

BRIEF COMMUNICATION

**THE STRUCTURE AND DEVELOPMENT OF INCOMPLETELY CLOSED  
CARPELS IN AN APOCARPOUS SPECIES, *SAGITTARIA TRIFOLIA*  
(ALISMATACEAE)<sup>1</sup>**

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- **Premise of the study:** Carpel closure is commonly considered as a key innovation in angiosperms; however, some families continue to exhibit a variety of forms of incomplete carpel closure. The carpel of *Sagittaria* species contains an unusual structure. In this study, we confirm the closure type of the carpel of *Sagittaria trifolia* and discuss its development and evolution.
- **Methods:** Scanning electron microscopy and light microscopy of semithin sections were used to observe the development and the mature structure of the carpel. Pollen tube growth in the carpel and seed germination in the achene was also studied.
- **Key results:** During late carpel development, the middle parts of the carpel margins underwent postgenital fusion. However, at maturity the lowest and uppermost parts of the carpel margins remained open. The mature carpel was incompletely closed and contained a secretion-filled canal, which extended from the stigma to the opening at the carpel base. At that site, pollen tubes could either grow to the ovule or exit the carpel and grow to other carpels. The basal opening also served as an exit point for the seedling to emerge.
- **Conclusions:** Incomplete carpel closure by *S. trifolia* differs from the closure types recorded in previous studies because two entrances link the ovary in the carpel to the outside environment. This type of carpel closure occurs as a result of the lack of fusion of the carpel margins at the base of the carpel and could improve the seed set and seedling germination of *S. trifolia*.

**Key words:** Alismataceae; carpel closure; carpel development; postgenital fusion; *Sagittaria trifolia*.

The carpel is a female reproductive organ that is unique to angiosperms and is thus often associated with their origin. Consequently, considerable research is focused on its structure and evolution, but the origin of the carpel still remains elusive. Originating in the flower center as open structures, carpels develop into closed sac-like structures that protect the ovules inside an ovary chamber (locule) that becomes sealed by epidermal appression and fusion of the carpel margins (Gasser and Beers, 1993; Taylor and Kirchner, 1996; Doyle, 1998; Endress and Igersheim, 2000). In addition to protecting ovules and seeds against herbivores and pathogens, carpel closure also plays an important role in increasing pollen competition and selection; therefore, the process of carpel sealing could have impacted the early history of angiosperms (Stebbins, 1974; Mulcahy, 1979; Haig and Westoby, 1989; Endress, 2001).

According to Endress and Igersheim (2000), four types of carpel can be distinguished based on their mode of closure. The true closed carpels are of angiospermy type III or IV and are also called “solid carpel” because the margins are partially and

totally fused and form a solid pollen tube transmitting tissue. In contrast, the margins of carpels of angiospermy type I or II are either unfused or only partially fused such that a hollow secretion-filled pollen tube pathway is formed (for details, see Endress and Igersheim, 2000). Carpels of type I and II are found among the earliest-diverging families of flowering plants, and occasionally among families that diverged later (Kaplan, 1967; Crepet, 2000; Raven and Weyers, 2001), but the carpels of angiospermy type III and IV have truly been driving the diversification of pollen-tube pathways and fruit structures early in their history (Doyle and Endress, 2000; Bobrov et al., 2005; Williams, 2009; Williams et al., 2010; Romanov et al., 2013). This categorization provides an effective way to help researchers understand the evolution of carpel structure (Endress and Igersheim, 2000; Endress, 2001), but the question remains as to whether carpel closure of angiosperms contain more patterns and whether some small variation in development process of carpel could impact the reproduction of plants.

The genus *Sagittaria* is distributed worldwide and has the richest species composition in the basal monocotyledonous family Alismataceae (Bogin, 1955; Chen, 1989). Kaul (1976) indicated that in *Sagittaria* carpels tend to be closed at anthesis by observation, but some recent reports have shown these carpels are probably incompletely closed (Wang et al., 2002, 2012). Here we studied the carpel structure and development of *Sagittaria trifolia*, an aquatic weed that mainly grows in paddy fields in China, to (1) re-evaluate the type and (2) the mechanism of carpel closure in *S. trifolia*, and (3) understand the impact of this carpel closure on the reproductive process of *S. trifolia*.

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## MATERIALS AND METHODS

**Study species and sites**—Plants of *Sagittaria trifolia* were collected from a paddy field in Xinyang, Henan Province, China (31°47'N, 114°02'E) in May 2012. They were transplanted to the greenhouse of Wuhan University, China (30°54'N, 114°36'E), where all experiments were carried out during July and August of 2012.

**Carpel development**—Floral buds, flowers, and fruits were collected at different stages and were preserved in formalin–acetic acid–70% alcohol (FAA; 5:5:90, v/v). The fixed material was dehydrated through a series of alcohol solutions ranging from 50 to 95%. The carpels were collected and passed through an iso-pentanol acetate series (SCR, Shanghai, China), and then critically point-dried, sputter-coated with gold, and viewed using a scanning electron microscope (Tescan VEGA-3-LMU, Brno, Czech).

**Observations of mature carpel structure**—Some fresh carpels were rapidly removed from the receptacles of anthetic flowers and immediately frozen in liquid nitrogen for 5 min, then observed with a cryo-scanning electron microscope (Tescan Quorum-S-3400N, Brno, Czech). To delineate the structure of individual carpels, we cut semithin transverse sections at six levels of the mature carpels. Some carpels from mature gynoeceia were fixed in FAA, dehydrated, and embedded, then sectioned with a glass knife to a thickness of 1  $\mu$ m. Sections were mounted on slides and stained with 0.5% toluidine blue (w/v in 0.2 mol/L  $\text{Na}_2\text{HPO}_4$ ), and observed with a light microscope (Nikon E100, Tokyo, Japan) and bright field optics (Wang et al., 2006).

**Observation of pollen-tube growth and seed germination**—Some female flowers of *S. trifolia* were bagged before anthesis, then hand-pollinated with a small quantity of pollen after blooming. The gynoeceia were picked from those hand-pollinated flowers 6 h later and fixed in FAA. These fixed gynoeceia were longitudinally sectioned by hand and cleared in NaOH (2%) until most tissues had become transparent, then stained with aniline blue (0.1% w/v in 0.03 mol/L  $\text{K}_3\text{PO}_4$ ) for 2 h (Kho and Baer, 1968). All sections were squashed and observed with a fluorescence microscope excitation at 395 nm (Olympus BX-43-U, Tokyo, Japan).

Mature aggregate fruits were collected from plants of *S. trifolia*. Achenes were peeled from these fruits and placed in a beaker of distilled water to promote germination. We observed changes in achene structure using a stereomicroscope (Olympus SZX2-ILLT, Tokyo, Japan) during seed germination.

## RESULTS

**Carpel development**—In *S. trifolia*, hundreds of carpel primordia were initiated successively in a spiral pattern on the receptacle (Fig. 1A). During the early stages of development, the young carpel is noticeably peltate, with an ovule primordium forming near at the base of the convex ventral side, just above the cross zone (Fig. 1B, C). The developing carpel becomes oblong and more concave, and its margins gradually grow over the ovule (Fig. 1D, E) until they touch each other on the ventral side and form a median longitudinal suture line from the tip of the carpel down to the cross zone below the point of ovule insertion (Fig. 1F). The carpels are thus conduplicate, although the ascidiate base of the ovary remains very short and the carpel is entirely plicate above the cross zone (white arrow, Fig. 1G). As the carpels develop, the ovaries become flattened laterally and bulged dorsally, and a ventral median longitudinal suture line extends from the tip of the stigmas almost down to the bases of the carpels. At anthesis, the suture appears incompletely closed above the cross zone at the base of the carpel (Fig. 1H, 1I).

**Structure of the mature carpel**—The ventral suture line was visible on the adaxial side of conduplicate carpels under SEM (Fig. 2A). The stigmas are minute and covered with many unicellular papillae arranged at the tip of the carpels around the

distal end of the ventral suture at the tip of the tip. At the proximal end, the ventral suture is still incompletely closed, and there is a small opening at the ascidiate base of the ovary, which had a glabrous epidermis (Fig. 2A).

Through serial transverse sections of a mature carpel of *S. trifolia* (Fig. 2A), we found that the plicate tip of the carpel, which forms the stigma was unfused, and that a secretion filled the ventral area (Fig. 2B). Below the stigma, however, the margins of the carpel are postgenitally united. Only a very thin canal is present in the center of its cross section, in which the secretion was distributed (Fig. 2C). The pollen tubes were able to grow distributed in the extracellular matrix (Fig. 2C'). At the top of carpel, the ovarian cavity is visible in cross section, and to the right, the canal with secretion is located between the ovarian cavity and ventral suture line (Fig. 2D). In the middle of the carpel representing the largest and the most basal region, the margins of the carpel remain fused, but the tiny unfused ventral canal disappears (Fig. 2E) and some secretions are present on the inner ventral surface of the ovarian cavity (Fig. 2E'). At the base of the carpel, the margins are incompletely fused above the cross zone (Fig. 2F) at the transition from the ascidiate base of the ovary (Fig. 2G) to the plicate upper part and clearly form an opening that connected the ovarian cavity with the outer part of the carpel (Fig. 2F). This opening corresponds to what we will refer to as the basal ventral outlet.

**Pollen-tube pathway and function of basal ventral outlet**—Pollen grains germinated on the stigmas, and pollen tubes grew to the base of the stylar canal. Then they could follow two avenues: turning to the ovule in the ovarian cavity or exiting from the carpel through the basal ventral outlet (Fig. 3A). When a pollen tube exiting from one carpel and growing in the extragynoeceal area arrived at the basal ventral outlet of another carpel in the gynoeceum, this pollen tube could enter this carpel and reach the ovule within it (Fig. 3B). By extragynoeceal growth on the receptacle surface, pollen tubes from a stigma of one carpel could end up entering other carpels of the gynoeceum (Fig. 3C). After the mature achene was placed in the distilled water, the seed in the achene was clearly seen (Fig. 3D); 3 or 4 d later, the seed germinated, and the radicle was observed to pass through the basal ventral outlet of the achene (Fig. 3E).

## DISCUSSION

**Carpel development and typification in *Sagittaria***—Previous studies have described the free carpels in the gynoeceum of *Sagittaria* as being conduplicate and assumed that they were closed, because the carpel margins appear to be adequately fused together (Singh and Sattler, 1973, 1977; Kaul, 1976). However, in the present study, we demonstrated that the carpel closure of *S. trifolia* is incomplete and that angiospermy type II, a postgenitally fused periphery is present, but fusion is incomplete in the stigmatic area. Meanwhile, in the carpel of *S. trifolia*, the stylar canal extended from the stigma down to the base of ovary, and in this area fusion was also incomplete such that a basal ventral outlet was formed at the ventral base of the carpel. Hence, in contrast with carpels of angiospermy type I, and type II of carpel closure in previous studies, in which access to the ovule is pronounced only via the stigma. The carpels of *S. trifolia* also provide access to the locule for the pollen tubes via the stigma, but also via the morphological and functional innovation of a basal ventral outlet.

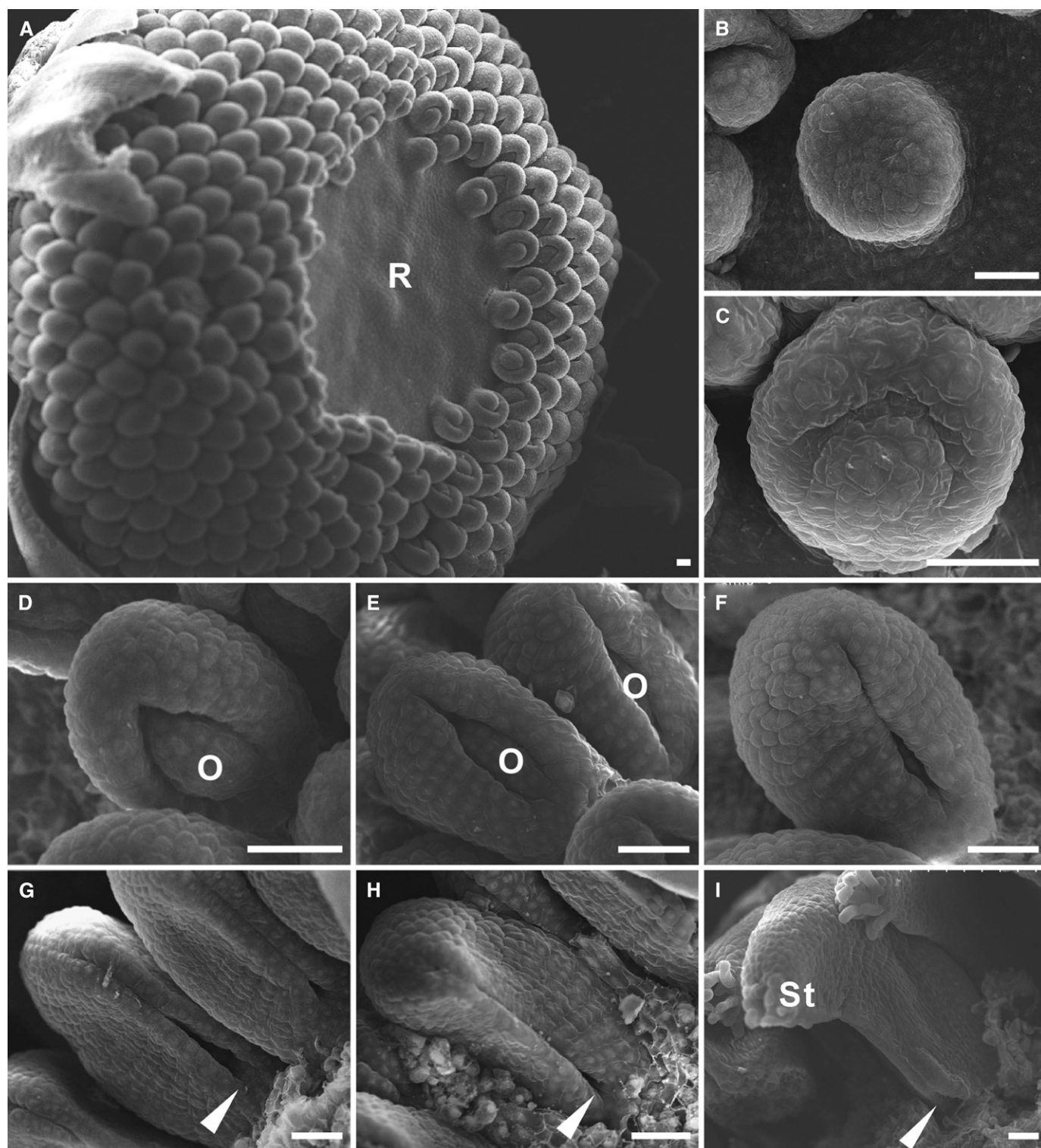


Fig. 1. Development of the carpel in *Sagittaria trifolia*. (A) Carpels are seemingly spirally arranged on the receptacle. (B) Carpel primordium. (C, D) Ventral side of young carpel with an ovule primordium initiated just above the cross zone at the transition from the ascidiate to the plicate zone. (E) Carpel growth and formation of the locule around the ovule. (F) Carpel with closed locule and plicate margins. (G) Carpel with bulged dorsal side of the ovary and a ventral suture line between the plicate margins closing from their tips down toward their bases. (H) Carpel with ventral suture line almost entirely closed down to the base of the ovary. (I) Carpel shortly before anthesis. White arrowheads indicate the cross zone of the carpels at the transition between its very short ascidiate ovary base and its plicate upper parts. Bars = 50  $\mu$ m. O, ovule; R, receptacle; St, stigma.



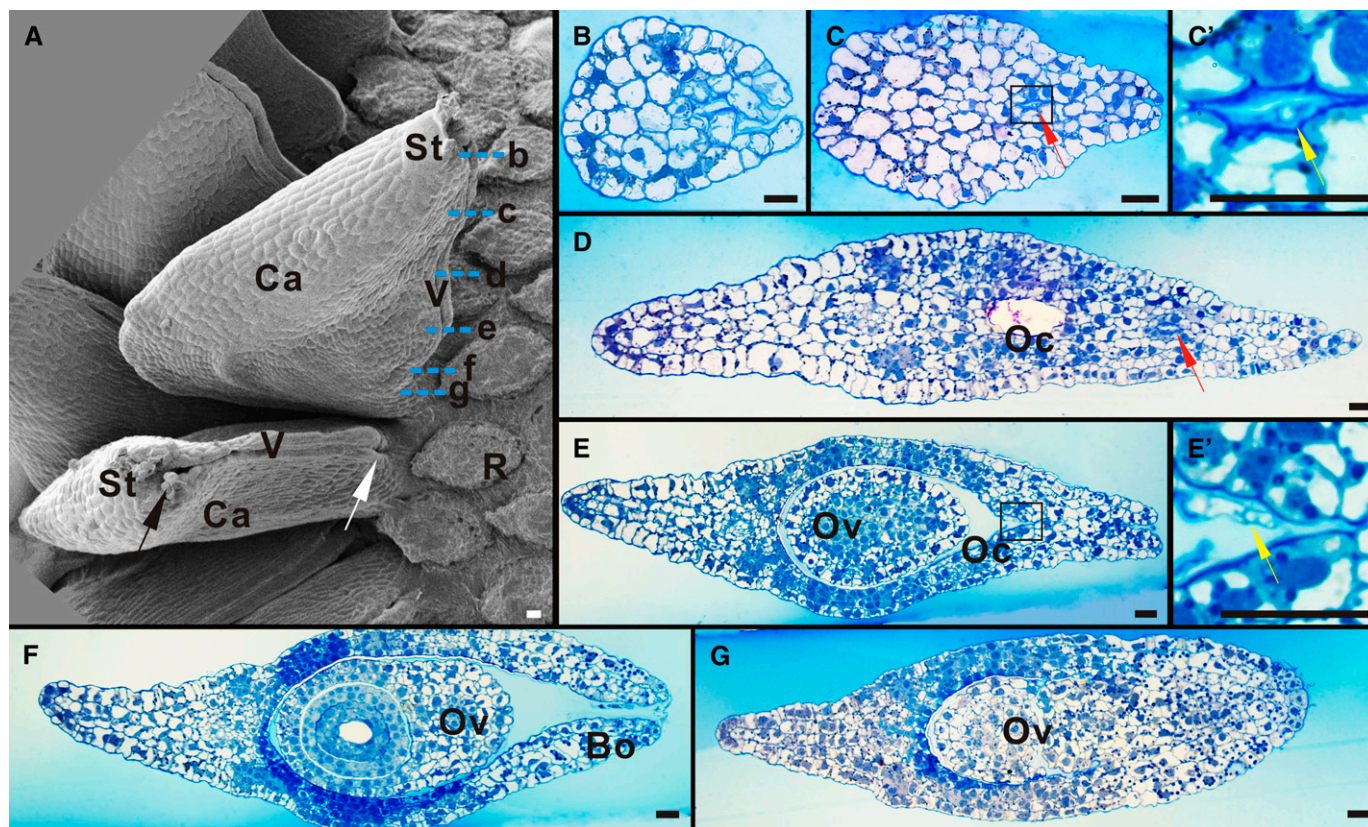


Fig. 2. Mature carpel in *Sagittaria trifolia*. (A) Scanning electron micrograph of two carpels on the receptacle in the gynoecium, which show the ventral side and lateral side of the carpel, respectively. Black and white arrowheads indicate the stigma and the basal ventral outlet, respectively. Blue dotted lines with lowercase letters indicate levels of (B–G). (B–G) Semithin transverse microtome section series of a carpel stained with toluidine blue arranged from the tip (B) to the cross zone of the carpel (I). (B) Stigma, showing plicate but not postgenitally fused carpel margins. (C) Midway between the stigma and the top of the ovary (D); box: region where carpel margins do not fuse postgenitally. (C') Magnification of boxed area in (C). (E) In the plicate portion of the ovary where the carpel margins are postgenitally fused on the ventral side; box: region shows inner ventral walls of the ovarian cavity. (E') Magnification of boxed area in (E). (F) Base of the ovary where the plicate margins of the carpels are not fused and form the basal ventral outlet. (G) Cross zone just below the basal ventral outlet. Red arrowheads indicate the style canal, filled with secretion; yellow arrowheads indicate the pollen tubes in secretion. Bars = 25 μm. Bo, basal ventral outlet; Ca, carpel; Oc, ovarian cavity; Ov, Ovule; R, receptacle; St, stigma; V, ventral suture.

Compared with the four types of closure described in previous studies, carpel development in *Sagittaria* is most closely related to type II. Angiospermy type II can be divided into two groups: type II<sub>a</sub> of carpel closure only contains one opening at the stigma; and type II<sub>b</sub> of carpel closure consists of two openings respectively at the stigma and carpel base. Thus, the carpel sealing in *Sagittaria* belongs to type II<sub>b</sub> (Fig. 4). The genetic basis for this pattern of incomplete postgenital fusion at the periphery of the carpel margins is unknown, but our developmental study suggests that it could be related to the extreme growth of the dorsal side of in the carpel of *S. trifolia*. For instance, carpel bulging causes the length of the ovary to be greatly reduced on the ventral side, resulting in the outlet of the ovarian cavity on the ventral side greatly reducing in size (Igersheim et al., 2001; Endress, 2011). The development of the ovule potentially interferes in the closure and fusion of the carpel margins in the conduplicate carpel, with the bulging favoring the fusion of the ventral slit in the middle region, but not the fusion at the periphery of the basal ventral outlet.

**Evolutionary implication of carpel type of *Sagittaria* and other angiosperms**—The apocarpous gynoecium of *S. trifolia* comprises numerous free carpels that are densely packed on the

center of the floral apex (Fig. 3C). Its fruit set could thus strongly be affected by an uneven pollination on the stigmas of the flower like in other species with apocarpous gynoecia and multiple individual stigmas (Carr and Carr, 1961; Wang et al., 2002, 2006; Huang, 2003). However, in *S. trifolia*, we clearly show that pollen tubes can grow between the bases of the carpels on the surface of the floral apex, which functions like an extragynoecial compitum, and could enter each locule via the basal ventral outlet. This extragynoecial pollen-tube growth has been shown to raise the fruit set of *S. trifolia* (X.-W. Wang et al, unpublished data). In addition, when the carpel becomes the achene after fertilization, the area corresponding to the basal ventral outlet of the carpel becomes the most fragile part of achene, which is able to allow the seedling leave from pericarp.

In other Alismataceae, the closure of the carpel on its ventral side and the bulging of the dorsal side are functionally linked with a decrease in ovule number per carpel and an increase in the numbers of carpels per flower (Kaul, 1976; Charlton, 1991; Igersheim et al., 2001; Lehtonen, 2009). Also, as in *Sagittaria*, the most specialized flowers of the family have similar apocarpous gynoecia with a large number of uniovulate carpels and tend to be monoecious or occasionally dioecious (Kaul, 1967, 1976; Wang and Chen, 1997; Igersheim et al., 2001). While Armbruster

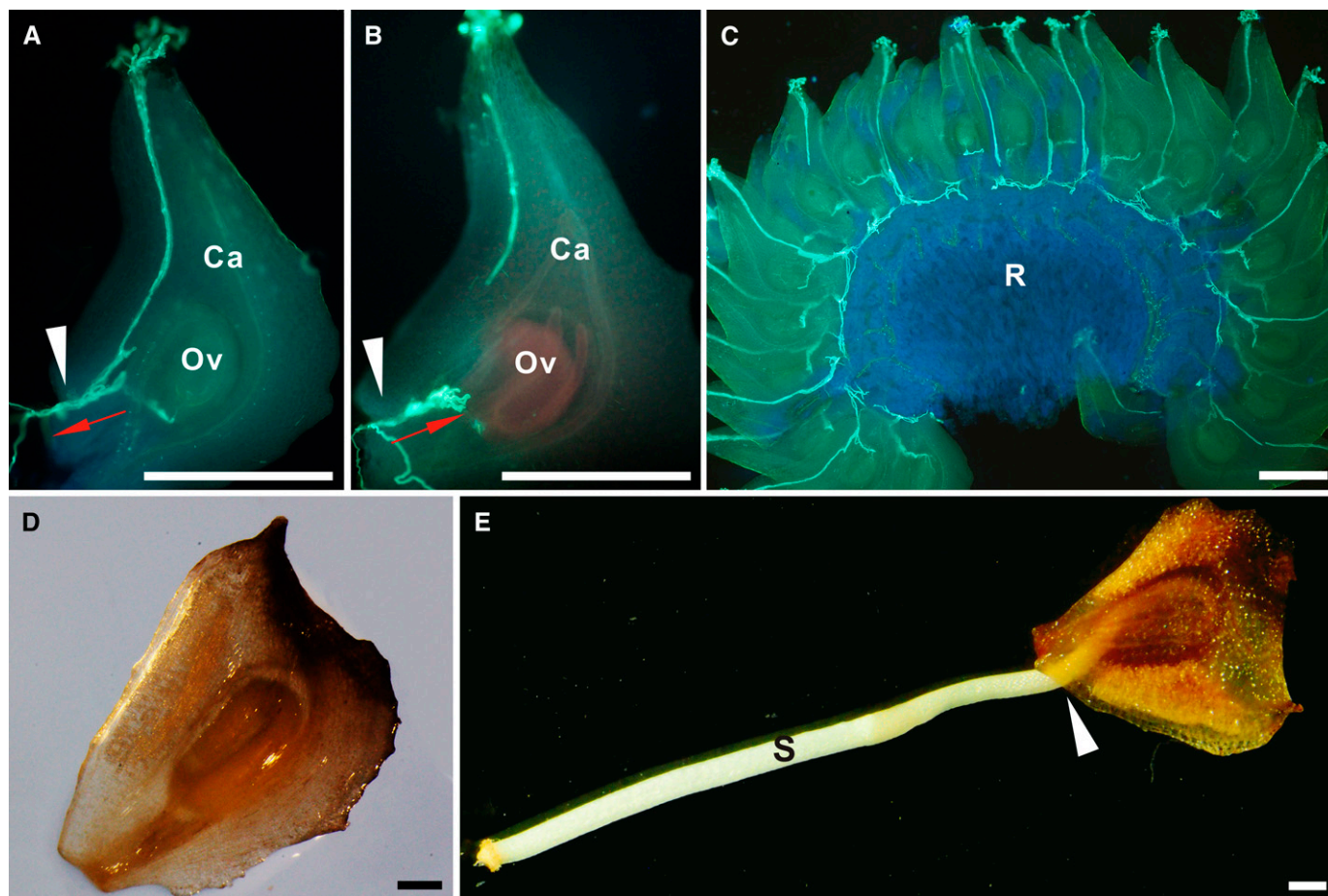


Fig. 3. Fluorescence (A–C) and stereoscope (D, E) micrographs showing (A–C) pollen tube growth and (D, E) seed germination of *Sagittaria trifolia*. White arrowheads point to the basal ventral outlet. Red arrows indicate the directions of pollen tube growth through the basal ventral outlet. (A) Pollen tubes leave the carpel by the basal ventral outlet. (B) Pollen tubes from the outside enter into the ovary of a carpel through the basal ventral outlet. (C) Pollen tubes fluorescing on stigmas, inside carpels, and in between the bases of the carpels of the apocarpous gynoecium. (D) Morphology of the achene and the seed within the carpel before germination. (E) The radicle emerges from the achene through the ventral base of the pericarp during germination. Bars = 500  $\mu$ m. Ca, carpel; Ov, ovule; R, receptacle; S, seedling.

et al. (2002) suggested that an increase in free carpel number per flower would influence offspring quantity and quality of apocarpous species, the presence of an extragynoecial compitum like in *Sagittaria* and other species of Alismataceae and other flowering plants with an apocarpous gynoecium would thus eliminate this effect and could help plants produce high fruit set even under uneven pollination conditions (Carr and Carr, 1961; Endress, 1982; Williams et al., 1993; Wang et al., 2006, 2012; Lyew et al., 2007). In addition, the extragynoecial pollen tubes can be protected from herbivores and pathogens by the dense packing of the individual ovaries in the gynoecium (Endress, 1982). The combination of densely packed carpels with a basal ventral outlet and an external compitum in *Sagittaria* might thus not be accidental. However, further detailed comparative studies of carpel development and the functions of the basal ventral outlet in apocarpous gynoecia in other Alismataceae are required to understand their evolutionary significance and the innovation of the incomplete carpel closure in *S. trifolia*.

Type II<sub>b</sub> carpel closure is present not only in Alismataceae. For instance, some authors have found an incompletely closed carpel in *Illicium*, a genus that belongs to Austrobaileyales, and has a similar structure to that found in *Sagittaria* (Williams et al., 1993;

Endress, 2001). The carpel of *Illicium* contains a postgenitally fused periphery and a secretory canal, along with several free carpels that are grouped around a central hump with some secretion (Keng, 1965; Endress, 2001). At its base, the carpel of *Illicium floridanum* also has a basal ventral outlet, which is covered by the central hump during anthesis. Pollen tubes have been observed between the free carpels in the groove between the carpel base and the apical residuum in the gynoecium of *I. floridanum* (Williams et al., 1993). The extragynoecial pollen tubes could be protected by both the carpel and the central hump. We might find more examples of type II<sub>b</sub> carpel closure in other apocarpous angiosperms with conduplicate carpels in future studies. However, there should be an extragynoecial compitum in the gynoecium containing carpels of angiospermy type II<sub>b</sub>, which requires a sufficient constant water supply (Endress, 1982, 1994). Therefore, plants with type II<sub>b</sub> carpel closure might only be found in species occupying aquatic or moist habitats.

**Conclusions and perspectives**—Although the carpel of angiosperms must be sealed by secretion or postgenital fusion, the carpels in some species (e.g., *S. trifolia* and *I. floridanum*) have morphological innovations as a result of variations in their



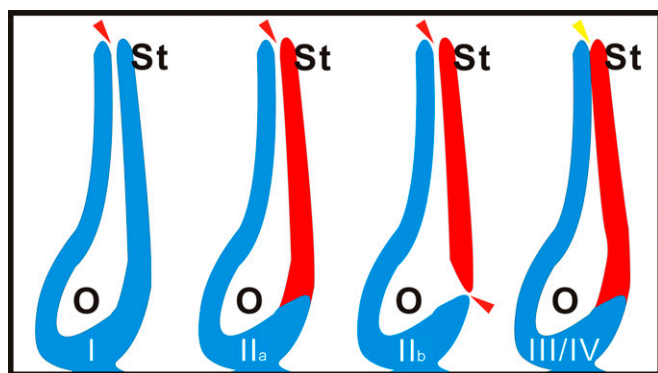


Fig. 4. Diagrams of carpel closure in *Sagittaria trifolia* (type II<sub>b</sub>) compared with the four types of carpel closure described by Endress and Igersheim (2000). Each pattern is illustrated with an ideal median longitudinal section of the mature carpel. Red areas: ventral slit closed by postgenital fusion. Red arrowheads point to the passages from the outside to the inside of the carpel; the yellow arrowhead points to the solid stylar canal. Type I: The carpel with a single opening is entirely ascidate and closed by secretion. Type II<sub>a</sub>: The carpel with a single opening is a combination of postgenital fusion at the periphery and with occlusion by secretion but no fusion in the center. Type II<sub>b</sub>: The carpel with two openings is sealed by partial postgenital fusion at the periphery and secretions. Type III/IV: The carpel, which has no opening, is closed by complete postgenital fusion. O, ovary; St, stigma.

developmental processes. Such morphological innovation may lead to changes in function. In the future, we will pay more attention to understanding the development of the carpel and the relationship between the carpel structure and reproductive process of plants.

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