Pak. J. Bot., 43(4): 1853-1859, 2011.

THE MORPHOLOGY AND ANATOMY OF THE HAUSTORIA OF THE HOLOPARASITIC ANGIOSPERM *CUSCUTA CAMPESTRIS*

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Abstract

The morphology and anatomy of the haustoria of the holoparasitic angiosperm *Cuscuta campestris* parasitizing itself and different tissues of *Mikania micrantha* were studied under scanning electron microscope, confocal laser scanning electron microscope and light microscope. *C. campestris* has a low stomatal density on the stem and there is a nonfunctional conical protuberance with a unique elliptic pore at the apex, which has not been reported before and we call it pseudo-haustorium. The pseudo-haustorium originates from the cortical parenchyma just external to the pericycle. Its initial cells divide anticlinally and periclinally, and then develop into an endophyte primordium, which consists of file cells and meristematic cells. When *C. campestris* infects host stem, petiole, leaf lamina and itself, it prefers host stem and has the least choice for leaf lamina. The development of the haustoria invading different tissues reveals that the haustorium in the leaf lamina region without veins is initially flat and its search hyphae does not differentiate into xylem and phloem hyphae, which differs from the haustoria with the annular vessel and phloem hyphae in host stem, petiole and its own stem. These indicate that the haustoria might differentiate vascular tissues only when their search hyphae come in with the contact the vascular tissues of the host or itself.

Introduction

Cuscuta campestris Yuncker (Convolvulaceae) is one of the most widespread parasitic weeds (Parker & Riches, 1993; Dawson *et al*., 1994). It is a stem holoparasitic plant without roots and leaves, but it can grow absorptive organ haustoria that provide physical and physiological bridge between itself and its host (Kujit, 1969; Kushan *et al*., 2006). It can infect diverse host species (Dawson *et al*., 1994), self-parasitize and hyper-parasitize (Liao *et al*., 2005), and it causes vast damage in agriculture (Kujit, 1969; Malik & Singh, 1979). It has also been reported that the parasite infects an invasive plant *Mikania micrantha* and restrains its growth and photosynthesis (Shen *et al*., 2005, 2007).

Kuijt (1977) divided the functional and mature haustorium of phanerogamic parasites into two parts, the upper haustorium and the lower endophyte. The upper haustorium lies external to the host, whereas the endophyte penetrates into host tissues. Some studies have elucidated the basic processes of haustorium development of *Cuscuta* invading host stems. Most of these studies concentrated on the endophyte, such as the studies on *C. epilinum* (Koch, 1874), *C. americana* (Peirce, 1893), *C. campestris* (Macleod, 1961), *C. pentagona* (Tripodi & Pizzolongo, 1967), *C. odorata* (Dörr, 1969), *C. reflexa* (Forstreuter & Weber, 1984), and *C. australis* (Lee & Lee, 1989). Especially, the unique "finger-like" hyphae of *Cuscuta* spp. were well studied (Dörr, 1968*a*, 1968*b*, 1969; Lee & Lee, 1989; Vaughn, 2003). Additionally, Heide-Jorgensen (1987) revealed the change of epidermal cells and the cement materials in *C. gronovii* and *C. reflexa* at the early stages. Lee (2007) described the cytological features of the upper haustorium of *C. japonica*. Lee and Lee (1989) reported some haustoria of *C. australis* were free from the host surface. We call these nonfunctional haustoria pseudo-haustoria since they are unable to draw resources from hosts. But there have been no further studies on such haustoria. The anatomical characteristics of the infection of *Cuscuta* on the host leaf lamina, petiole and its own stem have not been well studied. Only Vaughn (2002, 2003) reported limited structural and immunocytochemical characteristics of *C. pentagona* attaching and invading its host leaf lamina and petioles. It is known that parasites draw resources effectively from hosts by osmolarity or high transpiration rate (Press *et al*., 1990), but the morphology of the parasite stem and the stomata distribution is not clear for holoparasites, and it is uncertain whether the stems of the leafless holoparasite *C. campestris* has high transpiration.

In this study, the morphology of *C. campestris* and the characteristics of its haustoria were investigated by scanning electron microscope, confocal laser scanning electron microscope and light microscope. In the present study the origin of the upper haustoria in the holoparasitic association *Cuscuta-Mikania*, the structure of nonfunctional haustoria and the different characteristics of the haustoria attaching to the host stem, petiole, leaf lamina and itself.

Materials and Methods

Plant material: Seeds of *C. campestris* and *M. micrantha* were collected from a *M. micrantha* population infected by *C. campestris* and an uninfected *M. micrantha* population, respectively, in Guangzhou (23°8'N, 113°17'E, 8 m a.s.l.), Guangdong Province, China in January 2007 and stored at room temperature. On 20 March, the *M. micrantha* seeds were sowed in pots (about 400 ml in volume) at a depth of about 1 cm in a growth chamber with a photoperiod of 12 h and a temperature of 25°C. Seedlings were supplied with full-strength Hoagland nutrient solution daily and 55 days later they were removed into a glasshouse with an average temperature of 30.7°C and a relative humidity of 90.3%. On 6 June, when *M. micrantha* plants were about 120 cm tall, 20 *C. campestris* seeds were sown around each host plant.

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On the 20th day (10 July) after parasitization when the parasite grew vigorously and its branches infected the stems, petioles and leaf laminae of *M. micrantha* and the parasite itself, these parasitic associations were harvested. Meanwhile, several individual *C. campestris* stems without hosts were collected after every two hours from 8:00 to 18:00 on the harvest day.

Scanning electron microscopy: Large tissue pieces of the functional haustoria with endophytes in the stems of *M. micrantha*, *C. campestris* stems, and the *C. campestris* stems with the nonfunctional haustoria, were fixed in 2.5% (v/v) glutaraldehyde and paraformaldehyde in 0.1 mol L^{-1} phosphate buffer at pH 7.4 over 12 h. Samples were subsequently washed three times in the same buffer and then three times in distilled water. Afterwards, they were dehydrated in a series of graduated ethanol (30%, 50%, 70%, 80%, 90%, and 100%) and tert-butyl alcohol (TBA). At the last step of dehydration, samples in TBA were frozen at -20°C and then freeze-dried in a JFD-310 free drying device (JEOL Ltd., Tokyo, Japan). After mounted on aluminum stubs with silver paint, the samples were coated with gold-palladium in a JFC-1600 Auto Fine Coater (JEOL Ltd., Tokyo, Japan). All samples were observed under a JSM-6360LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Confocal laser scanning microscoy: Thin and tiny tissue pieces of the parasite with nonfunctional upper haustoria were fixed in 4% (v/v) glutaraldehyde and paraformaldehyde in 0.1 mol L^{-1} phosphate buffer at pH 7.4 over 12 h. The samples were washed in distilled water six times for 20 minutes each and subsequently dehydrated with graduated ethanol (50%, 70%, and 90%). Then, they were dyed with 0.03 ml 5% Eosin B, 0.01 ml 5% Orange and 0.96 ml 95% ethanol and kept overnight. The dyed samples were thoroughly washed by distilled water and dehydrated with a series of ethanol (50%, 70%, 90%, and 100%). Finally, they were immersed in Methyl salicylate for transparent treatment. All tissues were observed and photographed under LSM 510 META confocal laser scanning microscope (ZEISS, Germany).

Light microscopy: The stems, petioles and leaf tissues of *M. micrantha* infected by *C. campestris* and the self-parasitized *C. campestris* tissues were fixed in FAA (formalin: glacial acetic acid: 70% ethyl alcohol = 5:5:90 v/v) over 48 hours, then dehydrated in the following series (85%, 95%, 100% ethanol, 1/2 ethanol and 1/2 xylene, xylene), and then embedded in paraffin. Both transverse and longitudinal serial sections were cut on a microtome at 8 μm and mounted on slides. Some sections were stained with haematoxylin and others with safranine-fast green. All sections were observed under an OLYMPUS BHS-2 microscope and photographed with OLYMPUS PM-10AD photomicrographic system.

Results

Morphological characteristics of *C. campestris*: The surface of the parasite stem is smooth and its epidermal cells are slender and arranged tightly, and the density of stomata on the stem is about 2.6/mm^2 (Fig. 1*A*). The stomatal apparatus consists of a stoma and eight subsidiary cells (Fig. 1*B*). The guard cells are not obvious and their edges around the pore are rough (Fig. 1*C*). The stomata were open all day from 8:00 to 18:00.

On the surface of the dodder stem that is not attached to a host tissue, some conical protuberances were observed and we named them pseudo-haustoria (Fig. 1*D*). They are exposed in the air. When they stop expanding, the pseudo-haustorium is about 97.70 μm in height, 131.88 μm in width in transverse axis and the slope is about 121.97 μm in length. There is a unique pore at the apex of the pseudo-haustorium (Fig. 1*E*). The pore is about 7.12μm in length and 3.21 μm in width, with an irregular brim. The epidermal cells around the pore are elongated.

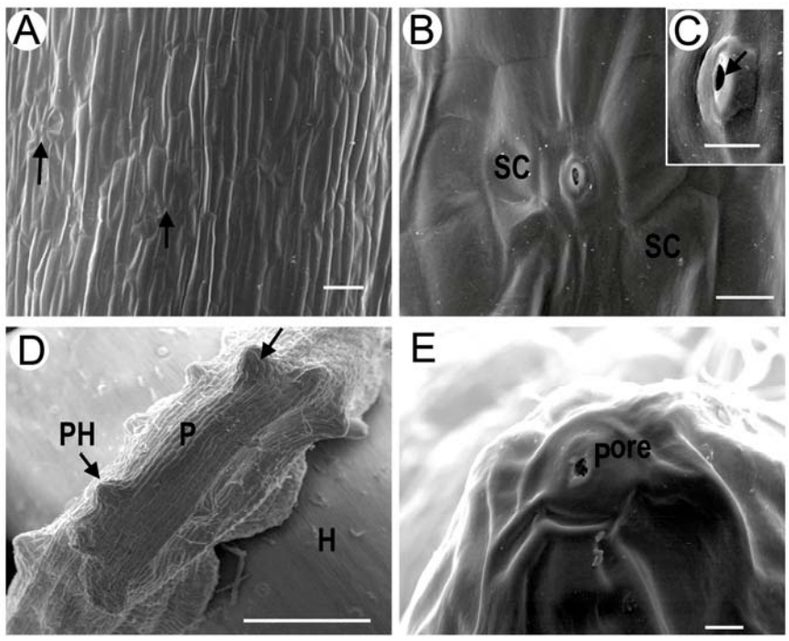

Fig. 1. Scanning electron micrographs of the *C. campestris* stem. *A*, Epidermal cells of *C. campestris* stem with few stomata marked with arrows. Bar=100μm. *B*, Stoma and subsidiary cells (SC) of the parasite stomatal apparatus. Bar=20μm. *C*, Rough edges around the stoma marked with an arrow. Bar=10μm. *D*, Nonfunctional haustoria (pseudo-haustoria (PH)) on the back of the parasite *C. campestris* (P) infecting the host stem (H). Bar=500μm. *E*, The unique pore at the apex of pseudo-haustorium. Bar=10μm.

Anatomical characteristics of the pseudo-haustorium: The structure of the parasite *C. campestris* stem consists of a one-layer epidermis, six- or seven-layer cortex and a central stele. In transverse section, vascular tissue is distinct. There are phloem cells outside, and xylem elements with vessels inside the vascular bundle (Fig. 2*A*).

When a pseudo-haustorium is initiated, several epidermal cells and cortical parenchyma just external to the pericycle of the parasite differentiate. The epidermal cells increase by anticlinal division (Fig. 2*B*). The cortical cells divide anticlinally and periclinally and develop into a group of meristematic cells. These differentiating cells have conspicuous nuclei, densely stained cytoplasm and abundant starch grains, and they are often gathered in the protuberance-initiated polar (Fig. 2*C*). The meristematic cells divide and develop into pseudo-haustorium. At the early development stage of the pseudo-haustorium, there is a clear break of the cortical cells external to the pericycle at its base (Fig. 2*D*). As it stops growing, the round epidermal cells become rectangle. The pseudo-haustorium at this stage is full of parenchymatous cells indicated as an endophyte primordium (EP) (Fig. 2*E*). In the inner region proximal to the stele of EP the cells are elongate and arranged regularly in row from the stele toward the tip and they are named file cells (FC). In the central region the cells contain dense cytoplasm and are active meristematic cells (MC). These two types of cells compose the mature pseudo-haustorium.

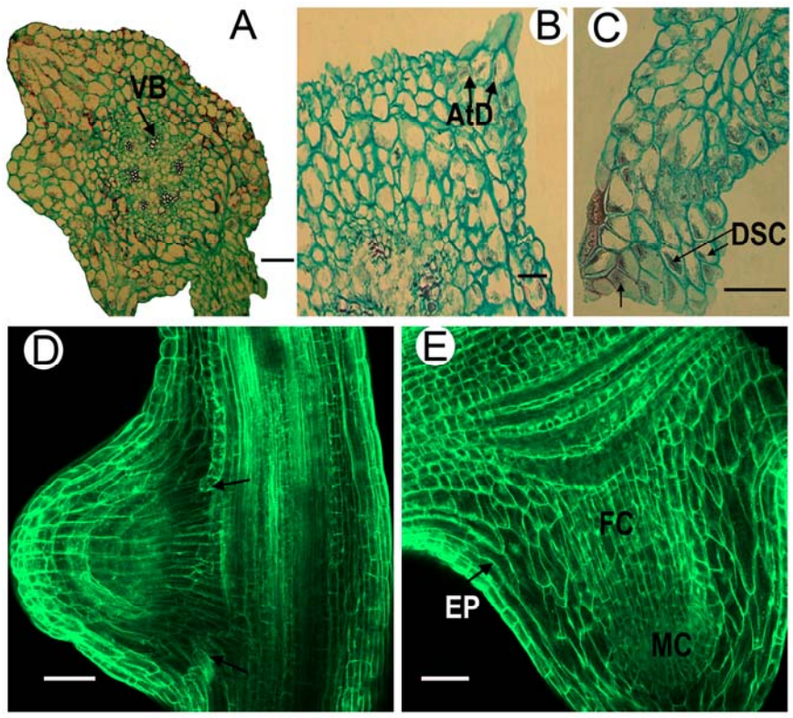

Fig. 2. Light (Fig. 2*A*-2*C*) and confocal laser scanning micrographs (Fig. 2*D*, 2*E*) of *C. campestris* stem and pseudo-haustorium. *A*, Vascular tissues of *C. campestris* in its transverse section with vascular bundle (VB). Bar=100μm. *B*, Anticlinally dividing epidermal cells (AtD). Bar=50μm. *C*, Cell inclusion has a polarity distribution towards the attached aims. DSC, densely strained cytoplasm. Bar=50μm. *D*, A break (between two arrows) of the cortical cells external to the pericycle at the base of the newly initiated pseudo-haustorium. Bar=100μm. *E*, The pseudo-haustorium at this stage is referred to as an endophyte primordium (EP) consisting of two kinds of cells: elongated file cells (FC) toward the tip in the region proximal to the stele of EP and meristematic cells (MC) in the central region. Bar=100μm.

Morphological characteristics of the haustoria on the host stem: At the initial attachment, the haustoria are in loose contact with the host stem and are easily detachable. The surface of initial haustoria touching the host stem is flat and their tip cells are congested (Fig. 3*A*). The cells in the center are gathered into a group, while the ones around the center are separated one by one and some have concave surfaces. The epidermal cells of the functional haustoria are elongated (Fig. 3*B*). As the contact between the host and the parasite becoming tighter and tighter, the surface of the stem of the parasite is modified and forms a tight seal with the host epidermis by invagination and protrusion of both their cell walls (Fig. 3*C*). After the haustoria invade the host stem successfully, the tip cells are connected to the vascular tissue of the host stem and the peripheral cells compress the host cortical cells and are combined with them (Fig. 3*D*).

Anatomical characteristics of the haustoria invading different tissues: The young dodder at its initial parasitization stage prefers infecting the host stem late it grous vigorously and produces many branches that infect the host leaf lamina, petiole and itself when the host stem is not available. According to our observations, the probability of host tissues infected is as follows: host stem > host petiole > parasite itself > host leaf lamina.

The development of endophyte primordium in functional haustoria is observed to be similar to that of the pseudo-haustoria. After the successful attachment, a functional haustorium like a peg develops and the endophyte is composed by axial cells and tip cells. When the parasite attaches to the host stem (Fig. 4*A*) and petiole tissues (Fig. 4*B*), the tip cells penetrate into the host tissues and develop into search hyphae. As the search hyphae contact the host xylem, they deposit secondary walls and become xylem hyphae with one kind of distinct annular vessel (Fig. 4*C*). At the tip of the haustoria, some search hyphae are highly vacuolated, reaching the host phloem and growing into the phloem hyphae (Fig. 4*C*). When the parasite attaches its own stem (self-parasitization) (Fig. 4*D*), the haustoria grows from one dodder stele to another and connects the two dodder stems. The vascular tissues between them are connected by the vessels of haustorium and phloem hyphae.

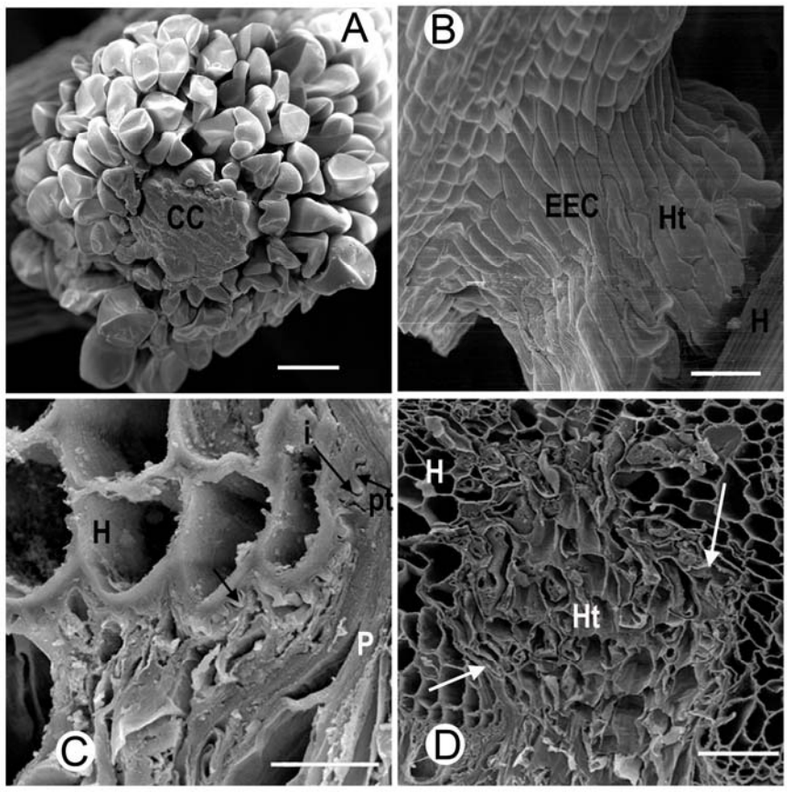

Fig. 3. Scanning electron micrographs of haustoria invading the host stem. *A*, Flat and congested central cells (CC) and concaved surrounding cell of a detached haustorium. Bar=100μm. *B*, A functional haustorium (Ht) attached onto a host stem (H) with elongated epidermal cells (EEC). Bar=100μm. *C*, A tight seal between the walls of the host (H) and the parasite (P) formed by invagination (i) and protrusion (pt). Bar=10μm. *D*, Haustorium (Ht) compressing the host cells and connecting with host plant (H). Bar=50μm.

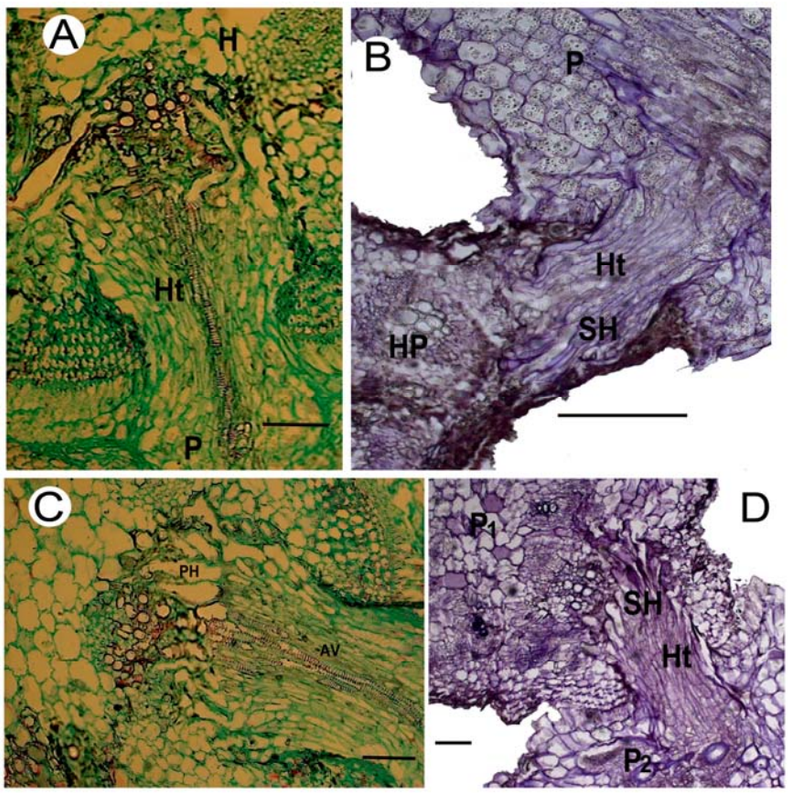

Fig. 4. Light micrographs in two different dyeing methods (haematoxylin and safranine-fast green) of the haustoria invading different host tissues and itself. *A*, A haustorium (Ht) of *C. campestris* (P) penetrates into a host stem (H). Bar=100μm. *B*, *C. campestris* (P) invades the host petiole (HP) by the haustorium (Ht); SH, search hyphae. Bar=200μm. *C*, Phloem hyphae (PH) and xylem hyphae with distinct annular vessels (AV). Bar=50μm. *D*, The parasite (P2) invades its own stem (P1) and self-parasitization happens by the haustorium (Ht). Bar=100μm.

When the parasite attaches to the lower epidermis of the host leaf lamina, a similar upper haustorium develops. The surface of the haustorium attaching to the leaf lamina becomes flat (Fig. 5*A*). Its meristematic region is not clear and the file cells divide periclinally (Fig. 5*B*). The elongate tip cells of the endophyte penetrate into the host leaf tissues from the lower epidermis and keep extending till to the upper epidermis without reaching the veins or the vascular tissues (Fig. 5*C*). The tip cells grow into search hyphae but do not differentiate into the xylem vessel and phloem hyphae.

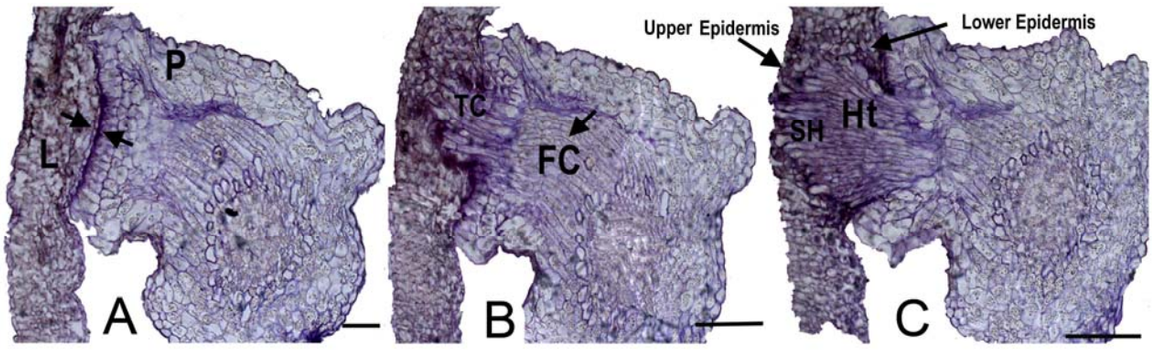

Fig. 5. Light micrographs of the haustoria developing in the host leaf lamina. *A*, Flat interface (marked with two arrows) of the invading haustorium and the host leaf lamina at the early stage of attachment. Bar=100μm. *B*, Periclinally file cells (FC) and the tip cell (TC) dividing in a developing haustorium. Bar=200μm. *C*, Endophyte extends from the lower epidermis to the upper epidermis and tip cells develop into the search hyphae (SH). Bar=200μm.

Discussion

In parasitic angiosperms transfer of water and solutions from hosts to parasites may be facilitated by high osmolarity and low water potential (Kujit, 1969; Fisher, 1983; Press *et al*., 1990). In hemiparasitic plants, high transpiration rate and durative opening stomata can sustain the flux of resources in parasitic association (Ullmann *et al*., 1985; Tuohy *et al*., 1986; Press *et al*., 1988, 1990). In this study, we observed that the stomatal density of *C. campestris* stem is extremely low, 2~3/mm^2, compared with about 400/mm^2 in the lower epidermis of olive leaf (Guerfel *et al*., 2009). The low stomatal density is associated with a low rate of transpiration (Xu & Zhou, 2008; Fraser *et al*., 2009). Thus, it is more likely that the holoparasitic *C. campestris* depends on the osmolarity and water potential to extract resources from its host rather than high transpiration. Raven (1983) reported that predominantly phloem feeding holoparasites such as *Cuscuta* might also derive organic compounds via xylem connections with the host and this flux may be limited by the relatively low rates of transpiration.

Dodder has the ability of choosing its host plant and can infect the host stem, petiole, leaf and itself (Kelly, 1992; Liao *et al*., 2005). Except for the resource conditions (Kelly, 1992) and the attachment orientations (Lee & Lee, 1989), the parasite might also choose its attaching tissues by recognizing different types of surface shapes. In the present study it is observed that *C. campestris* seems to prefer the linear, round interface, such as the stems and petioles of *M. micrantha* and its own stems, to flat surface, such as the host leaf lamina. Perhaps, the stems and the petiole are upstanding and can supply more effective support than the leaf lamina. Additionally, the shapes of the linear interface might be easier to be coiled for dodder than the plane of the flat leaf lamina.

Contact stimuli and natural light (including far-red light) can produce chemical signals and induce the development of haustorium in *C. japonica* (Tada *et al*., 1996). The endophyte primordium and the functional upper haustorium would not develop in *C. australis* and *C. japonica* when the parasites were not in contact with hosts (Lee & Lee, 1989; Lee, 2007). In this study it was observed that *C. campestris* produced two kinds of haustoria, pseudo-haustorium and the functional haustorium. It seems that when a stem of *C. campestris* attaches to tissues of *M. micrantha*, the parasite receives some kind of signal and initiates endophyte primordia. As the primordia grow bigger and bigger over time, if they do not attach to host tissues, they stop development and become pseudo-haustoria; if they do, they develop into functional haustoria. On the top of the pseudohaustorium is a unique elliptic pore with irregular brim, which has not been observed before. The irregular brim might suggest that the pore is formed by cell-breaking or degrading, but, the causes of formation and the function of the pore need further studies.

The functional haustoria have different anatomical structures depending on the host tissues they attach to. When *C. campestris* attaches to the host stem, a tight connection between them is established by invagination and protrusion to each other. The structure of invagination and protrusion is due to the malleability of the epidermal cell walls of the parasite by cell-wall-loosening complexes, which has been revealed as an important step of the successful attachment in *C. pentagona* (Vaughn, 2002). Lee and Lee (1989) observed and discussed that search hyphae of the functional haustoria of *C. australis* in the host stem would finally become xylem and phloem hyphae, which is consistent with our observation on the functional haustoria of *C. campestris* in the host stem, petiole and its own stem. However, the micrographic observation on the haustorial development in the host leaf lamina has not been well reported. Vaughn (2002, 2003) observed the conformations of the epidermal cell wall and the extracellular and intracellular growth of the haustorial hyphae in *C. pentagona* attaching and invading leaf lamina and the petiole. In this study we observed when *C. campestris* attaches to host leaf tissue without veins, the search hyphae keep growing through the mesophyll, but do not differentiate into the xylem or phloem hyphae. It seems that the search hyphae do not differentiate into the vascular hyphae unless they contact the host vascular tissues. There might be some kind of signals in the vascular elements that trigger the differentiation of the parasite hyphae, which still needs to be identified in future. In this study, we did not have samples of the parasite invading the vein of the leaf lamina, may be because such invasion is rare and our samples were limited. Thus, further relevant studies on the parasitization in the region of leaf lamina with veins might reveal if the parasite invades the veins; and if it does, whether the search hyphae can differentiate into xylem and phloem hyphae.

The differentiation and the section break of the cortical cells near the pericycle in the endophyte primordial (Fig. 2*B*, 2*D*) indicate that the haustoria of *C.*

campestris may originate from the cortical parenchyma just external to the pericycle, which is consistent with the origination of haustorium in *C. americana* (Peirce, 1893). However, the haustoria in *C. australis* (Lee & Lee, 1989) and *C. epilinum* (Koch, 1874) originate from the middle layers of cortical cells. Therefore, the origins of haustoria might differ from species to species. The stem of *C. campestris* had a clear vascular bundle in the transverse section, contraty to the observations of Lee & Lee, 1989 who did nto find vascular bundle in *C. Australis.*

The developmental paths of endophyte primordium in *C. campestris* are summarized in Fig. 6. The initials of all haustoria originate from the cortical parenchyma external to the pericycle. These initial cells differentiate and develop into the endophyte primordium composed by file and meristematic cells. When the host is unavailable, endophyte primordium stops growing and is thus named as pseudo-haustorium. The file and meristematic cells consist of the nonfunctional pseudo-haustorium. Otherwise, when the parasite invades the host stem, petiole and self-parasitizes, the file and meristematic cells develop into the axial and tip cells, respectively, and tip cells grow into the search hyphae, then differentiate into the xylem and phloem hyphae. The axial cells, xylem and phloem hyphae consist of the functional haustoria, which is similar to those in *C. australis* (Lee & Lee, 1989). When the parasite attaches to the host leaf lamina, the meristematic cells are not distinct, but they grow into the tip cells that then develop into the search hyphae. However, the search hyphae do not come in contact of the vascular tissues of the vein and do not differentiate into the xylem and phloem hyphae. The axial cells and search hyphae consist of the functional haustoria in the host leaf lamina.

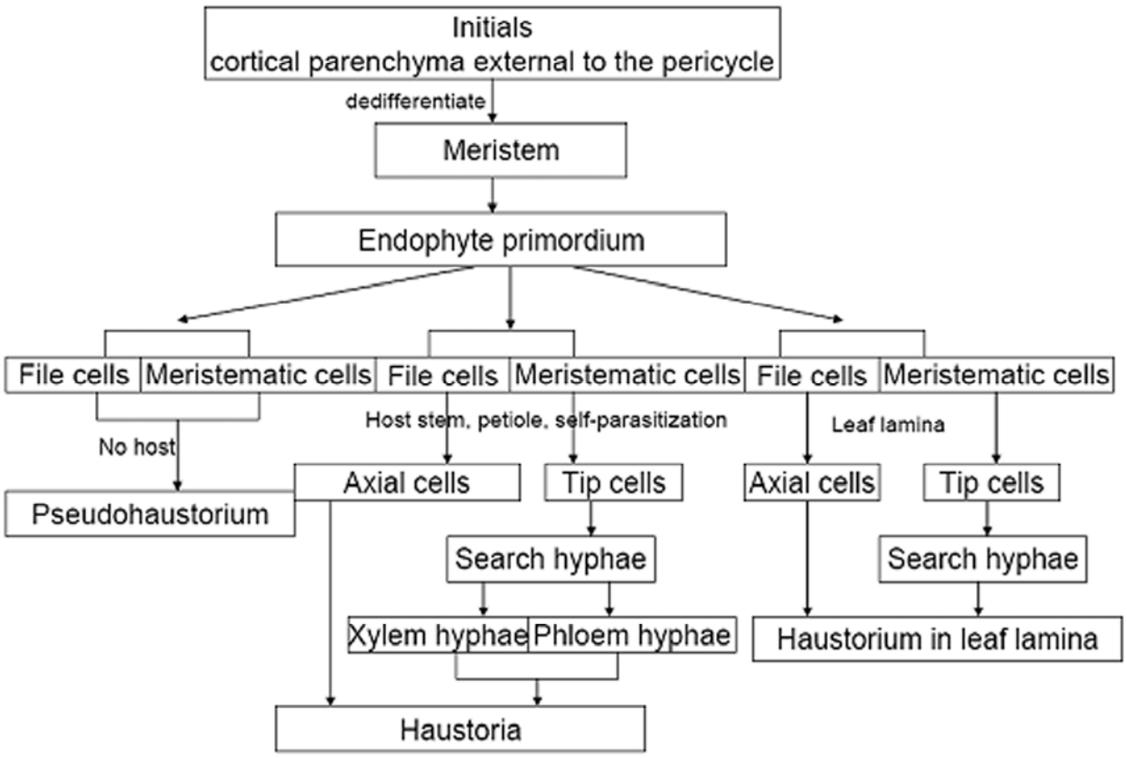

Fig. 6. Schematic summary of the nonfunctional and functional haustorial development of *Cuscuta campestris*.

Conclusions

The leafless and rootless holoparasite *C. campestris* has only a very few stomata in the shoot surface, thus the parasite is unlikely to capture resources from its host by transpiration. The nonfunctional haustorium (pseudo-haustorium) had the similar endophte primordium to the functional haustorium. The endophte primordium originates from the cortical parenchyma external to the pericycle. The pseudo-haustorium is full of parenchyma cells and at the apex there was a cryptic elliptic pore. The parasite infects host stem, petiole, leaf lamina and itself. Somehow, it could recognize the different interfaces to choose its host tissues (host stem > host petiole > itself > host leaf lamina). The development and structure of the haustoria of the parasite in *M. micrantha* stem, petiole and the parasite itself are all similar, but in host leaf lamina the haustorium neither invade the veins and nor develop into the xylem and phloem hyphae.

Acknowledgments

This work was supported by the Technology Innovation Program of Functional Development of Instrument and Equipment of the Chinese Academy of Sciences (Grant No.: yg2010079), the Knowledge Innovation Program of the Chinese Academy of Sciences, the National Natural Science Foundation of China (30470345), and the Natural Science Foundation of Guangdong Province, China (021536).

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(Received for publication 26 July 2009)