent materials for study of flower metamorphosis revealed in nature due to its series transformations of stamens into various metamorphosed organs reported over a century ago by the Russian plant taxonomist Maximowicz [34]. However, due to the geographical limitations of plant materials, only the foliar metamorphosis of the calyx was reported with both stamen and pistil development considered normal. Based on our extensive field work in the natural habitats of these plants, it has been evidently revealed that the sepals, stamens, and pistils of this species could develop into leaflike organs, showing the morphological variations at the extremely high levels [23,35].

The plants of *A. barbulata* are perennial herbs of Ranunculaceae and are endemic to China [39]. This taxon has long been treated as *A. rivularis* Buch.-Ham. ex DC. var. *flore-minore* Maxim. [40]. Recently, Wang [41] restored its name *A. barbulata* originally established by Turczaninow [39], which was adopted in this study. In China, these plants are mainly distributed in Sichuan, Shaanxi, Gansu, Qinghai, and Ningxia, frequently located at altitudes of 1200–3100 m and in riparian grasslands or hillside wetlands. Our extensive field studies revealed the abnormal flowers of plants distributed in Shaanxi, Sichuan, and Gansu [23,35].

In our previous studies, the morphological variations of sepals in *A. barbulata* were explored to provide strong morphological evidence to support the hypothesis that the sepals were derived from the bracts in angiosperms [23]. In the present study, the morphological characteristics of the metamorphosis in stamens of *A. barbulata* were investigated using morphological, histological, and scanning electron microscope (SEM) analyses. The objectives of this study were to (1) reveal the morphological characteristics of the metamorphosis in stamens of *A. barbulata* and to (2) provide potential morphological evidence to support the theory that the flowers of angiosperms were derived from metamorphic leaves.

2. Materials and Methods

2.1. Plant Materials and Environmental Characteristics of Collection Sites

Our field studies showed that the transformation patterns of abnormal flowers in *A. barbulata* were consistent in different geographic locations (Table 1). The high frequency of abnormal flowers was observed in the Mt. Zhegushan, Maerkang County, Sichuan,

China. Therefore, the flower samples were collected at the Mt. Zhegushan (altitude 3100–3200 m). For comparison, flower samples were also collected in Taibai Mountain (altitude 1800–2100 m) and both Meixian and Foping Counties (altitude 1900–2000 m), Shaanxi, China, from July to August 2017 to 2019. To investigate the effects of environmental conditions on the floral variations among all four collection sites, the multivariate statistical analysis, i.e., the linear mixed model analysis and analysis of variance (ANOVA), were performed using SPSS version 20.0 with the environmental conditions as the concomitant variables (Table 1). The voucher specimens of various metamorphosed flowers were deposited in the Herbarium of the College of Life Sciences at Northwest University, Xi'an, China (accessions Chang Hong-Li 201,701 to 201,720 and 201,901 to 201,915).

Table 1. Characteristics of the four collection sites (i.e., Mt. Zhegushan, Taibai Mountain, Meixian County, and Foping County) and floral organs of *Anemone barbulata*.

Characteristics	Mt. Zhegushan	Taibai Mountain	Meixian County	Foping County
Environmetnal condition				
Area (m^2) **	300	150	100	50
Altitude (m) **	3100–3200	1800–2100	1900–2000	1900–2000
Average annual temperature (°C) **	6.1	6.2	14.6	11.6
Average annual rainfall (mm) **	770	875	610	940
Drought season	May to June	November to February	November to February	November to February
Average annual humidity (%) **	50	75	65	55
Floral characteristics				
Number of plants examined	125	48	34	16
Number of plants with only normal flower *	19	10	6	3
Number of plants with abnormal flower/Number of plants examined (%) *	85	79	82	81
Number of abnormal flowers/Number of total flowers per plant (%) *	72	56	66	60
Number of abnormal flowers/Number of total flowers per collection site (%) *	80	73	72	75
Number of sepals of 35 flowers (range per flower) *	189 (5 to 7)	184 (5 to 7)	188 (5 to 7)	189 (5 to 7)
Number of sepals per flower ± SD (n = 35) *	5.4 ± 0.6	5.3 ± 0.6	5.4 ± 0.6	5.4 ± 0.7
Number of stamens of 35 flowers (range per flower) *	1752 (25 to 90)	1725 (27 to 88)	1786 (29 to 83)	1696 (29 to 95)
Number of stamens per flower ± SD (n = 35) *	50.1 ± 15.8	49.3 ± 13.9	51.0 ± 12.6	48.5 ± 14.3
Number of carpels of 35 flowers (range per flower) *	1264 (22 to 64)	1329 (25 to 54)	1294 (18 to 58)	1280 (21 to 54)
Number of carpels per flower ± SD (n = 35) *	36.1 ± 9.3	38.0 ± 7.6	37.0 ± 9.0	36.6 ± 8.2
Observation/collection date	3–9 July 2017; 6–8 July 2019	18–20 July 2017	26–28 July 2017; 1–4 July 2018	10–13 July 2019
Voucher specimen	Chang Hong-Li 201,701 to 201,714 and 201,901 to 201,910	Chang Hong-Li 201,715 to 201,717	Chang Hong-Li 201,718 to 201,720	Chang Hong-Li 201,911 to 201,915

Notes: No signficant differences ($p > 0.05$) are detected in the floral characteristics (marked with a symbol "*") based on the environmental conditions (marked with symbols "**") among the four collection sites. SD, standard deviation.

2.2. Morphological Observations of Floral Organs

The fresh floral materials (i.e., about 40–50 normal and 40–50 abnormal flowers at different developmental stage) were collected from all four collection sites (Table 1) and immediately fixed in FAA solution containing formalin (Kermel, Tianjin, China), acetic acid (Kermel, Tianjin, China), and 50% ethanol (Kermel, Tianjin, China) in the ratio of 5:5:90 by volume. The color photographs of the variant flowers were taken in the field with a digital camera (Nikon Coolpix 990, Tokyo, Japan). Different types of metamorphosed stamens, i.e., white sepaloid structures and leaflike organs, were collected from the flowers in the laboratory. Floral materials used for morphological observations were given transparent treatment with 5% NaOH (Aladdin, Shanghai, China) for 5–7 d, rinsed in distilled water, stained with safranin (Haokebio, Hangzhou, China), and photographed with a Nikon Coolpix 990 digital camera under a dissecting microscope (Olympus SZX9, Tokyo, Japan).

2.3. Scanning Electron Microscope Observations of Floral Organs

The small floral samples (less than 1 cm in diameter), i.e., the representative metamorphic or abnormal stamens selected from the 30–40 normal and abnormal flowers collected

from all four collection sites (Table 1) under an Olympus SZX9 dissecting microscope with a cold external light source, were observed with SEM (Hitachi S-800, Tokyo, Japan). The stamen materials were first dehydrated in an ethanol and iso-amyl acetate series (Aladdin, Shanghai, China) and then treated with critical point drying with liquid CO_2 (Guanghuang, Nanjing, China). The floral materials were then mounted on aluminum stubs, coated with gold-palladium, and observed and photographed under SEM.

2.4. Histological Examinations of Floral Organs

The floral materials (i.e., 20–30 normal and abnormal flowers at different developmental stages collected from all four collection sites; Table 1) used for histological analysis were immediately fixed in FAA solution, as described above, and dehydrated in an alcohol series, infiltrated with xylene, embedded in paraffin wax, sectioned at 8 µm thickness using a Leica microtome (RM2125, Leica Biosystems, Beijing, China), stained with safranin and fast green (Aladdinn, Shanghai, China), and observed and photographed with a compound microscope (Olympus CX 40, Tokyo, Japan).

3. Results

The morphological characteristics of normal flowers and stamens in *A. barbulata* are shown in Figure 1. The morphological patterns of abnormal flowers and both morphological and histological characteristics of abnormal stamens in *A. barbulata* are displayed in Figures 2–4, respectively. The results of multivariate statistical analyses revealed no significant difference in the floral variations among the four collection sites under various environmental conditions (Table 1).

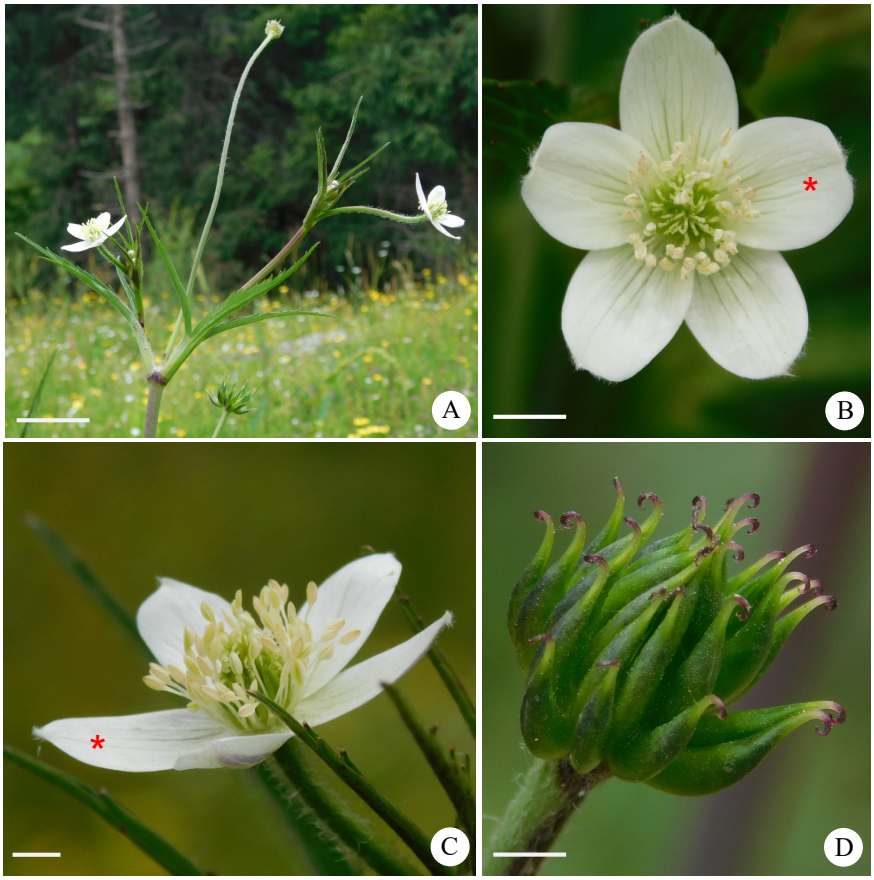


Figure 1. Mature and normal flowers and young fruit of *Anemone barbulata*. (**A**) Inflorescence (cyme). (**B**) Top view of a normal flower. A red star indicates one of the five sepals. (**C**) Side view of a normal flower. A red star indicates one of the five sepals. (**D**) Side view of young fruit. Scale bar = 1.5 cm (**A**) and 0.3 cm (**B**–**D**).

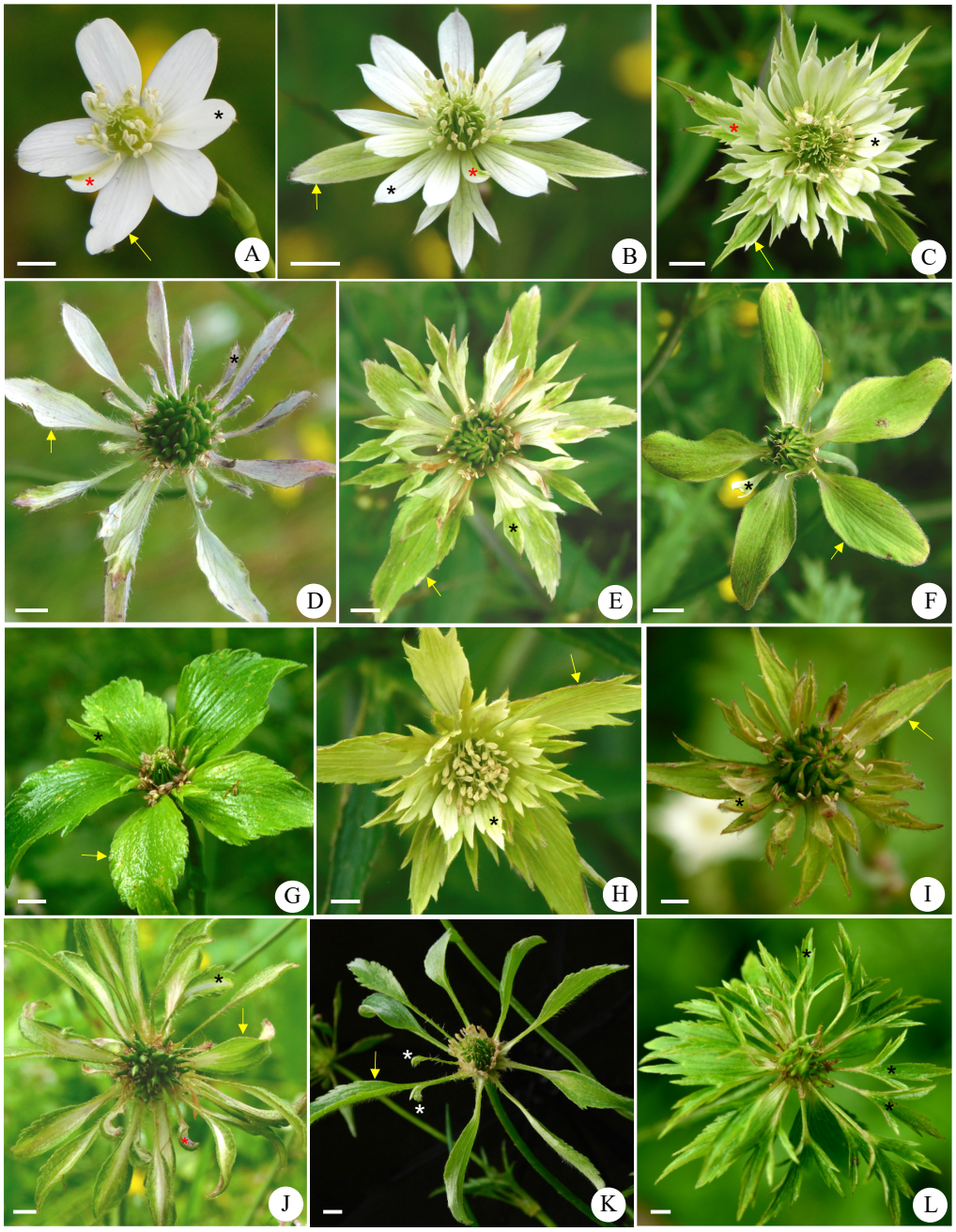


Figure 2. Mature and abnormal flowers of *Anemone barbulata*. (**A**) Several stamens metamorphosed into the white sepaloid structures (black star) with some containing the pollen sacs to generate pollen grains formed on one side (red star). (**B**) A part of the androecium metamorphosed into white sepaloid structures (black star) with some containing the pollen sacs to generate pollen grains formed on one side (red star). (**C**) Many stamens metamorphosed into white structures showing the entire edge (black star) or 3-lobed structures (red star). (**D**) Many stamens transformed into white structures with many of them containing long stalks (black star). (**E**) A typic whorl of stamens transformed into 3-lobed white or green structures with some of them containing short stalks. (**F**) Several stamens metamorphosed into green structures containing a residual anther (black star). (**G**) Several stamens transformed into 3-lobed green structures. (**H**) Many stamens transformed into 3-lobed light green or white structures. (**I**) Many concave abnormal stamens arranged in whorls. (**J**) Many stamens transformed into either entire or 2–3-lobed white structures with long stalks and green edges. (**K**) Several leaflike abnormal stamens containing the fist-shaped anthers (next to white stars) and a long stalk. (**L**) Many transformed stamens of 3-lobed leaflike structures. Metamorphic sepals are indicated by yellow arrows; metamorphic stamens are indicated by black stars. Scale bar = 0.4 cm.

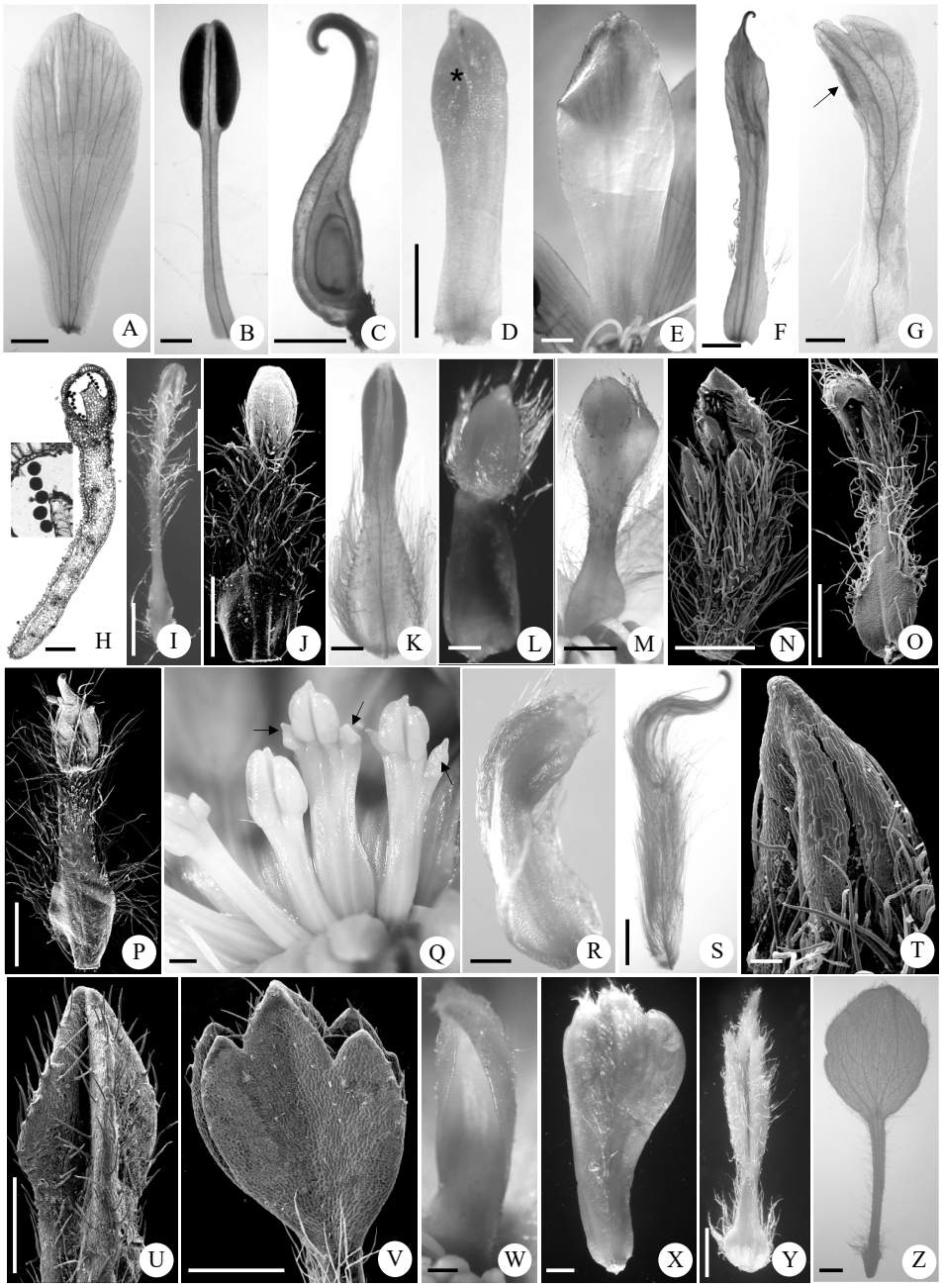


Figure 3. Normal and metamorphic floral organs of *Anemone barbulata*. (**A**) Normal sepal. (**B**) Normal stamen. (**C**) Normal carpel. (**D**) A metamorphosed stamen of white sepaloid structure containing a residual anther on the top (black star). (**E**) The metamorphosed stamen of a white sepaloid structure morphologically similar to the normal sepal. (**F**) The metamorphosed stamen of sepaloid structure showing the hooked tip and the basal three veins. (**G**) The white sepaloid structure containing a residual pollen sac on one side (arrow). (**H**) Histological observation of the cross-section of the sepaloid structure displayed in (**G**) showing the pollen sac and pollen grains. (**I**–**Q**) Various types of structural stamens. (**I**) A slightly metamorphic structural stamen. (**J**) A highly metamorphic structural stamen with a basal sheath. (**K**) A metamorphic structural stamen with the enlarged base and a sharp tip. (**L**) A metamorphic structural stamen containing the long trichomes between the filament and anther. (**M**) A metamorphic structural stamen containing a residual anther at the tip. (**N**) A 3-lobed curled metamorphic structural stamen. (**O**) A metamorphic structural stamen with adaxially concave top portion. (**P**) A metamorphic structural stamen with a 3-pointed bump at the tip. (**Q**) A metamorphic structural stamen with pointed bumps (black arrows) at the base of the anther.

(**R**–**V**) The processal stamens. (**R**) A metamorphic processal stamen with the anther forming a depression and the filament expanded into a stalk. (**S**) A metamorphic processal stamen with the upward and reversed connective and the filament covered with dense trichomes. (**T**) A metamorphic processal anther showing the opened thecae and pollen grains. (**U**) A metamorphic anther with two pollen sacs on both sides separated into four leaflike semi-slices. (**V**) A metamorphic anther with two 3-lobed leaflike organs connected by the connective. (**W**–**Y**) The leaflike stamens. (**W**) A metamorphic leaflike stamen with the adaxially concave depression. (**X**) A metamorphic leaflike stamen with three shallow lobes at the top. (**Y**) A curled metamorphic leaflike stamen showing a 3-lobed structure on the top. (**Z**) A metamorphic leaflike stamen with an entire edge and a long stalk. Scale bar = 1 mm (**A**,**B**,**D**–**S**,**U**–**Y**), 3.5 mm (**C**), 0.1 mm (**T**), and 2 mm (**Z**).

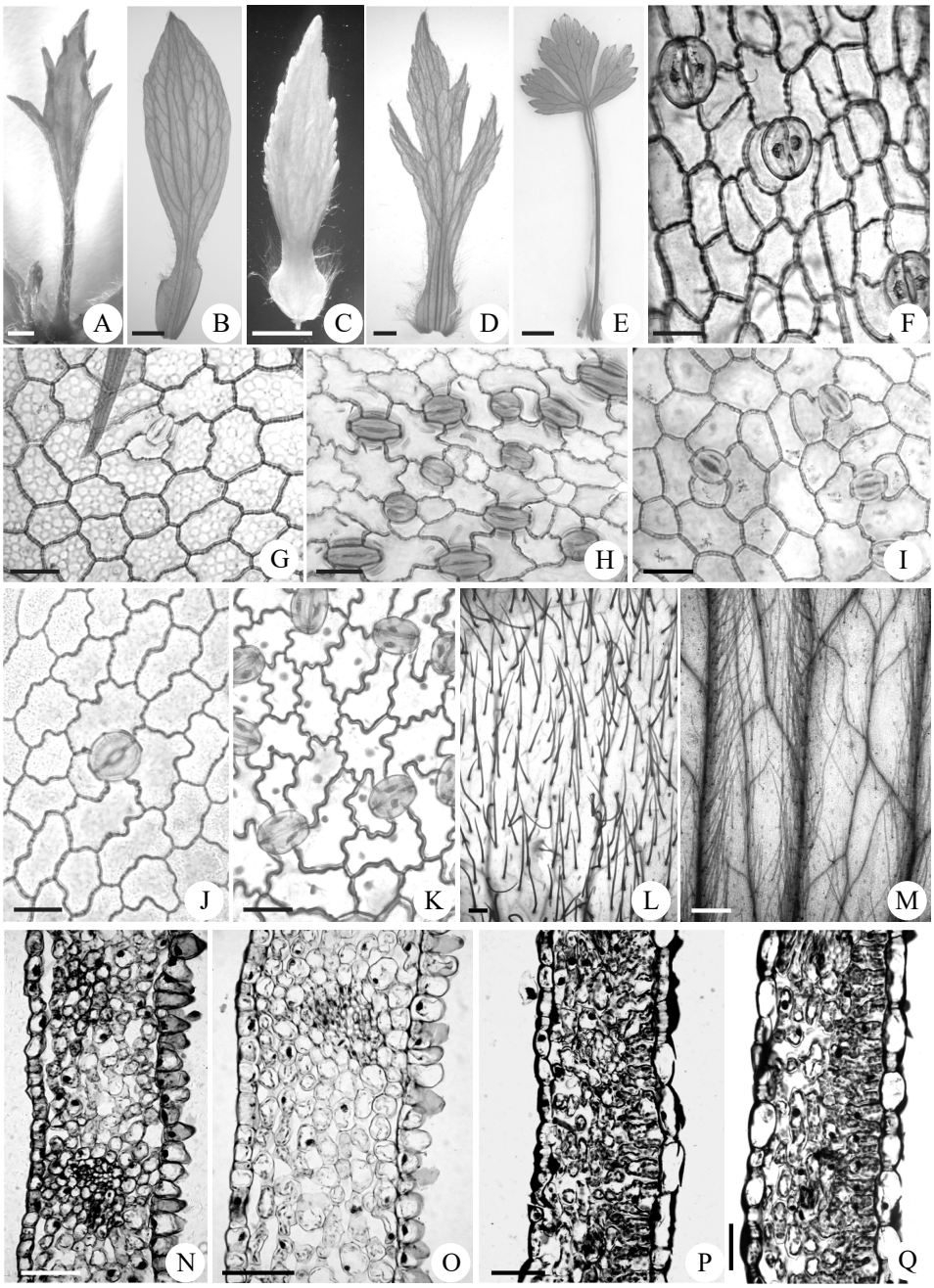


Figure 4. The metamorphic stamens and their microscopic characteristics of *Anemone barbulata*. (**A**–**E**) Five types of leaflike stamens, showing a 3-lobed edge and a long stalk (**A**), an entire edge and a broad stalk (**B**), a dentate edge and broad stalk (**C**), 3–4 shallow lobes (**D**), and three deep lobes (**E**), respectively.

(**F**) The dorsal surface with a few stomata of the metamorphosed stamen displayed in Figure 3K. (**G**) The ventral surface with a few stomata of the metamorphosed stamen displayed in Figure 3R. (**H**) The dorsal surface of the completely leaflike stamen with a large number of stomata. (**I**) The ventral surface of the completely leaflike stamen with less number stomata than that shown in (**H**). (**J**) The ventral surface of a normal leaf showing the pattern of stomata. (**K**) The dorsal surface of a normal leaf showing more stomata than the ventral surface displayed in (**J**). (**L**) The upper epidermis of a completely leaflike stamen showing the trichomes evenly distributed. (**M**) The lower epidermis of a completely leaflike stamen showing the distribution patterns of the trichomes, denser along the venation than those in the inter-venation areas. (**N**) Longitudinal section of a transformed stamen of white sepaloid organ showing no differentiation in mesophyll. (**O**) Longitudinal section of a white metamorphic sepal showing no differentiation in mesophyll. (**P**) Longitudinal section of a green leaflike metamorphosed stamen showing the differentiation of palisade and spongy parenchyma tissues in mesophyll. (**Q**) Longitudinal section of a normal leaf showing the differentiation of palisade and spongy parenchyma tissues in mesophyll. Scale bar = 2 mm (**A**–**E**,**M**), 0.5 mm (**F**–**L**), and 1 mm (**N**–**Q**).

3.1. Morphological Characteristics of Normal Flowers and Stamens in Anemone barbulata

The flowers of *A. barbulata* were attached to the compound cymes located in the axils of the uppermost leaves (Figure 1A). An involucre was formed with a total of three bracts under the cyme. These flowers were actinomorphic each with 5–7 sepals (Table 1), which were white and elliptic or elliptic-obovate in shape (Figure 1B,C and 3A). Each flower contained a total of 35–100 stamens (Table 1), smooth and glabrous with no stomata (Figure 1B,C). The stamen was 0.5–1.5 cm long (0.8 ± 0.6 cm; Table 1), consisting of a filiform filament and an anther of ellipsoid in shape (Figure 3B). Each anther was composed of two halves (thecae) connected by the connective with each theca composed of two fused pollen sacs and opened by a longitudinal slit to release pollen grains. The connective showed no protrusion. Each flower contained a total of 25–70 carpels (Table 1), each with the style hooked (Figure 3C). All floral organs were free and arranged spirally on a swollen receptacle (Figure 1B,C). The achene body was ovoid or fusiform in shape, slightly compressed, 7–8 mm long (7.3 ± 0.5 mm; Table 1), with persistent style hooked (Figure 1D).

3.2. Morphological Characteristics of Abnormal Stamens in Anemone barbulata

The transformational patterns of the normal stamens into abnormal stamens were diverse. In general, the metamorphosed stamens were observed in two routes of transformation, i.e., the stamens were transformed into either a white sepaloid structure or a green leaflike organ, as further separately described (below).

The transformation of stamens into sepaloid structures was relatively simple, with the anther and filament gradually flattened and expanded into the sepaloid structure (Figure 2A,B and 3D–I). Stomata and trichomes were generally not observed on the sepaloid structure, whereas the dichotomous veins were evident (Figure 3F,G), similar to those on the normal sepals (Figure 3A). The tips of the sepaloid structures were occasionally hooked (Figure 3F), morphologically similar to the stigma of the carpel (Figure 3C). During the flattening process, the pollen grains were observed in the external remnants of the pollen sacs on one or both sides of the anther (Figure 2A,B and 3D,G,H). It was noted that some abnormal white stamens with 3-lobed tip (Figure 2C) or covered with both stomata and trichomes (Figure 2C–E), which tended to occur in phyllome structure, were not considered sepaloid of metamorphosed stamen.

The metamorphosed stamens of leaflike structures were categorized into three groups ranked from low to high levels of transformational degree: (1) the structural stamens characterized with the presence of filament, anther, and pollen sacs (Figure 3I–Q); (2) the processal stamens showing signs of losing pollen sac and pollen (Figure 3R–V); and (3) the leaflike stamens with the absence of the morphological characteristics of a regular stamen (Figure 3W–Z and 4A–E).

3.2.1. Variations in Number, Size, and Color of Abnormal Stamens

Although the results revealed trivial difference between the number of normal and abnormal stamens (Figures 1 and 2), a significant difference was observed in size between the normal and abnormal stamens (Table 1). The normal stamens were 0.5–1.5 (0.8 ± 0.6) cm in length (Figure 3B), whereas the abnormal stamens were generally larger in size than the normal stamens, with some extremely large variants of abnormal stamens up to 7 ± 0.6 cm long (Table 1). In general, the similar type of variant stamens showed the similar size, suggesting that the evident patterns were observed in the whorled arrangement of the abnormal stamens based on the degree of metamorphosis. Both normal and abnormal stamens were observed in different colors. The filaments and anthers of the normal stamens were white and yellowish, respectively (Figure 1B,C), whereas the metamorphosed stamens were either gradually transitioned from white to green (Figure 2C,D) or directly transformed into green and occasionally with both white and green tissues detected in the same organ (Figure 2).

3.2.2. Variations in Shape of Abnormal Stamens

The shape variations were identified as the most complex morphological character in comparison between normal and abnormal stamens (below) based on the three types of transformed stamens as categorized above, i.e., the structural stamens (Figure 3I–Q), the processal stamens (Figure 3R–V), and the leaflike stamens (Figure 3W–Z and 4A–E).

In the structural stamens (Figure 3I–Q), the shape variations were evident and diverse, but the degree of changes was not conspicuous in size. The filaments were generally flat, either stripe or flaky in shape with the base of the filament expanded into a sheath and covered with long or short trichomes along the edges or on the dorsoventral surfaces (Figure 3I–M). In the anther, the connective was generally flattened to further elongate and protrude sharply (Figure 3K,L). Alternatively, the anthers were flattened upward, while the filament was flattened and expanded into a stalk (Figure 3M). The connective continued to elongate and separate from the pollen sacs, forming the middle lobe (Figure 3N,O). After the pollen sacs on both sides were separated, they were opened to form two dentate teeth or lobes, though the process leading to this condition was not observed (Figure 3N,O). Among these three dentate teeth or lobes formed on the anther, the middle one was always the largest, while the 3-lobed structure initially formed was generally flaky and curled (Figure 3N,O). The pollen grains were generally present in the pollen sacs remained in the structural stamens. Two additional conditions were occasionally observed, i.e., (1) the connective and pollen sacs on both sides formed the 3-pointed bumps (Figure 3P) and (2) the stamens formed the pointed bumps on one or both sides of the anther (Figure 3Q).

In the processal stamens (Figure 3R–V), the morphological variations were more diverse in comparison to those observed in the structural stamens. The stamens were flattened in one of the four different ways: (1) continued from the morphological observations of structural stamens, i.e., probably as those shown in Figure 3K, the anther was flattened outward along the connective, while the outer side of the anther was maintained as the sac-like structure to varying degrees, causing the anthers to form a depression, while the filament was flattened and expanded into a stalk (Figure 3R); (2) the connective was extended upward and reversed to form a hook-like structure, the anthers were partially flattened longitudinally and slightly coiled inward, and the filament was enlarged and covered with dense trichomes (Figure 3S); (3) each theca was opened along the longitudinal slit to release the pollen grains and the two pollen sacs on each side were separated from each other to form four leaflike semi-slices, while the filament was enlarged into a flaky or stripe structure (Figure 3T,U); and (4) each pollen sac was opened along the dorsoventral side to form two 3-lobed leaflike organs, connected by the central connective (Figure 3V). These metamorphosed stamens no longer contained the basic anther and filament structures, i.e., the connective, and the pollen sacs, and pollen grains, and thus completely degenerated morphologically and anatomically.

In the leaflike metamorphosed stamens (Figure 3W–Z and 4A–E), some stamens were rolled inward to form depressions (Figure 2G,H and 3X). These leaflike stamens were commonly found and largely located in the middle of the androecium but still far away from the carpel compared with the structural and processal stamens. These leaflike stamens showed some shared morphological variations with the processal stamens, e.g., those displayed in Figure 3R, showing shrinking pollen sacs and enlarging anthers to form deep flaky depressions as well as those with three shallow lobes at the top (Figure 2C,G and 3X), which were probably the continued development from those displayed in Figure 3N, showing the gradual disappearance of pollen sacs and formation of 3-lobed structures on the top, and occasionally involuted with 3- or 5-lobe and often stalked (Figure 3Y), with the latter type sharing the morphological variations with some of the processal stamens (e.g., Figure 3N,O). Ultimately, the stamens developed into the flat leaflike structures. The margin patterns of the leaflike stamens were also morphologically diverse, showing either entire (Figures 3Z and 4B), 3- or 5-lobed (Figure 4A), crenate to denticulate (Figure 4C), or one or two rounds of three shallow or deep lobes (Figure 4D,E). The common shapes of the leaflike stamens were usually 3-lobed, occasionally 5-lobed. The most extremely transformed stamens were morphologically the same as the leaves of *A. barbulata*, showing the long petioles with 3 lobes (Figures 3Z and 4E) and located in the outmost of the androecium (Figure 3K,L).

3.2.3. Variations in Venations, Stomata, Trichomes, and Persistence Patterns of Abnormal Stamens

In the normal stamen, there was only one vascular bundle extended from the receptacle into the stamen and then into the connective (Figure 3B). With the intensified degrees of metamorphosis in the stamens, the number of basal veins was gradually increased from 1 (Figure 3K) to 3 (Figures 3F and 4B) or even 5 (Figure 4D). In the structural stamen, venation was generally not formed (Figure 3K), whereas a complex network of veins was gradually established once the green leaflike structure was formed (Figure 4B,D,E).

The stomata were not observed on the epidermis of normal stamens. In the abnormal stamens, when the filament began to flatten but had not yet formed a sheet-like structure (Figure 3K), the stomata were observed on the dorsal surface but not on the ventral side (Figure 4F). When the anthers were flattened (Figure 3R), stomata began to appear on the ventral surface (Figure 4G). The highest number of stomata (1.5 ± 0.6 mm^{-2}) was observed in the completely leaflike stamens (Figure 4H,I). Similar to the leaves of these plants, more stomata were revealed on the abaxial surface than the adaxial surface of the metamorphosed stamens (Figure 4J,K).

The normal stamens contained no trichomes. As the degree of leaflike feature increased in the metamorphosed stamens, the trichomes were observed, either abundant and messy in both structural and processal stamens (Figure 3I–K,M,R,S) or uniform in the leaflike stamens, i.e., the upper epidermal trichomes were evenly distributed (Figure 4L), while the lower epidermal trichomes showed a consistent distribution pattern with denser trichomes along the venation than those in the inter-venation areas (Figure 4M).

Our observations showed that the normal stamens of *A. barbulata* fell off after the pollination was completed, whereas the leaflike metamorphosed stamens remained on the plants.

3.3. Histological Analysis of Abnormal Stamens

The results of the histological studies showed that in the white sepaloid organs, no differentiation was observed in mesophyll into either palisade or spongy parenchyma tissues. The cells of the mesophyll were largely round in cross-section (Figure 4N), which was similar to that of the normal sepals of these plants (Figure 4O). In the green leaflike metamorphosed stamens, the upper surface was composed of a layer of the palisade parenchyma, which was the main photosynthetic tissue, while the lower half of the green leaflike organ

was composed of open, loose aerenchyma tissue, which was spongy mesophyll (Figure 4P), showing the similar structure to that of the normal leaves (Figure 4Q).

4. Discussion

In this study, a large number of flower buds (up to 40–50 collected from all four collection sites) at early developmental stages were observed to determine the temporal initiation of metamorphosis of stamens in *A. barbulata*. The results revealed largely normal development at the ontogenetic stages of stamens of these flower buds. These results were consistent with those previously reported [42], with the earliest observation of metamorphosis in stamens of *A. barbulata* determined at the late stage of flower bud. We further explored the morphological and histological characteristics of the metamorphic stamens in the floral organ development and evolution.

4.1. Metamorphic Variations of Stamens in Anemone barbulata

4.1.1. Characterization and Variation Patterns of Metamorphic Stamens

Our findings were consistent with those previously reported in *A. barbulata* [42], showing that the total number of both normal and abnormal stamens ranged from 35 to 100, based on the observations of flower samples at different developmental stages. Studies haves shown that the significant differences were observed in the number of normal and abnormal stamens in two genera of Ranunculaceae, *Delphinium* and *Aquilegia* [43].

In the first relatively simple metamorphic process, i.e., the transformation of stamens into white sepaloid structures, which were generally not covered with any stomata or trichomes. In the second metamorphic process, the stamens of *A. barbulata* were transformed into green leaflike structures via diverse and complex routes, showing successive variations of size, color, shape, venation, stoma, trichome and persistence pattern. In general, the metamorphosed characteristics of stamens included: (1) the varied morphological characteristics and transformational degrees of the abnormal stamens were detected in the same flower; (2) the varied transformation processes of stamens ranged from wrapping to open patterns; (3) the outer whorl of the stamens showed more variations than the inner whorl of the stamens of the same flower; (4) the variant stamens with similar degree of transformation were arranged in the same whorl on the receptacle of the flower; (5) the microscopic morphological traits of metamorphosed stamens, such as trichomes, stomata, and venations, were developed to the same levels of those in the phyllome structures, whereas the metamorphosed stamens themselves did not develop into true leaves, suggesting that these morphological characteristics developed faster in leaflike organs than the normal morphological changes in stamens; and (6) as the degree of metamorphosis increased, the transformation level of stamens into leaflike organs was enhanced, while the microstructure of leaflike stamens was largely the same as that of the normal leaves. The transformations of these characteristics were generally consistent with the metamorphosis of sepals in *A. barbulata* [23]. For example, the trichomes were denser on the adaxial surface than that of the abaxial side of the transformed stamens. These results were consistent with those previously reported, showing that the adaxial side was covered with dense trichomes of the genuine cauline leaves in *Arabidopsis* [17]. Furthermore, in the mutant flowers in cultivars of *Delphinium* and *Aquilegia*, the abnormal floral organs were revealed with features of both petals and stamens or stamens and carpels, respectively [43], suggesting the morphological transformation and homeotic variation between petals and stamens or between stamens and carpels.

Together with the comprehensive analysis of the metamorphosis of sepals [23] and stamens in *A. barbulata*, it was concluded that (1) the transformation of floral organs in *A. barbulata* started from the outside of the receptacle to the inside of the flower, i.e., an anamorphosis process; and (2) in flowers with metamorphosis of only tepals, the tepals were not deeply divided, showing either entire edge or inter-dentate teeth or small lobes, while the 3-lobed metamorphic tepals were often observed in flowers with metamorphosed stamens [23]. These characteristics revealed the inward transformation pattern of the ab-

normal stamens in *A. barbulata*, suggesting that the translocation of homologous structures could be acropetally completed [7]. Furthermore, in both *Delphinium* and *Aquilegia*, the floral organization, vascularization, symmetry, and organ number of the abnormal flower caused the formation of mosaic organs compared with the normal flowers [43], whereas our study showed that in *A. barbulata*, the mutant changes were reflected in the same floral organization and vascularization but not in the symmetry and organ number of the flower.

4.1.2. Metamorphic Characterization of Anthers

Each of the normal stamens of *A. barbulata* contained two anthers and four pollen sacs. Our extensive observations revealed two flattening routes in the process of metamorphosis in stamens developing into leaflike organs. First, the anthers appeared to be flattened from the base upward into a smooth and flat dorsoventral structure with no thickening or depression formed, whereas the pollen grains were commonly remnant. It was expected that this type of foliar structure was probably transformed into the sepaloid organs. The second flattening route was rather more complex and varied than the first route with the longitudinal flattening of anthers started from the connective to transform into a leaflike organ. In this process, the depression or circinate stages were evidently observed, which were not detected in the metamorphosis of sepals in *A. barbulata* [23]. This variation was probably caused by the shared features of both foliar organs. Although it was difficult to determine the relationship between depressions and circinate organs, it was speculated that both of these organs represented important stages in the transformation of stamens, with each or both being the necessary processes in the transformation of axial to foliar organs. It is commonly known that in both angiosperms and ferns, some leaves go through a circinate stage before opening, suggesting that the characteristics of depression and circination could probably be the common approaches used by plants to transform from axial to foliar organs. Furthermore, it was also worth noting that the 3-lobed structure was the most common feature revealed during the metamorphosis of stamens in *A. barbulata*. It was speculated that the connective and pollen sacs on both sides of connective were transformed into these three lobes, respectively. This speculation could be supported by the characteristics of stamens in the basal genus of angiosperms *Chloranthus*, showing the androecium composed of a 3-lobed structure with four thecae [44,45], even though it was still unknown whether this 3-lobed structure was evolved from either the subdivision of one stamen [46,47] or the fusion of three stamens [48]. Therefore, we proposed two alternative hypotheses explaining the formation process of anthers in angiosperms. First, the leaflike organs could thicken the edges or depressions and gradually enlarged into pollen sacs. Second, the ancestral state contained a 3-lobed leaflike structure, with the central lobe forming the connective and the lobes on both sides curled to form pollen sacs. However, it was noted that the results were not sufficient to determine whether these two processes were parallel or sequential. Further studies were needed to provide evidence to support these hypotheses and their developmental modes. It was noted that our observations suggested that some of the abnormal stamens were still functionally reproductive because they still generated pollen grains. In particular, pollen grains were generated in both structural and processal stamens, whereas leaflike stamens did not generate pollen grains. These variations were observed on the same individual plant. Further studies are needed to quantify the impact of the overall reproduction of the individual plants, i.e., this impact might be recovered by alternative vegetative growth of rhizomes. To conclude, from the morphological perspective, the essential nature of the leaflike stamens in *A. barbulata* was probably the recurrence of the foliar stage of the ancestral stamens of angiosperms. Therefore, the metamorphosis of flower in *A. barbulata* with all floral organs capable of transforming into leaflike organs provided strong evidence to support the "foliar theory" stating that floral organs are modified leaves [2,16].

4.1.3. Homeosis in Floral Organs

Although the transformed processes of the stamens in *A. barbulata*, in particular the anthers, appeared to be diverse and arbitrary, the common morphological patterns reflecting the homeotic transformations among the floral organs were evident, e.g., the sepaloid organs, the leaflike structures, the retention of pollen sacs in the variant stamens, the 3-lobed leaflike structures, and the hook-like recoiling at the tips of the transformed stamens. All of these structures showed that the morphological nature of the metamorphic organs could be revealed in either flowers or leaves, suggesting the homological variants among floral organs and between floral organs and leaves. The homeotic transformation is commonly known between petals and stamens in Ranunculales [12,21,49–51] with the exceptions identified in *Sanguinaria* and *Macleaya* of Papaveraceae, showing the nonhomologous structures of petals and stamens [21]. Furthermore, the homeotic transformations are also found between vegetative and reproductive organs in angiosperms [52–54]. For example, the stamens in wheat were revealed with foliar origin [52].

It was claimed that the most extreme form of the abnormal flowers is that any floral organs could be replaced by foliage-type of leaves, except for the stamens, which are the least likely to transform into leaflike organs [8]. However, our study showed that the stamens of *A. barbulata* was evidently transformed into either sepaloid or leaflike organs. To date, it seemed that *A. barbulata* was the only example showing the homeotic transformation of all floral organs into leaflike organs found in nature. Morphological studies have shown that the most primitive types of stamens are flat and wide bars in shape, leaflike organs with 3-base veins, with no differentiation of filament, anther, and connective [8,55–59]. Indeed, the data in our study showed that this foliar structure proposed in these studies was morphologically similar to the intermediate 3-lobed circinate structure with residual pollen sacs and pollen grains identified in *A. barbulata.* The leafy nature of stamens was known as early as in Goethe's time [2]. Therefore, it could be indeed assumed that the progressive transformation of stamens into 3-lobed circinate structure and further into flattened leaflike organ represented a major developmental transformation in the stamens of angiosperms, while homeosis was probably the result of a gradual or a sudden evolutionary process which is effective in the evolution of floral diversification in triggering either structural or functional changes [21]. If the "foliar theory" is supported by the development of the floral organs, then homeotic development could be the result of a progressive differentiation of floral organs. Therefore, it is evident and reasonable to conclude that the observations of all floral organs transformed into leaves in *A. barbulata* were supportive of the progressive transformation with the homologous characteristics retained in both floral organs and leaves.

It was proposed that if one type of floral organ is found in the position of another type of floral organ or two types of floral organs switch their positions, then homeosis is the result of a sudden evolutionary process [21]. This proposal was supported by the flowers of *Lacandonia* showing that carpels and stamens switched their positions [60] and flowers of *Capsella bursa-pastoris* showing that four petals were transformed into stamens [29,31]. These results suggested that the metamorphosis of the floral organs in both *A. barbulata* and *Lacandonia*/*C. bursa-pastoris* were two alternative developmental modes of floral organs. It was noted that our data presented in this study, i.e., the floral organs were normal during the early developmental stages, whereas the metamorphotic variations were observed only during the later floral developmental stages, and these variations were commonly and consistently observed in different geographical collection sites, were not sufficient to provide any strong evolutionary insights to the floral evolution in angiosperms. Further studies, e.g., identification of the genes responsible for these transformed stamens, are necessary to identify the explicit evolutionary significance of the metamorphosed stamens in the floral evolution of angiosperms.

4.2. Possible Mechanisms of Metamorphosis in Stamens of Anemone barbulata

Our observations and a previous study [42] revealed both the normal and abnormal stamens arose as elliptical primordia at the beginning of the organ initiation stages, while

the homeotic variation of the stamen likely occurred in the later developmental stages. Therefore, it was assumed that the metamorphosed stamens were initiated with the primordia similar to those of normal stamens at inception but subsequently followed the developmental patterns similar to those of sepals or leaves. This assumption would be supported by the observations in both *Delphinium* and *Aquilegia*, showing that the floral mutants were formed after their initiations of these organs [43]. Similarly, the genetic switch was demonstrated in both *Clarkia tembloriensis* [61] and *Arabidopsis* [62], revealing an early development similar to that of floral primordia with a differentiation identified during the later developmental stages.

Although the transformation of sepals into leaflike organs in *A. barbulata* has been reported [23], our results showed that the metamorphosis of stamens was much more complex as the stamens could either entirely or partly develop into sepaloid or leaflike organs, even part of the pistil, i.e., the style tissue. The formation of these metamorphic organs was probably caused by all five classes of floral genes. Indeed, the molecular mechanisms regulating the metamorphosis of *A. barbulata* have been explored. For example, the floral variations of *A. barbulata* were attributed to the difference between two *AP3-3* paralogs of the normal and abnormal flowers of *A. barbulata* [63], while the expressions of *FUL1*, *SEP1*, *SEP3*, and *AGL6* genes were significantly up-regulated in variant plants in comparison with those in normal flowers of *A. barbulata* [64]. Alternatively, the external stimulations, e.g., low temperature induction, nutrient or soil conditions, parasitic insect pests, or chemical treatment, could be the possible causes of the metamorphosis of floral organs [8,65]. Furthermore, studies showed that the physical constraints could condition the phenotypic outcome of the floral organs, including the size and shape of a floral meristem and the de novo leaf primordia [65–68]. The results of multivariate statistical analyses revealed no significant variations in the floral characteristics among the four collection sites (Table 1), suggesting that the varied environmental conditions played insignificant roles in the process of metamorphosis in flowers of *A. barbulata*.

4.3. Persistence Patterns of Stamens in Anemone barbulata

Abscission of the normal stamens of *A. barbulata* was observed after pollination, whereas the metamorphosed stamens showed varied persistence patterns. The slightly abnormal stamens still contained the pollen sacs with pollination functions and abscissed after pollination. Similarly, the white sepaloid stamens abscissed after pollination. However, in the leaflike metamorphic organs, the stamens persisted after pollination, consistent with the physiological and functional characteristics of normal floral organs and leaves. Similarly, the metamorphosed sepals also persisted in *A. barbulata* [25]. Given the biological and agricultural importance of the persistence patterns in plants [69–71], it is important to explore the morphological and molecular mechanisms regulating the developmental andpersistence patterns of the metamorphic stamens and sepals in *A. barbulata* after pollination.

5. Conclusions

This work provided a general view of the morphological metamorphosis in stamens of *A. barbulata*, showing the transformation of the stamens into either white sepaloid structures or green leaflike organs, highlighting the morphological characteristics of the stamens of the ancient flowers. In particular, the depression or circinate stages, the connective, and two pollen sacs of the anther represented the apical 3-lobes of the unfolding leaflike organs. These characteristics revealed the stamen development from axial to foliar organs and the homeotic transformations among leaf, sepal, stamen, and carpel. Our study provided morphological evidence to support the "foliar theory" stating that floral organs were derived from modified leaves. The metamorphosis of stamens in *A. barbulata* was probably an example representing the progressive development mode in the formation of floral organs in angiosperms. Future studies are necessary to identify the genetic and molecular factors involved in the formation of these metamorphic organs in *A. barbulata*.

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