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SCANNING ELECTRON MICROSCOPIC STUDY OF THE INFECTION PROCESS OF *PHYTOPHTHORA CAPSICI*

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Abstract

The infection process of *Phytophthora capsici* on susceptible and resistant pepper cultivars were studied by scanning electronic microscopy. The development of *P. capsici* within 24 h post inoculation (hpi) was quite similar between susceptible cv. early Calwonder (EC) and resistant cv. Criollo de Morelos 334 (CM334). Zoospores encysted on the leaves and formed adhesive cysts at 3 hpi. Cyst germinated by producing two germ tubes at 12 hpi. The hyphae penetrated both abaxial and adaxial epidermis of pepper leaves without forming appressoria-like structures or via stomata even when they passed over it at 24 hpi. Up to the first three steps of infection, no apparent difference was observed between susceptible and resistant cultivars. However, differences were detected at the third day post inoculation (dpi) as mycelia grew out from the abaxial surface of the leaf of the susceptible cv. EC, but not from CM334. Small water soaked lesions appeared at 4 dpi, and sporulation occurred at 5 dpi on the susceptible cv. EC, while on the resistant cv. CM334, no disease symptom developed and no hyphae was observed on the leaf surface even at 12 dpi. The disease symptom and the sporulation appeared first on the abaxial leaf surface of cv. EC, followed by on adaxial leaf surface 24 h later. The new mycelia from leaves were observed both leaf surfaces through the ruptured epidermis, leaf veins and the stomata. The emergence of new mycelia through stomata appeared to be random rather than a preferred route. At 10 dpi the mycelium spread all over the surface of susceptible pepper leaf and the hyphal colony could be observed at 12dpi.

Introduction

The oomycete plant pathogen *Phytophthora capsici* can infect leaves, fruits, stems and roots of pepper () which cause devastating disease (Saleem *et al.*, 1998). It caused billions of dollars annual loss in damage to crops in America (Oelke *et al*., 2003). *Phytophthora capsici* can attack many species of pepper, tomato, and other members of the solanaceae family as well as watermelon and other members of the plant family Cucurbitaceae (Bonnie *et al*., 2008; Fatima *et al*., 2009). The disease occurs optimally under cool and moist weather conditions at about 25-30°C and the RH (relative humidity) over 85%. The disease can happen through the whole growing season of the crop. The symptoms caused by *P. capsici* vary on different hosts (Lamour, *et al.*, 2012; Quesada-Ocampo & Hausbeck, 2010; Khan & Cheng, 2010). Leaf symptoms appear firstly around lesions with dark green in the middle and pale green around and finally formed water soaked lesions that expand rapidly resulting in total destruction of the plant in a few days (Yin *et al.*, 2012; Lamour *et al.*, 2012). Lesions also appear on the stems as black, greasy areas and stem lesions may girdle the stem then crimple and kill the foliage above the lesions (Yin *et al.*, 2012). The symptom begins on pedicle with dark green water soaked lesions, then the lesions turns gray and soft rot, when there is enough humidity layers of white mold could be observed (Lamour *et al.*, 2012). The disease spread greatly when the soil is flooded and the zoospores are the vital route of infection of roots (Tyler, 2002). Zoospores could also spread to the leaves and stems of the plant by splashing.

There are three common ways to control the *P. capsici*, including cultural practices, fungicide applications and using genetically resistant varieties. However none of these methods is completely satisfactory for controlling the disease. As chemical control can bring problems to the environment, selection of cultivars with higher levels of resistance is the major interest for plant breeders (Cora Boedo *et al*., 2008). As a result, the study of plant-pathogen interaction mechanism is crucial and plenty of researches investigated about the infection process of fungal pathogens. The different steps of *Alternaria dauci* infection and development on carrot leaves were studied (Cora Boedo *et al*., 2008; Dugdale *et al*., 2000). The infection process of *Fusarium graminearum* in wheat spikes and the response of spike tissues to the resistance were investigated (Kang *et al*., 2008; Ribichich *et al*., 2000). The developments of *Cercospora henningsii* on Cassava Leaves were studied by scanning electron microscopy (Ahmed *et al*., 2009). However, data on plant pathogenic oomycetes is scarce. Except for the studies about the infection process of the *Phytophthora clandestine* (Ma *et al*., 2010) and the *Phytophthora sojae* (Zuo *et al*., 2005), few literature is currently available describing interaction processes between oomycetes and their hosts. Further work is thus needed to characterize these processes using microscopic approaches. A scan of literature did not reveal any detailed study on the germination, penetration and the development of the *P. capsici* on pepper plant and little is known about the infection process on the foliage. The present study was carried out to examine the different steps of *P. capsici* infection on pepper leaves leading to the disease by scanning electron microscope.

Materials and Methods

***P. capsici* isolate:** *P. capsici* isolate HX-9 was isolated from Hebei province, China and confirmed by genomic PCR with specific primers PCAP and ITS1, according to the method described by Ristaino *et al*., (1998). The physiological race was identified as 3, and mating type was identified as A1A2 according to the method described by Bonnie R (2008). It was maintained on PDA medium and kept at 4°C in dark, and sub-cultured on fresh PDA plate and incubated at 25°C for 7 days before infection assay.

The plant cultivars and culture condition: The susceptible pepper line was EC and the resistant pepper line was CM 334. Seeds were planted in 7.7cm×11cm×5.5cm plastic potting cells and thinned to 2-3 plants/cell. The

plants were maintained in a green house at 28±2°C, 75±5% RH and 30000-32000lx light intensity. The leaves were chosen from the growing point and counted backward from the fifth to the sixth expended true leaves.

The zoospore suspension: The *P. capsici* isolate Hx-9 was cultured on PDA plates in dark at 25°C for 7 days and then the mycelia were incubated with distilled water at 25°C for 48h. Decanted the distilled water and replaced with a sterile soil suspension, and kept the plates at 25°C for another 48h (Bonnie *et al*., 2008). After checking the sporulation, plates were incubated at 4°C for 60 min then at 25°C for 60min. The zoospore suspension was filtrated with two layers of gauze into a flask, counted by hemacytometer, and adjusted to a concentration of 1×10^5 zoospores per mL (Zhijun *et al*., 2007; Akhtar *et al*., 2012; Saleem *et al*., 2011).

Sample collection for the scanning electron microscopy study: The zoospores suspension was sprayed on both surfaces of the leaves of 8-week-old healthy potted plants. Leaf samples were collected at 6, 12, 24h, then 2d, 3d, 4d, 5d, 7d, 10d, 12d from the inoculated plants. For each stage leaf samples were collected from 10 plants; 5×5mm leaf samples were cut and randomly fixed. The specimens were fixed for 24h in 3% glutaraldehyde (100mmol/L, pH 6.8) at 4°C. Then the specimens were rinsed using phosphate buffer (pH 6.8) for 2-3h, and dehydrated in a graded ethanol-acetone series. The dehydration started in 30% ethanol, passed through 50%, 70%, 80%, and 90% ethanol; then two changes each through 100% ethanol, and then after through pure acetone for twice, keeping the tissues for 30s at each stage. The specimens were then critically dried using CO_2 as transition fluid, and the dried samples were mounted over copper stubs using double-stick tape and coated with about 20nm gold particles in a sputter coater. Specimens were observed with a JSM-6360LV electron microscope fitted with a scanning attachment at 20kV. At least 10 samples at random were observed from every batch to confirm the findings.

Results

The Hx-9 isolate tested with the *P. capsici* -specific PCR showed the expected 172 bp product, supporting identified the isolates' as *P. capsici*. The infection process was divided into six steps: including (I) the encystment of the zoospores; (II) the germination of cyst; (III) the penetration of the germ tubes; (IV) growing of new mycelia; (V) the sporulation and (VI) the spread of the mycelium.

The encystment, germination and penetration of *P. capsici* on leaf surface of both the susceptible and resistant cultivars: The epidermis of pepper leaves from both the susceptible and resistant cultivars was smooth and the stomata were rare. On both sides of the leaves from susceptible and resistant lines there was no wax on the surface, and the stomata were clean and closed (Fig. 1a). Zoospores quickly differentiate to form adhesive cysts at about 3 hpi on both susceptible (Fig. 1a) and resistant cultivars (Fig. 1d). The cysts started to germinate on both surface of pepper leaf about 12 hpi and out of the 20 germinated cysts observed, each cyst produced two germ tubes and the germ tubes did not branch (Fig. 1b, c and e). The germ tubes started to penetrate the pepper leaf at about 24h after inoculation, and they did not enter the stomata even when they passed over them, instead, they entered the leaf tissue directly without forming appressoriums (Fig. 1c). All of the zoospores could encyst quickly on the leaf surface, but very few of the cysts germinated to form germ tubes. The zoospores encysted, germinated and penetrated the leaf surface of the resistant cultivars similarly as on the susceptible cultivars. There was no apparent difference between two different cultivars during the first three steps.

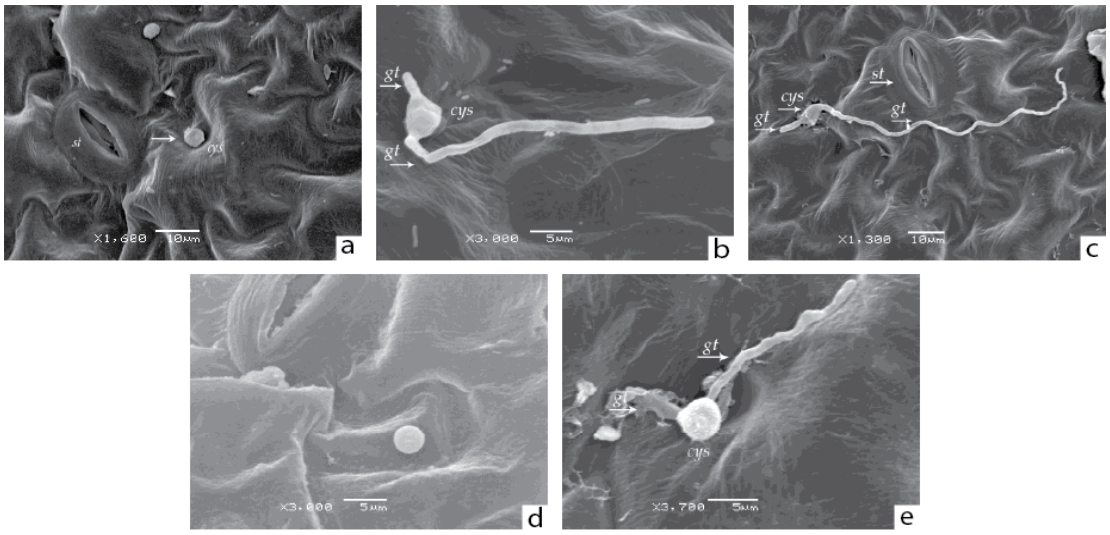

Fig. 1. Cyst of P. capsici isolate Hx-9, germinating and penetrating the leaf surface of the susceptible pepper cv. EC and the resistant cv. CM334. **a.** A zoospore encysted on the leaf surface (arrow) at 3h postinoculation (hpi); **b.** The cysts (cys) germinated and developed 2 germ tubes as seen 12h post-infection (hpi.); **c.** Germ tube (gt) developed and passed over a stoma (st) without entering it. The penetration is direct from the longer germ tube without developing appressorium as seen 24 hpi; **d.** A zoospore encysted on the leaf surface of the resistant cultivar CM334 as seen 3 h post-inoculation (hpi); **e.** The cysts on the resistant leaf surface germinated and developed two germ tubes at about 12h post-infection (hpi); cys = cyst, gt = germ tube; st stomata.

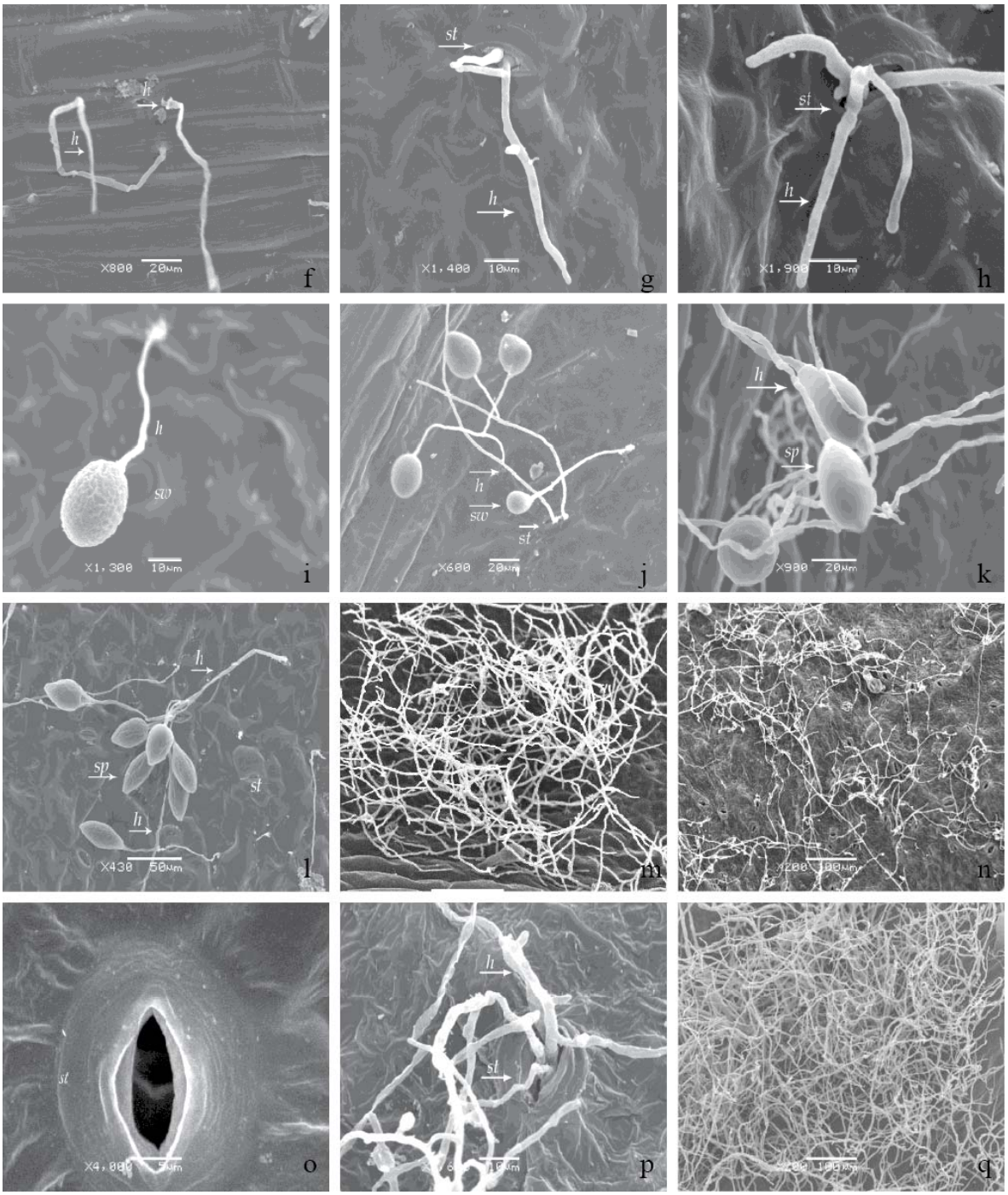

Fig. 2. Scanning electron microscopy images of the development of hypha of *P. capsici* on the leaf surface of the susceptible pepper cv EC. **f.** Release of mycelium of *P. capsici* through veins on the abaxial epidermis of pepper leaf as seen 3 day post inoculation (dpi).; **g.** Two mycelium grew out of the pepper leaf surface through stomata, and the mycelium branched vertically (3dpi); **h**. Group of the mycelium started to grew out of the adaxial epidermis through stomata as seen 4 day post inoculation (dpi); **i**. The mycelium on the abaxial epidermis swelled terminally as seen 4 day post inoculation (dpi); **j**. On the abaxial epidermis of the pepper leaf two mycelium grew out of the surface through stomata, and one through direct penetration, while all of the hypha swelled terminally and the shape ranged from spherical to oval (4dpi); **k.** The formation of sporangium of *P. caisici* on the abaxial surface of susceptible pepper leaf as seen 5 day post inoculation (dpi); **l**. A cluster of sporangium of of *P. caisici* on the abaxial surface of pepper leaf as seen 5 day post inoculation (dpi) **m.** A dense cluster of hypha emerged around the leaf vein as seen 7 day post inoculation (dpi); **m**. Hypha spread on leaf surface, as seen 10 day post inoculation (dpi); **o**. The stomata with no mycelium observed on the 12 day post inoculation (dpi); **p**. *P. caisici* release mycelium from stomata on the leaf surface of pepper as seen on the 12 day post inoculation (dpi).; **q**. The mycelium permeated on the leaf surface as seen on the 12 day post inoculation (dpi); cys cyst; gt germ tube; h hypha; sp sporangium; st stomata; sw swelling.

The infection process of *P. capsici* on the leaf epidermis of susceptible cultivar EC (early calwonder): Differences in the infection process of the two cultivars became visible from 3 day post inoculation (dpi). The disease symptom started to develop 4 dpi on the leaves of the susceptible pepper as small water soaked lesions on the abaxial leaf surface. The water soaked lesions appeared on the adaxial epidermis 1 day later. Then the lesions enlarged and spread over the leaf 7 days after inoculation, during the time the leaf turned yellow and end up with a darkish brown 12 days after inoculation. The white mildew layer was visible to the naked eye on the samples fixed 10 days and 12 days after inoculation. However, no evident symptoms were observed on the leaves of the resistant pepper line. The abaxial epidermis of the susceptible pepper leaf started to release mycelium either through direct penetration (Fig. 2f) or through stomata at the 3 day post inoculation, and the mycelium branched vertically (Fig. 2g).

New hyphae on the adaxial epidermis emerged one day later than that on the abaxial epidermis. At the 4 dpi the new mycelia started growing out of adaxial epidermis as seen in Fig. 2h that group of hypha grew out of the stomata. The new hyphae grew out through epidermis on the both abaxial epidermis and adaxial epidermis and at different part of the leaf surface, such as stomata (Fig. 2g, h, and q), leaf vein (Fig. 2f) and the smooth leaf surface (Fig. 2i, j). At the same time the mycelia on the abaxial epidermis began to swell terminally and the shape of swellings varied from spherical to oval (Fig. 2i, j).

Interestingly, according to the observation of 129 regenerated mycelia on the samples fixed 3-4 dpi and on the both surfaces of pepper leaves, about 64.3 of the mycelia grew out of the leaf surface through direct penetration like veins and smooth areas, while 35.7 were through stomata. However, most of the penetrations through stomata have a group of mycelia (Fig. 2g, h, j). These indicate that the regeneration of mycelia through stomata was not the preferable way for the *P. capsici*. The swellings developed and formed sporangia of *P. capsici* on the abaxial leaf surface as seen 5 dpi (Fig. 2k, l). Few hyphae could develop a bunch of swellings and then the swellings formed a cluster of sporangia (Fig. 2l). Among the hundreds of mycelia that observed only two developed in this way, most of the hypha branched and swelled terminally forming only one sporangium on each branch of the mycelium. At 7 dpi, a dense cluster of hyphae emerged in large clusters in abundance from mid rib as well from smaller veins (Fig. 2m), while at 10 dpi the mycelia ramified all over the leaf surface and the stomata opened widely (Fig. 2n). The cells on the leaf surface crimpled from the 10 dpi. The stomata which did not generate any mycelium opened widely and the leaf surface crimped greatly around on the 12 dpi (Fig. 2o). Clusters of mycelia were generated and branched and spread all over the epidermis (Fig. 2p, q). Even though the regeneration of mycelium occurred through both surfaces of leaves, the density of clusters and sporangia were more abundant on abaxial surface. No mycelium grew out from the leaves of the resistant pepper line even on the specimens fixed 12 dpi.

Discussion

This study using the scanning electron microscope studied the infection process of the *P. capsici* on susceptible and resistant cultivars of pepper leaves and divided the infection process into six steps. The zoospores of *P. capsici* formed adhesive cysts within 3 hours. The cysts germinated and produced two germ tubes which penetrated the epidermis of the pepper leaves directly without forming appressorium. It was observed that the cyst of *Phytophthora sojae* germinated and formed appressorium before penetrated the epidermis of hypocotyls (Zuo *et al*., 2005), and Ma, *et al.*, observed that the cyst of the *Phytophthora clandestine* germinated and formed one germ tube, and sometimes the tip of the germ tubes became swollen and formed an appressorium before penetrated the root. Kebdani *et al*., (2010) observed that all the cyst of the *Phytophthora parasitica* formed one germ tube and then differentiated a round appressorium before the structure penetrated the epidermis. However, the formation of appressorium was not observed in this study and all of the germinated cysts formed two germ tubes. No apparent differences in the process of the cyst attachment, germination and the hyphal penetration were evident between susceptible and resistant cultivars (Kang *et al*., 2008; Ribichich *et al*., 2000; Ma *et al*., 2010; Zuo *et al*., 2005). Similar results in this research. Zoospores can encyst, was got were also obtained germinate and penetrate both the susceptible and the resistant leaf surface. When the mycelium penetrated the host epidermis, fungal development in the resistant or incompatible cultivar was restricted due to putative plant defence reactions (Cora Boedo *et al*., 2008). When the mycelium invaded the host plant, the resistance genes of cv. CM334 expressed, and produced specific proteins, leading to a series of physiological and biochemical reaction, and finally reduced the spread of hypha in the host plant and showed no symptoms visually. In order to get a clear knowledge about the resistance mechanism of the resistant cultivar CM334, further studies need to be taken.

The germ tubes penetrated the leaf surface randomly, but no penetration was observed from the stomata, as the tube did not entered the stomata even when they passed over it, similar result was observed by Ahmed (2009) on *Cercospora henningsii* (Ma *et al*., 2010). For the abaxial and adaxial surface of pepper leaf, there was no difference in the stages before penetration. However, symptoms were observed one day earlier on the abaxial leaf surface and so did the regeneration of the mycelium and the swelling of the hyphae. Mycelia were developed in clusters inside the leaf tissue (Cora Boedo *et al*., 2008; Zuo *et al*., 2005), and they generated through different part of the leaf epidermis on both sides of the leaves, either through stomata or by rupture of leaf surface on different part of the epidermis including the veins. The growing mycelia from ruptures of leaf surface and the stomatal openings were 64.3% and 35.7% in this study. It shows that the emergence of mycelia through stomata was not a preferred route. However, more mycelia were observed from the abaxial epidermis than the adaxial surface, and more sporangia were also formed. This maybe because of the abaxial epidermis of the pepper leaf have more stomata, as more mycelia grew out from the stomata. Hence the symptoms started on abaxial epidermis earlier. More mycelia were observed around the

leaf vein, may be because the mycelia spreaded inside the leaf tissue firstly through veins. While for the highly resistant cultivar CM334 no mycelium was observed from the pepper leaf surface and no symptom developed either. In order to get accurate knowledge about the plant-microbe interaction in these two cultivars further studies may be under taken to discover the different responses of resistant cultivar and the susceptible cultivar that are infected by *P. capsici*.

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