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ORIGINAL PAPER

Correlation between total hypha length and haustoria number of *Pseudoidium neolycopersici* in type I trichome cells of tomato leaves

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Abstract

Powdery mildew haustoria are easier to be observed by light microscopy in trichome cells compared to epidermal cells of infected leaves. The objective of this study was to explore the relationship between the hyphal length and the number of haustoria in type I trichome cells of tomato (*Solanum lycopersicum* Mill.) leaves. The trichome cells of tomato cv. Moneymaker were inoculated with conidia of tomato powdery mildew (*Pseudoidium neolycopersici* L. Kiss), isolate KTP-04. On these cells, the *P. neolycopersici* isolate produced a maximum of four vigorously elongated hyphae per conidium. At 12 days after inoculation, KTP-04 formed two to five haustoria per conidium. Field-emission scanning electron microscopy showed that the haustorium consisted of a haustorial body and several lobes embedded in an extrahaustorial matrix. The number of haustoria per hypha and hyphal length on trichomes were positively correlated. Also, the tips of one to four hypha per conidium (excluding germ tubes and primary appressoria) were injured using a minute glass needle installed on micromanipulator under a high-fidelity digital microscope, and their total hyphal lengths were compared. Wounded hyphae possessed the same number of haustoria in trichome cells as non-wounded hyphae, and total hyphal lengths were similar between treatment groups. In this study, a new model was developed to study the infection mechanisms of powdery mildews that will be useful in future gene expression studies.

Keywords Conidial germination · Haustorial formation · Hyphal elongation · *Solanum lycopersicum* · Tomato powdery mildew

Introduction

Powdery mildew infections of tomato are caused by three species: *Pseudoidium neolycopersici* L. Kiss and *Leveillula taurica* (Lév.) G. Arnaud, two pathogens known from many parts of the world (White et al. 1997; Kiss et al. 2001; Zheng et al. 2013), and *Golovinomyces lycopersici* (Cooke & Massee) L. Kiss, reported only from Australia (Kiss et al. 2001; Braun et al. 2019). All these species are obligate biotrophs that infect leaves and stems, and reduce the yield of the infected tomato plants. In Japan, *P. neolycopersici* occurs regularly on hydroponically cultured tomato (*Solanum lycopersicum* Mill.) (Matsuda et al. 2001; Shimizu et al. 2007; Nonomura et al. 2008). Since 1998, we isolated five *P. neolycopersici* isolates from infected leaves collected in our greenhouse (Kashimoto et al. 2003; Nonomura et al. 2010, 2013; Seifi et al. 2012). The infection cycle of *P. neolycopersici* begins with conidial germination, elongation of an appressorial germ tube and formation of an appressorium after the conidium landed on a plant leaf surface. The appressorium helps the adhesion to plant leaf surface and fungal penetration of the plant cell wall. Matured haustorium forms after penetration into the host epidermal cell; it begins

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to draw nutrients and water from the host cell, and new epiphytic hyphal growth leads to an expansion of the colony on the leaf surface (Jones et al. 2001; Kashimoto et al. 2003; Jacob et al. 2008). Thus, the establishment of functional haustoria is essential for successful fungal colonisation on the leaf surface; effector molecules must be delivered from fungal haustoria to host cells to suppress plant defences and promote fungal establishment (Lindeberg et al. 2012).

The leaves of wild and cultivated *Solanum* species have morphologically distinct trichomes (types I to VII) (Lemke and Mutschler 1984; Glover 2000; McDowell et al. 2011). These trichomes act as biological antennae, responding to physical and chemical stimuli or producing chemicals after detecting changes in environmental factors (Kennedy and Sorenson 1985; Duffey 1986; Peter and Shanower 1998; Kessler and Baldwin 2001; Pichersky and Gershenzon 2002; Simmons and Gurr 2005; Nonomura et al. 2009b; Kang et al. 2010; Tooker et al. 2010). Morphological characteristics of leaf type I trichomes of tomato cv. Moneymaker (MM) were 1.5–2.5 mm in length on a multicellular base with a small glandular tip, and abundant and larger/longer than other types of trichomes. In a previous study, we used the leaf type I trichomes of MM to elucidate the interaction between trichomes and *P. neolycopersici*, as well as the infection processes of powdery mildew pathogens on trichomes, by high-fidelity digital microscopy (DM) (Suzuki et al. 2018). We found that trichome cells induce the same cytological responses as leaf epidermal cells following inoculation with *P. neolycopersici* conidia, and demonstrated that trichomes provide experimental material to be used as infection sites for powdery mildews. MM leaf epidermal and trichome cells induce hypersensitive cell death (HR) following invasion by the barley powdery mildew pathogen (*Blumeria graminis* f. sp. *hordei* Marchal race1) and the melon powdery mildew pathogen (*Podosphaera xanthii* Pollacci), and produce papilla-like structures at red clover powdery mildew pathogen (*Erysiphe trifoliorum* Greville) invasion sites (Matsuda et al. 2005; Nonomura et al. 2010; Seifi et al. 2012; Takikawa et al. 2011a, 2015). We also demonstrated that the hyphal development of specific tomato powdery mildew isolates (*P. neolycopersici* KTP-03 and -04) was not suppressed in the cells of trichomes on powdery mildew-resistant wild tomato lines (*S. peruvianum* LA2172). Therefore, we compared the resistance and virulence of isolates using type I trichomes of tomato powdery mildew-susceptible and resistant lines, by simultaneously observing the infection processes of powdery mildews on leaf trichomes and the cytological responses of powdery mildew-invaded trichome cells.

Generally, chemical treatments such as chlorophyll removal (discoloration), fixation, and staining are required for clear observation of HR or papillae in epidermal cells by epifluorescent microscopy (EM) (Li et al. 2007; Nonomura et al. 2010), and cell haustoria by light microscopy (LM) (Huang et al. 1998; Dyki 2003; Li et al. 2012). In contrast, we were able to easily and directly observe cytological responses and mature haustoria in powdery mildew-invaded trichomes by microscopy without chemical treatment, because trichomes consist of transparent cells growing from the leaf epidermis (Suzuki et al. 2018). Therefore, trichomes were excellent and advantageous sites for morphological and cytological analysis of the interactions between plant cells and pathogens under natural environmental conditions. The physiological relationships among infection structures of tomato powdery mildew pathogens, in or on trichome cells, have not yet been analysed and reported. In this study, our main objectives were to clarify (1) the morphological characteristics of functional haustoria of tomato powdery mildew pathogens in both leaf epidermal and trichome cells using field-emission scanning electron microscopy (FE-SEM), (2) the relationships between total hypha length and haustorium number per hypha on trichome cells, and (3) the trichome infection processes of intact and wounded hyphae. To our knowledge, this is the first study described about developments of a new method to study haustorial formation of powdery mildew fungi in leaf trichome cells with light microscopy, without chemical treatment.

Materials and methods

Plant materials

MM tomato seeds were germinated on water-soaked filter papers in a Petri dish for 3 days in a growth chamber (LH-240N; Nippon Medical & Chemical Instruments, Osaka, Japan) under continuous illumination (19.8–40.3 μmol m^{-2} s^{-1}; 400–700 nm) from white (full-spectrum) fluorescent lamps (FL40SS W/37; Mitsubishi, Tokyo, Japan) at 25 ± 2 °C. Seedlings at the cotyledon stage were placed into polyurethane cubic sponge supports (3 × 3 × 3 cm^3). Sponge supports containing seedlings were inserted into 30-mL cylindrical plastic cases (diameter 3 cm, length 5 cm), each containing 20 mL hydroponic nutrient solution (4.0 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.66 mM $NH_4H_2PO_4$, 0.057 mM FeEDTA, 0.048 mM H_3BO_3, and 0.009 mM $MnSO_4$), and then incubated for 14 days in a temperature-controlled room under the following conditions: 25 ± 2 °C, 50–70% relative humidity (RH) and continuous illumination at 22.2 μmol m^{-2} s^{-1}. MM seeds were obtained from their self-pollinated progeny in our greenhouse.

Fungal materials

Isolate KTP-04 of tomato powdery mildew (*P. neolycopersici*) was used in this study (Nonomura et al. 2013). Mature conidia were collected from fungal mycelia on

infected leaves using an electrostatic spore collector, as previously described (Nonomura et al. 2009a), and transferred onto true leaves of 14-day-old tomato healthy seedlings by high-fidelity DM (KH-2700; Hirox, Tokyo, Japan). Inoculated seedlings were maintained for 14 days in growth chambers at 25 ± 1 °C and 50–70% RH under continuous illumination at 22.2 μmol m^{-2} s^{-1} (Nonomura et al. 2013).

Dynamic analysis of powdery mildew infection on tomato leaf type I trichomes

Conidia of KTP-04 were inoculated onto leaf epidermal cells and type I trichomes of 14-day-old MM plants. More than 100 conidia were used for inoculation in one experiment. Powdery mildew-inoculated plants were incubated in the growth chamber for 2–12 days at 25 ± 1 °C, 70–90% RH under continuous illumination at 22.2 μmol m^{-2} s^{-1}. The infection processes of powdery mildew isolates on leaves and type I trichomes were observed using the KH-2700 DM. KTP-04 hyphal development was photographed at 2–12 days after inoculation of a single conidium onto a type I trichome using the ½" Interline transfer charge-coupled device (CCD) camera of the KH-2700 DM. Micrographs were analysed using the Adobe Photoshop image-processing software (ver. 5.0; Adobe Systems, San Jose, CA, USA) to improve image contrast without changing the original information. For rates of germination and appressorium formation, data were presented as means and standard deviation (SD) of five replicates (where 20 times was equal to one replicate).

Microscopic observation of mature haustoria in tomato powdery mildew-invaded type I trichomes

We collected 25 leaf segments (approximately 1 × 1 cm^2 in area) from five powdery mildew-inoculated tomato plants. The experiments were conducted three times (total of 75 leaf segments). Samples were directly observed under the KH-2700 DM, and under a light and epifluorescence microscope (BX-60; Olympus, Tokyo, Japan) without chemical treatment. Other samples were fixed and their chlorophyll removed in a boiling alcoholic lactophenol solution (10 mL glycerol, 10 mL phenol, 10 mL lactic acid, 10 mL distilled water, and 40 mL 99.8% ethanol) for 1–2 min, and then stained with 0.1% Aniline Blue (Nacalai Tesque, Tokyo, Japan) dissolved in distilled water, as previously described (Sameshima et al. 2004). Samples were then observed under the BX-60 light and epifluorescence microscope with a dichroitic mirror at 400 nm (maximum excitation, 330–385 nm; barrier filter, 420 nm).

Tomato leaf pieces (1 × 1 mm^2) with tomato powdery mildew-inoculated trichomes were prefixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, pH 7.4) at 4 °C for 3 days, and postfixed with 1.0% buffered osmium tetroxide (Nisshin EM, Tokyo, Japan) for 1 h. After fixation, leaf pieces were washed for 5 min three times in ultrapure water, dehydrated in a graded ethanol series from 30–100%, and embedded in Quetol 651 resin mixture (Nisshin EM). Serial semithin sections (200 nm thickness) were cut from the resin blocks using a diamond knife, mounted on cover glasses (13 mm diameter), stained with 1.0% uranyl acetate for 20 min, and counterstained with lead solution (Sigma Aldrich, Japan) for 5 min. The sections were coated with 1.0 nm thick osmium using an osmium coater (Neoc-Pro; Meiwafosis, Tokyo, Japan), and then observed under a FE-SEM (SU8220; Hitachi, Tokyo, Japan) using a backscattered electron (BSE) detector with an accelerating voltage at 2.0 kV, as previously described by Koga et al. (2015).

Length measurement of hyphae on powdery mildew-inoculated type I trichomes

Mature KTP-04 conidia were inoculated onto young leaves and type I trichomes of 20 14-day-old MM plants. Powdery mildew-inoculated plants were incubated in the growth chamber for 2–12 days at 25 ± 1 °C, 70–90% RH under continuous illumination at 22.2 μmol m^{-2} s^{-1}. We examined 20 conidia from the isolate. The lengths of one to four colony-forming hyphae formed from conidia were measured separately using the KH-2700 DM and summed. Data were presented as means and SD of 20 measurements.

Relationship between haustorium number and length in hyphae from conidia on tomato leaf type I trichomes

Mature KTP-04 conidia were inoculated onto young leaves and type I trichomes of 60 14-day-old MM plants. Powdery mildew-inoculated plants were incubated in the growth chamber for 2–12 days under the conditions described above. The tips of hyphae (excluding germ tubes and appressoria) elongated from 60 conidia were injured by pricking with a tiny glass needle installed in a micromanipulator. We conducted five experiments to explore conidium infection processes, varying the number (zero to four) of injured colony-forming hyphae. We observed the haustoria in type I trichomes under a BX-60 LM instrument (Olympus), and counted the haustoria. The lengths of one to four colony-forming hyphae formed from each of the 60 conidia were measured separately using the KH-2700 DM and summed. Data were presented as means and SD of 20 samples (zero injured hyphae) and 10 samples (one to four injured hyphae). Standard curves were prepared by plotting total haustoria number against total hyphal length (zero to four injured hyphae).

Results

Comparable infection process of powdery mildew on both tomato leaf epidermal cells and type I trichomes

We monitored the process of KTP-04 infection on MM leaf epidermal and type I trichome cells. On the surface of both cells, KTP-04 conidia showed very high germination rates, exceeding 98% of inoculated conidia, and 40–50% of germ tubes grew downwards producing primary appressoria. The remaining conidia (50–60%) germinated upwards and germ tube elongation ceased completely within 24 h after germination, without forming primary appressoria on either epidermal cells (Fig. 1A) or type I trichomes (Fig. 1B). Next, we compared downward growing germ tubes. All conidia (100 conidia per plant cell) germinated on epidermal and type I trichome cells at 3–5 h and then produced primary appressoria at 6–8 h after inoculation, with germination rates of 97.8 ± 2.1% on epidermal cells and 98.1 ± 1.8% on type I trichomes. The rate of appressorium formation was 44.5 ± 5.1% on epidermal cells and 45.1 ± 4.7% on type I trichomes. Fungi produced elongating colony-forming hyphae from the conidia within 48 h after inoculation, and then successfully infected epidermal cells and type I trichomes. From a single conidium, and before hyphal development ceased completely at 12 days after inoculation, a maximum of five hyphae (containing germ tubes) formed on epidermal cells (Fig. 1C), whereas a maximum of four hyphae formed on trichomes (Fig. 1D). Conidiophores were produced on hyphae growing on epidermal cells (Fig. 1C), whereas no conidiophores formed on type I trichomes during the experimental period (Fig. 1D).

Powdery mildew haustoria in type I trichomes

KTP-04 haustoria were observed in trichome cells using the KH-2700 DM (Figs. 1D and 2A) and BX-60 LM instruments (Fig.

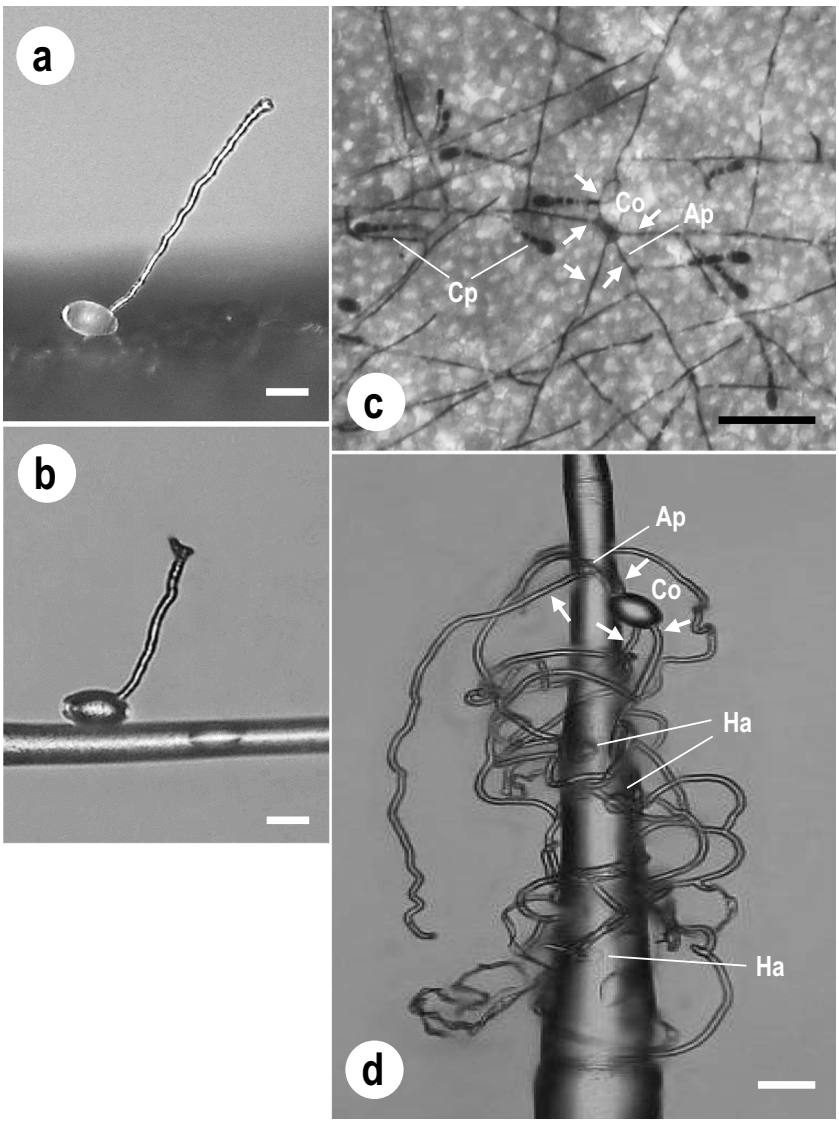

Fig. 1 Digital and light micrographs of germinated conidia and elongated hyphae of KTP-04 on leaves of MM tomato plants. **A** and **B**) Conidia germinated upward on leaf epidermal cells (**A**) and a leaf type I trichome (**B**) at 3–5 h post-inoculation (pi). Germ tube elongation ceased completely within 24 h pi. **C** and **D**) KTP-04 hyphae elongated vigorously on leaf epidermal cells (**C**) and the trichome cell (**D**). Micrographs were taken at 12 days pi with KTP-04 conidia. Five hyphae from a single conidium (arrows), conidiophores, and no functional haustoria were observed on or in epidermal cells, whereas four hyphae (arrows), no conidiophores, and functional haustoria were observed on or in the trichome cell at the same time point. Ap, appressorium; Co, conidium; Cp, conidiophore; Ha, haustorium. Bars represent 20 μm (**A**, **B**, and **D**) and 50 μm (**C**)

2B). We then observed papilla-like structures produced beneath primary and hyphal appressoria in type I trichomes using the BX-60 EM instrument (arrows in Fig. 2C), and the functional haustorium formed in them using the SU8220 FE-SEM; the transparent globular structure of trichome cells is shown in Fig. 2A. As shown in Fig. 2D, trichome cells were penetrated through penetration pegs arising from hyphal appressoria. These were surrounded by papillae consisting of thinner, electron-dense and much thicker, electron-lucent callose-like materials. Penetration pegs continued their intracellular development and gave rise to haustoria. The fully developed haustorium consisted of a central body and several lobes embedded in a haustorial matrix surrounded by an extrahaustorial membrane. Large spherical structures (up to 1–1.5 μm in diameter) resembling vacuoles were seen within the central bodies and lobes of haustoria. The first functional haustoria were observed in trichome cells at 24–36 h after inoculation.

Length of powdery mildew hyphae on type I trichomes

The lengths of all hyphae formed from conidia were measured under the KH-2700 DM when hyphal growth completely stopped on type I trichomes after inoculation with KTP-04 conidia. The average total hyphal length was approximately 2,637.5 ± 1,157.0 μm at 12 days after inoculation (Table 1). Hyphal injury was performed as shown in Fig. 3. There was no significant difference in either hyphal length or haustorium number among elongated hyphae produced by injury and non-injury treatments (Fig. 4A–E; Table 1). When all four hyphal tips were injured, a new (fifth) hypha appeared from the conidium (Fig. 4G), or occasionally from a germ tube (Fig. 4H) or appressorium (Fig. 4I), at rates of 93, 5, and 2%, respectively. Breaking the tip of the fifth hypha by the same method did not result in the appearance of a sixth hypha from the conidium (Fig. 4F).

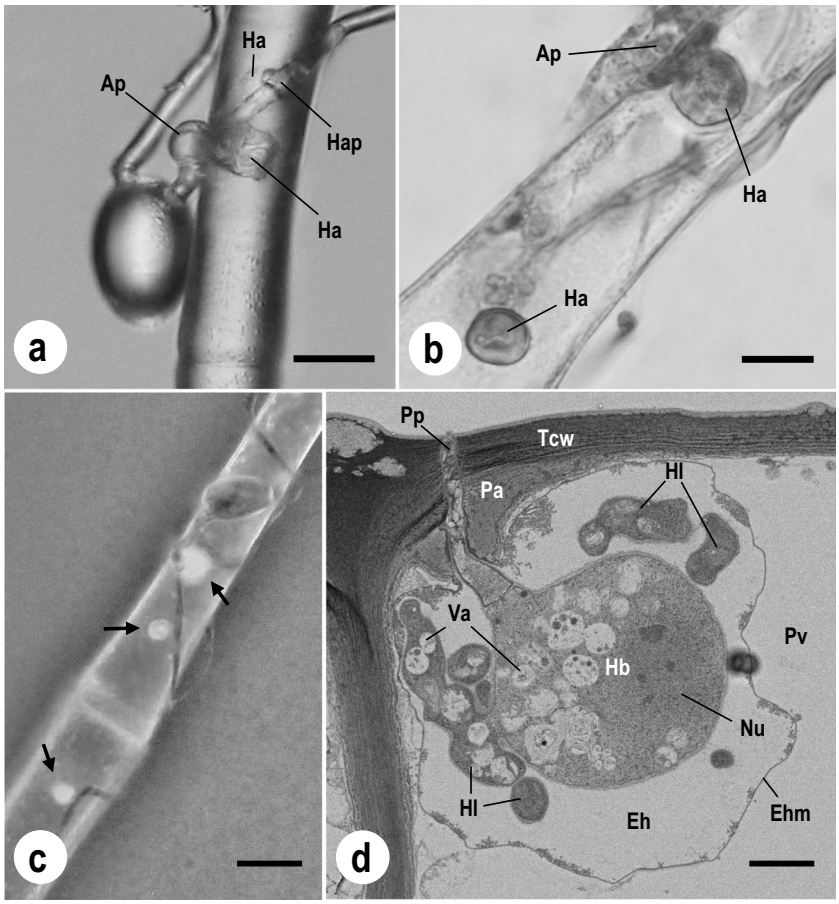

Fig. 2 Digital micrographs, (**A**), light micrographs (**B**) and field-emission scanning electron micrographs (**D**) showing haustoria of KTP-04, and epifluorescent micrographs (**C**) showing papilla-like structures in leaf type I trichome cells. KTP-04 formed haustoria (Ha) beneath appressoria (Ap) and hyphal appressoria (Hap) in the trichome cells. Without chemical treatment (**A**), haustoria were round, transparent structures; following chemical treatment (**B**), haustoria were globular and pale brown. (**C**) Papilla-like structures (arrows) produced beneath primary and hyphal appressoria. Micrographs were taken at 72 h pi with KTP-04 conidia. (**D**) A nearly longitudinal section through a penetration peg (Pp) emerging from an appressorium on a trichome cell wall (Tcw), surrounded by papilla-like structures (Pa). Haustorium consists of a haustorial body (Hb) and several haustorial lobes (Hl) embedded in an extrahaustorial matrix (Eh) and surrounded by an extrahaustorial membrane (Ehm). Vacuoles (Va) within the haustorial body and haustorial lobes are shown. Nu, nucleus; Pv, plant vacuole. Bars represent 10 μm (**A**, **B** and **C**) and 2 μm (**D**)

Table 1 Relationship between haustorium number and total hyphal length of type I trichomes on tomato (*Solanum lycopersicum* Mill. cv. Moneymaker) leaves

Number of hyphae injured	Number of hyphae produced from conidia	Number of conidiophores	Number of haustoria	Total hyphal length (μm)	Hyphal length per haustorium (μm)
0	4.0 ± 0.6^{a}	0^{a}	7.6 ± 2.9^{a}	2637.5 ± 1157.0^{a}	342.4 ± 73.0^{a}
1	3.9 ± 0.3^{a}	0^{a}	6.6 ± 2.1^{a}	2862.5 ± 1098.0^{a}	429.4 ± 68.4^{a}
2	3.9 ± 0.3^{a}	0^{a}	5.8 ± 2.3^{ab}	2311.0 ± 1214.3^{ab}	388.1 ± 109.1^{a}
3	4.0 ± 0^{a}	0^{a}	3.2 ± 2.6^{b}	1134.6 ± 975.9^{b}	371.8 ± 124.2^{a}
4	4.5 ± 0.5^{b}	0^{a}	4.0 ± 1.4^{b}	1765.4 ± 865.5^{ab}	430.1 ± 116.8^{a}

Different letters indicate a significant difference ($p<0.05$, Tukey's method).

Relationships between hyphal length and haustorium number on/in type I trichomes

When zero hyphae were injured, KTP-04 formed 3–14 functional haustoria in type I trichomes. As the number of injured-hyphae increased, the total number of haustoria in trichomes generally decreased, with 4 to 10, 2 to 10, 1 to 9, and 2 to 6 haustoria produced following injury to one to four hyphae, respectively (Table 1). Total haustorium number was correlated with total hyphal length in each of the four injury experiments (Fig. 5). Hyphal length per haustorium was similar between injured and non-injured hyphae (Table 1).

Discussion

In this study, we observed germination of tomato powdery mildew isolate KTP-04 conidia on MM type I trichomes, and successfully analysed the infection processes on leaf epidermis and trichome cells. In our previous study (Takikawa et al. 2011b), we reported that the direction of germ tube projection from conidia was potentially determined after inoculation of tomato powdery mildew conidia onto host and non-host plant leaves, as well as artificial membranes (e.g. Parafilm). Suzuki et al. (2018) reported that the direction of germ tube projection is a unique characteristic among powdery mildew fungi; tomato and red clover powdery mildew pathogens have non-catenated conidia, and barley and melon powdery mildew pathogens have catenated conidia. These characteristics are important for successful host plant infection by powdery mildews. We found that among tomato powdery mildew pathogens that germinated downwards onto type I trichomes, nearly all conidia formed non-lobed appressoria on the trichome cells (see Fig. 2A), successfully infecting cells upon the first penetration attempt by appressoria (Suzuki et al. 2018). Thus, we confirmed that tomato powdery mildews were capable of producing appressoria and primary haustoria in type I trichomes, successfully infecting the cells and vigorously elongating hyphae by repeated cell invasion.

Only a few histological studies have been conducted to fully observe the steps of the infection processes of tomato

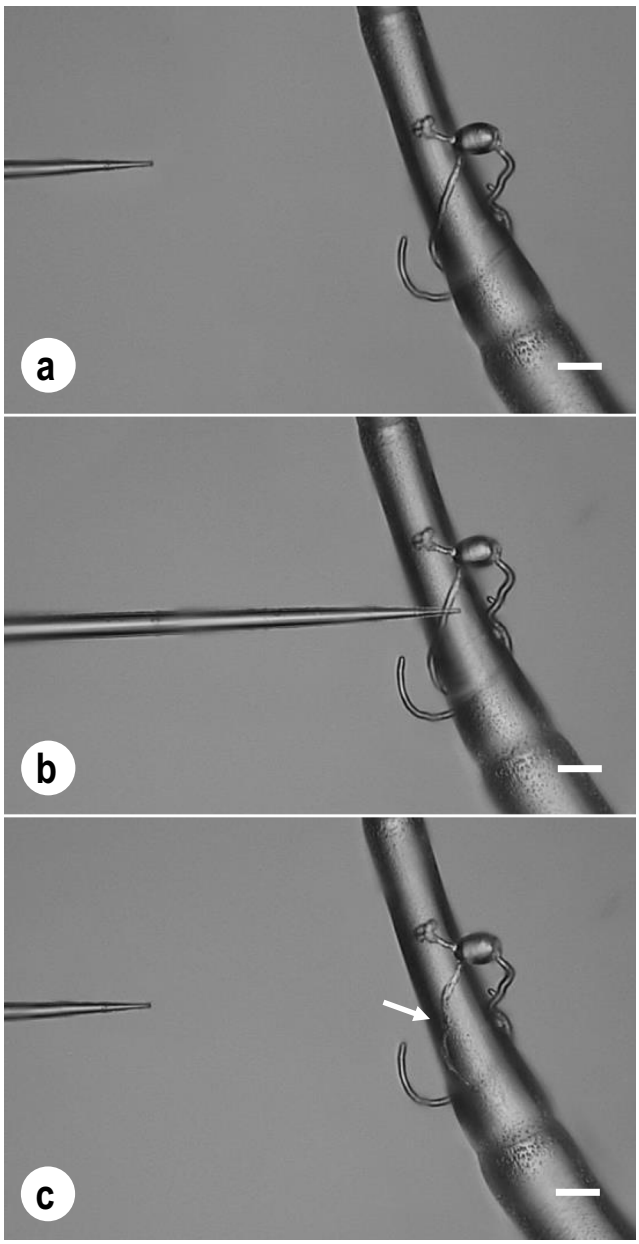

Fig. 3 Procedure for hyphal injury experiment. Conidium hyphae tips were pricked using a tiny glass needle installed in a micromanipulator. **A**, **B** and **C** are digital micrographs taken before, during, and after hyphal injury, respectively. Arrow indicates hyphal injury. Bars represent 20 μm

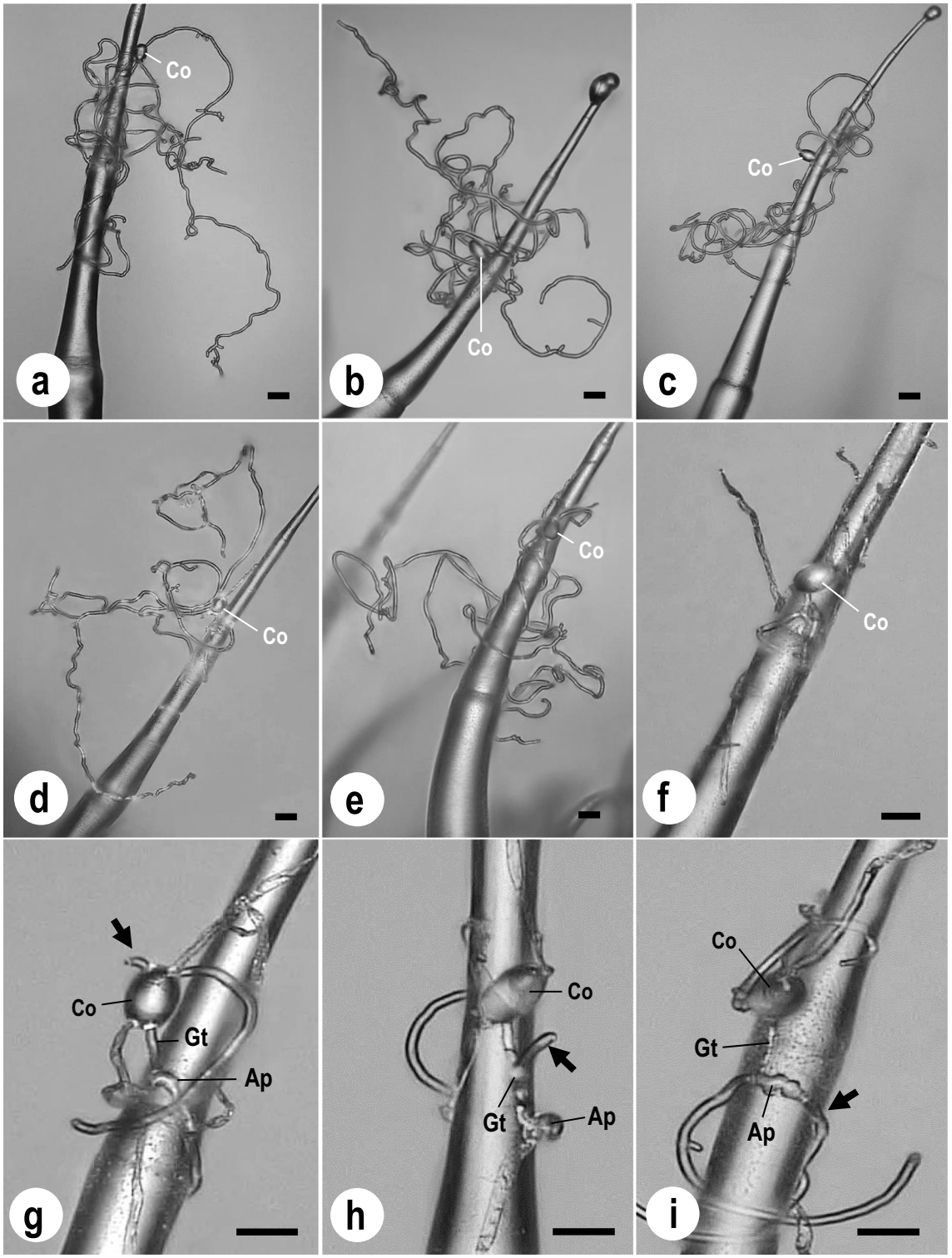

Fig. 4 Process of KTP-04 infection on type I trichome cells observed under a KH-2700 DM instrument. Tips of hyphae (excluding germ tubes and appressoria) elongating from conidia on type I trichome cells were injured as shown in Fig. 3. Six treatment levels were set: **(A)** four uninjured colony-forming hyphae, **(B)** one of four hyphae injured, **(C)** two of four hyphae injured, **(D)** three of four hyphae injured, **(E)** all four hyphae injured, and **(F)** five hyphae injured. **G**, **H**, and **I** show the appearance of a fifth hypha (arrow) from a conidium, germ tube, and appressorium, at 5 days pi with KTP-04 conidia, respectively. The emergence of a fifth hypha from a germ tube or appressorium was much rarer. In **A**–**E**), hyphae vigorously elongated on the trichome until 12 days pi, then ceased hyphal elongation without forming conidiophores. In **F**), fungi did not form a sixth hypha from a conidium. Ap, appressorium; Co, conidium; Gt, germ tube. Bars represent 20 μm

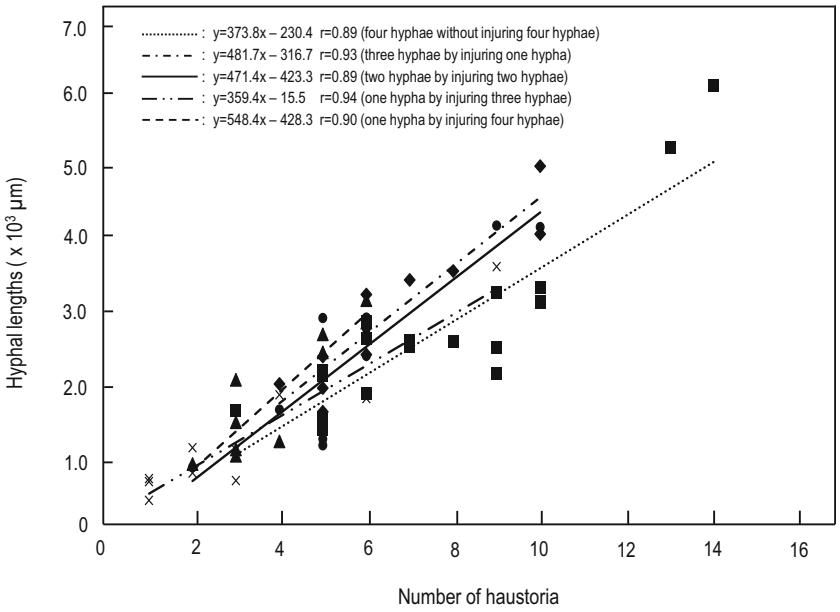

Fig. 5 Relationship between the number of haustoria and hyphal lengths in KTP-04 on leaf type I trichome cells at 12 days pi with KTP-04 conidia, when hyphal development completely ceased. Total hyphal length increased as the number of functional haustoria increased in type I trichome cells. Data for 0–4 injured hyphae are plotted as (■), (♦), (●), (x), and (▲), respectively

powdery mildew pathogens. These earlier studies focused on the morphology of pathogen features at the tomato leaf surface, including appressoria (Nonomura et al. 2010; Lebeda et al. 2014) and conidiophores (Whipps et al. 1998; Dyki 2003; Oichi et al. 2004, 2006). In the present study, we examined haustorial formation in powdery mildew-infected trichomes, because these are the most important infection structures for nutrient uptake from plant cells. To our knowledge, no studies of tomato powdery mildew haustoria have described their shapes and characteristics within infected plant cells in detail. Braun (1987) and Zeller (1995) reported that powdery mildew pathogen haustorial types are broadly divided into two groups, globose and digitate. Saenz and Taylor (1999) described *Erysiphe* haustoria as globose. LM observations of haustorial shapes have revealed round complexes 15–24 µm in diameter (Dyki and Staniaszek 1997; Li et al. 2012), circular complexes (LaMondia et al. 1999), and spherical to sac-like complexes 5–25 µm in diameter (Whipps et al. 1998). Segarra et al. (2009) examined micrograph sections of tomato powdery mildew (*Erysiphe polygoni*) haustorial bodies within tomato plant (cv. Roma) adaxial epidermal cells using TEM. However, they did not describe the haustorial shapes or characteristics in detail. In the present study, we applied DM, LM, EM and SEM to study infected trichomes, and confirmed previous reports of *P. neolycopersici* haustoria in trichomes at 24–36 h post-inoculation. Our results support the TEM findings of Segarra et al. (2009). Interestingly, tomato powdery mildew fungi formed functional mature haustoria (primary haustoria beneath appressoria and haustoria beneath hyphal appressoria) in a single trichome cell. Dyki and Staniaszek (1997) observed up to three haustoria within one cell. We observed up to six functional haustoria within a single trichome cell (data not shown); thus, appressorial formation allows fungi to produce functional haustoria within trichomes. Recently, Micali et al. (2011) demonstrated that papillae produced in epidermal cells of *Arabidopsis thaliana* infected with the epiphytic powdery mildew *Golovinomyces orontii* contain callose (*β*-1,3-glucan). Because the papillae examined in this work were morphologically similar to those observed in the *Arabidopsis–Golovinomyces* interaction (see Fig. 2D), we surmise that electron-lucent materials observed around penetration pegs are also made of callose. Also similar to the *Arabidopsis–Golovinomyces* interaction, membranes and vesicular structures were observed in the callose layer surrounding penetration pegs, as well as callose encasement of haustoria (see Fig. 2D); however, this structure was not as prominent as that seen in *Arabidopsis* epidermal cells invaded by *G. orontii* haustoria (Micali et al. 2011).

In our previous studies, we clarified that tomato leaf type I trichome and epidermal cells were able to induce the same response to fungal invasion i.e. papilla in response to *E. trifoliorum* from red clover and HR in response to *P. xanthii* from melon (Suzuki et al. 2018). We simultaneously monitored powdery mildew infection processes and trichome cytological responses using the KH-2700 DM and BX-60 LM instruments without histochemical staining (Suzuki et al. 2018). Following these observations, we suggest that trichome cells invaded by non-pathogenic and pathogenic powdery mildews are useful for the study of molecular interactions between plants and powdery mildews at the scale of a single trichome cell. It was much easier to collect only powdery mildew-inoculated single trichome cells at different powdery mildew developmental stages than on leaf epidermal cells. Little is known of the genetic factors and molecular mechanisms associated with non-host resistance, plant immunity to potential pathogens (Ellis 2006), and host

susceptibility. Our knowledge of non-host resistance is based on the zigzag model of plant immunity (Jones and Dangl 2006). Schweizer (2007) subsequently proposed two models for non-host resistance. The first of these postulates the absence of fungal effectors, leading to a non-compromised basal defence response due to the interaction between plant pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs); the second postulates non-host resistance (R)-genes and host susceptibility caused by fungal effectors. To further investigate the molecular mechanisms of non-host resistance and host susceptibility, dynamic cellular changes should be studied at the transcriptomic and/or proteomic level. In such studies, it is crucial to avoid intracellular contamination of differently responding plant cells and fungal materials. Therefore, the isolation of intracellular contents using powdery mildew-inoculated trichome cells by micropipette manipulation (Fujita et al. 2004) and laser microdissection (Chandran et al. 2010) will facilitate analysis of the molecular mechanisms of powdery mildew infections, such as differential gene expression during the establishment and proliferation stages of infection. Techniques developed for foliar trichomes can thus be used to study plant–pathogen interactions and communication at the level of individual trichome cells.

It is impossible to directly observe haustoria in tomato leaves without removing chlorophyll from leaf epidermal cells to confirm whether haustoria were formed beneath the hyphal appressoria. However, using the microscopic techniques applied in this study, we successfully observed haustoria in trichome cells. We also reported, for the first time, the relationship between the number of powdery mildew haustoria and total hyphal length, and demonstrated no significant effect of hyphal injury on hyphal length per haustorium. Our results indicate that trichome cells can be used to further study tomato host responses to pathogenic powdery mildews and host susceptibility to powdery mildew fungal effectors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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