Conidia of the tomato powdery mildew *Oidium neolycopersici* initiate germ tubes at a predetermined site

Yoshihiro Takikawa · Ling Xu · Koji Kakutani ·
Teruo Nonomura · Takeshi Sameshima ·
Yoshinori Matsuda · Hideyoshi Toyoda

Abstract The emergence of germ tubes from the conidia of powdery mildew fungi is the first morphological event of the infection process, preceding appressoria formation, peg penetration and primary haustoria formation. Germination patterns of the conidia are specific in powdery mildew fungi and therefore considered useful for identification. In the present study, we examined conidial germination of the tomato powdery mildew *Oidium neolycopersici* KTP-01 in order to clarify whether germ tube emergence site in KTP-01 conidia is determined by the first contact of the conidia to leaves (as found for the conidia of barley powdery mildew), or alternatively is predetermined and is unrelated to contact stimulus. Highly germinative conidia of KTP-01 were collected from conidial pseudochains on conidiophores in colonies on tomato leaves using two methods involving an electrostatic spore attractor and a blower. In the electrostatic spore attraction method, the conidia were attracted to the electrified insulator probe of the spore collector—this being the first contact stimulus for the conidia. In addition, the blowing method was used as a model of natural infection; pseudochain conidia were transferred to detached leaves by air (1 m/s) from a blower. Thus, landing on the leaves was the first contact for the conidia. Furthermore, conidia were also blown onto an artificial membrane (Parafilm-coated glass slides forming a hydrophobic surface) or solidified agar plates in Petri dishes (hydrophilic surface). Eventually, almost all conidia on the probe and on tomato leaves or artificial hydrophobic and hydrophilic surfaces synchronously germinated within 6 h of incubation, indicating that the first contact of the conidia with any of the aforementioned substrata was an effective germination induction signal. Germ tube emergence sites were exclusively subterminal on the conidia. Moreover, the germ tubes emerged without any relation to the sites touched first on the conidia. Thus, the present study strongly indicates that conidia of *O. neolycopersici* produce germ tubes at a predetermined site.

Keywords High-fidelity digital microscope · Germination

T. Nonomura (✉) · T. Sameshima · Y. Matsuda · H. Toyoda
Laboratory of Plant Protection and Biotechnology,
Faculty of Agriculture, Kinki University,
3327-204 Nakamachi, Nara 631-8505, Japan
e-mail: nonomura@nara.kindai.ac.jp

Y. Takikawa
Plant Center, Institute of Advanced Technology,
Kinki University, Wakayama 644-0025, Japan

L. Xu
School of Life Science, East China Normal University,
Shanghai 200062, China

K. Kakutani
Pharmaceutical Research and Technology Institute,
Kinki University, Osaka 577-8502, Japan

Introduction

One of the most remarkable morphological changes in powdery mildew conidia is the formation of appressorial germ tubes (Boesewinkel 1980; Braun et al. 2002). These changes are characterized by the initiation of initial germ tubes at terminal, subterminal and lateral positions on the conidium, the subsequent elongation of germ tubes, and appressorial development at the germ tube apex (Cook and Braun 2009), followed by primary haustoria production for the successful establishment of infection. Alternatively, microcyclic conidiogenesis (i.e., conidiophore development and sporulation) may also occur directly on the body

of the germinated conidia following nutrient uptake from the host plant tissues (Kiss et al. 2010).

In our previous paper (Nonomura et al. 2010), appressorial shapes (nonlobed, nipple-shaped and lobed appressoria) of the tomato powdery mildew (*Oidium neolycopersici*) were determined as a result of repeated unsuccessful attempts at penetration by the infection pegs generated from the appressoria. In addition, germ tube length was shown to be dependent upon the timing of the contact of the germ tube tip with the leaf surface (Nonomura et al. 2009). Previous observations of *O. neolycopersici* conidia infecting tomato leaves showed that appressorial germ tubes originated from subterminal (Whipps et al. 1998; Lebeda and Mieslerová 1999; Jones et al. 2000, 2001; Kiss et al. 2001; Matsuda et al. 2001; Kashimoto et al. 2003; Mieslerová et al. 2004) and terminal (Mlíčkova et al. 2004) positions on the conidium. These observations suggested that the site of emergence of germ tubes in tomato powdery mildew conidia could change and be determined after the conidia had made contact with the leaves, as found for barley powdery mildew (Wright et al. 2000). In the system of powdery mildew identification proposed recently by Cook and Braun (2009), the site of conidial germination is a very important factor. From this point of view, in the present study, we attempted to clarify whether the tomato powdery mildew conidia initiate germ tubes at a predetermined site or determine the site after they contact the leaves; we used highly germinative *O. neolycopersici* conidia collected from conidial pseudochains on conidiophores (Nonomura et al. 2009), and examined them with a high-fidelity digital microscope. This enables three-dimensional observations of nonfixed conidia inoculated onto leaf or artificial surfaces (Matsuda et al. 2005b; Nonomura et al. 2010).

Materials and methods

Plant and pathogen

Seeds of *Lycopersicon esculentum* Mill, cv. Moneymaker were germinated in vermiculite in a tray and grown in a growth chamber for seven days under the following environmental conditions: $25 \pm 0.5°C$, 95–100% relative humidity (RH), and continuous illumination of 3500 lux provided by fluorescent lamps. Once the cotyledon leaves had unfolded, the plants were transplanted to soil in 15 cm pots and grown in a temperature-controlled glasshouse ($25 \pm 3°C$) for one month before they were used in inoculation experiments.

To maintain the inoculum of *O. neolycopersici* L. Kiss (KTP-01), conidia were dusted onto leaves of fresh two-month-old tomato seedlings (cv. Moneymaker) with a paintbrush (dusting inoculation method) every two weeks, as described by Kashimoto et al. (2003). Moneymaker is highly susceptible to KTP-01 (Matsuda et al. 2005a). Conidia were collected from colonies ten days after inoculation and used for the following experiments.

Germination assay

Inoculated one-month-old seedlings were transferred to the growth chamber. The formation of conidiophores and conidial pseudochains was observed without detaching leaves using a high-fidelity digital microscope KH-2700 (Hirox, Tokyo, Japan), as described by Matsuda et al. (2005b). Conidia in pseudochains were collected after ten days using an electrostatic spore collector probe (Nonomura et al. 2009) held by a manipulator on the digital microscope. In this method, the conidia were electrostatically attracted to the probe without directly touching other conidia on conidiophores (electrostatic spore collection method); the probe tip was moved slowly toward colonies on leaves while viewing the attraction of the conidia under the digital microscope. In the present experiment, the amount of electricity at the tip surface was 5×10^{-1} nanocoulomb (nC) under an electrostatic voltage of 5.0 kV at 100 μm (distance between the probe tip and conidiophore apex). After collection, the probe was detached from the collector and incubated for 6 h under optimal germination conditions for KTP-01 conidia: $25 \pm 0.5°C$, 95–100% RH, and continuous illumination of 3500 lux (Kashimoto et al. 2003).

Additionally, as a model of natural infection, conidia and/or pseudochains on tomato leaves (at ten days after inoculation) were blown towards healthy detached leaves placed 20 cm from the inoculum plants, using the air blower (at 1.0–1.5 m/s) to transfer conidia without touching them directly (spore blowing method). Also, conidia were similarly blown onto an artificial membrane (Parafilm-coated glass slides, hydrophobic surface) or onto agar plates (1% w/v agar) (hydrophilic surface) in Petri dishes. Inoculated leaves, slides and plates were incubated for 6 h under the conditions described earlier.

During incubation, the conidia were examined to determine the positions of their germination sites, and these were plotted on maps of three conidia demarcating six zones on the basis of the parts touching the substrata: terminal, subterminal and lateral parts of the conidia (see Fig. 3). Twenty conidia were observed in each electrostatic conidium attraction or blowing conidium transfer experiment, and the experiments were repeated three times; thus, in total, 60 conidia from three replications were plotted.

For conidia in which germ tubes were not detected, their hidden sides were surveyed by rotating the probe under the digital microscope. Similarly, germ tube-free conidia on

leaves or artificial surfaces were gently reversed using a glass needle connected to the manipulator to check for germ tube production on their hidden sides.

Results

Powdery mildew pustules became visible on leaves four days after inoculation, and the first mature conidia were detected on conidiophores seven days after inoculation. During the following three days, the powdery mildew successively stacked mature conidia on conidiophores in a pseudochain formation, without releasing the first mature conidia. At 11 days after inoculation, some conidiophores stacked a maximum of four mature conidia which then fell off conidiophores on leaves as four-conidia pseudochains. In the present study, we therefore collected pseudochain conidia from conidiophores at ten days after inoculation. At this stage, the conidiophores produced differently developed pseudochains in the colonies; some carried mature conidia singly, and others had pseudochains with two or three mature conidia. All of these conidia were fully mature, independent of each other, and were collected as described above.

Table 1 shows the germination rates of conidia on the probe, tomato leaves, membrane-coated glass slides, and agar plates. Despite differences in spore collection and substrata, conidia initially germinated 2–3 h after incubation. The number of germinated conidia increased rapidly and reached a maximum at 6 h after incubation. The methods used for spore collection and the conidium-contacting substrata were not found to be detrimental to the successful germination of conidia. Figure 1 provides some digital micrographs of germinating conidia on the spore collector probe (Fig. 1a, b), tomato leaves (Fig. 1c, d), Parafilm-coated glass slides (Fig. 1e, f), and agar plates (Fig. 1g, h). Clearly, some conidia initiated germ tubes where they touched the substratum and others where they did not contact it. These results indicate that the site of
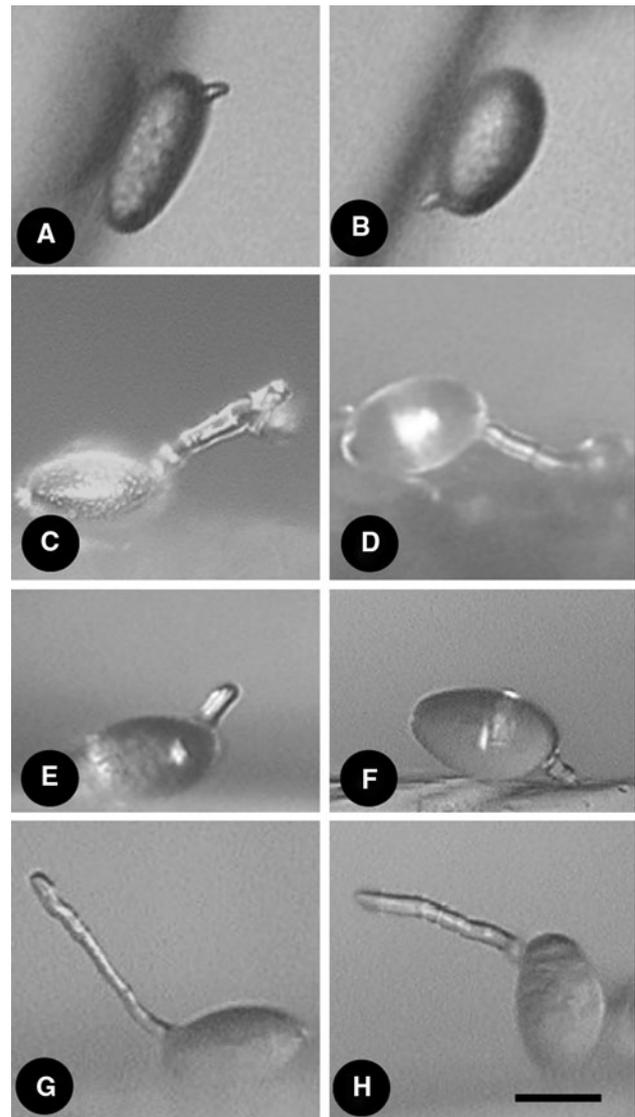

Fig. 1 Digital micrographs of germinating conidia of *O. neolycopersici* KTP-01. **a**, **b** Conidia on the spore collector probe (after 3 h incubation). **c**, **d** Conidia on tomato leaves (after 6 h). **e**, **f** Conidia on Parafilm-coated glass slides (after 3 h). **g**, **h** Conidia on agar plates (after 6 h). *Bar* 10 μm

Table 1 Germination percentages for conidia of *Oidium neolycopersici* KTP-01 on different substrata

Substrata used	Hours after inoculation					
	2	3	4	5	6	7
Spore collector probe	0 a	2.0 ± 1.2 b	22.7 ± 6.1 c	92.0 ± 4.0 d	96.7 ± 1.2 d	98.7 ± 1.2 d
Tomato leaves	0 a	3.2 ± 2.3 b	25.3 ± 1.2 c	91.3 ± 1.2 d	94.7 ± 4.2 d	96.0 ± 2.0 d
Parafilm-coated glass slides	0 a	3.6 ± 3.5 b	26.7 ± 1.2 c	91.3 ± 4.6 d	96.7 ± 2.3 d	99.3 ± 1.2 d
Agar plates	0 a	2.4 ± 2.0 b	23.3 ± 8.1 c	92.7 ± 4.2 d	96.0 ± 2.0 d	98.7 ± 1.2 d

Data are given as means and standard deviations of three replications

Different letters next the mean values indicate a significant difference ($p < 0.05$) according to Tukey's method
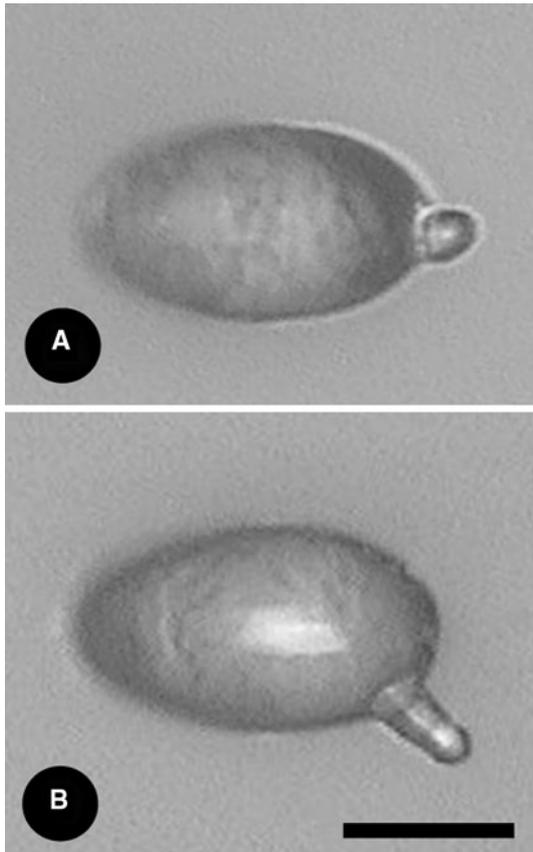
Fig. 2 Both sides of the same conidium of *O. neolycopersici* KTP-01 photographed 6 h after inoculation on a Parafilm-coated glass slide. An apparently terminal tube (**a**) was confirmed to be subterminal (**b**). *Bar* 10 μm

germ tube initiation had no direct relation to the side of contact with the substrata.

As noted earlier, almost all of the collected conidia could initiate germ tubes. In fact, conidia that produced no germ tubes on their observed side were shown to have them on the hidden side when reversed using a micromanipulator. Moreover, on both the probe and the tomato leaves, we observed conidia whose germ tubes seemed to be terminal (Fig. 2a). However, all of these were actually found to be subterminal (Fig. 2b). Thus, the present methods were effective for precisely determining the germination site in conidia.

Although the conidia used were highly germinative, the position of germ tube initiation seemed to vary. To clarify the relationship between the germ tube emergence site and the touched sides of the conidia, we recorded the germination site in each conidium and plotted the sites of all tested conidia on the same conidium-body maps demarcated with latitudinal lines (Fig. 3). The data were very clear, and the germination sites definitely assembled within two latitudinal zones, but not within either terminal or lateral (equatorial) zones, whether the conidia rested on

their ends (left column in Fig. 3), shoulders (middle column) or side (right column). Moreover, the distribution of sites was identical whether conidia were on the probe (Fig. 3a), tomato leaves (Fig. 3b) or artificial surfaces (Fig. 3c).

Discussion

An exogenous signal causing germination in powdery mildew conidia is their first contact with external biotic or abiotic substrata (Nielsen et al. 2000). This contact stimulus could also be produced by directly touching mature conidia on conidiophores with a glass needle (Wright et al. 2000; Oichi et al. 2006) or with an electrified insulator probe (Moriura et al. 2006; Nonomura et al. 2009). Interestingly, Wright et al. (2000) reported that the germination of barley powdery mildew conidia was triggered by touching them with a glass needle, and that the primary germ tube emerged from the touched side of the conidia. This type of signal recognition by conidia seemed to direct the elongation of the germ tube towards the leaf surface. In contrast, tomato powdery mildew conidia were shown to initiate germ tubes from both touched and untouched sides of conidia, suggesting that tomato powdery mildew conidia possess a predetermined site for this initiation (Nonomura et al. 2009). Hence, the major aim of the present study was to investigate this divergent germination phenomenon by means of intensive digital microscopic observations of germination.

In the present study, mature conidia of KTP-01 in pseudochains on tomato leaves were collected using an electrostatic spore collector probe because of their high viability (Nonomura et al. 2009). In addition, this method of collection under a digital microscope was effective for precisely determining the first contact stimulus of conidia at the time of attraction. Indeed, the findings confirmed that almost all touched conidia successfully germinated. More importantly, the present digital microscopic observation combined with the electrostatic spore collection enabled us to survey every side of the attracted conidia by rotating the probe. This approach was useful for surveying the initiation of germ tubes on hidden sides of conidia. We took photographs (see Fig. 2a) of conidia whose germ tubes seemed to emerge terminally. Actually, many studies have presented scanning electron micrographs (Jones et al. 2000, 2001; Matsuda et al. 2001; Kashimoto et al. 2003) and light micrographs (Whipps et al. 1998; Lebeda and Mieslerová 1999; Kiss et al. 2001; Mieslerová et al. 2004; Mlíčkova et al. 2004) of germinating and/or appressorium-forming conidia of *O. neolycopersici*. Of these reports, only the photographs provided by Mlíčkova et al. (2004) showed conidia apparently producing appressorial germ tubes

Fig. 3 Germination sites of
O. neolycopersici KTP-01
conidia plotted on demarcated
maps of conidia when viewed
on **a** probes of the electrostatic
spore collector, **b** tomato leaves,
c artificial surfaces. In **c**, 120
spots obtained from the conidia
on the Parafilm-coated glass
sides (hydrophobic surface)
(*dark circles*) and agar plates
(hydrophilic surface) (*open
circles*) were plotted on the
same conidial map
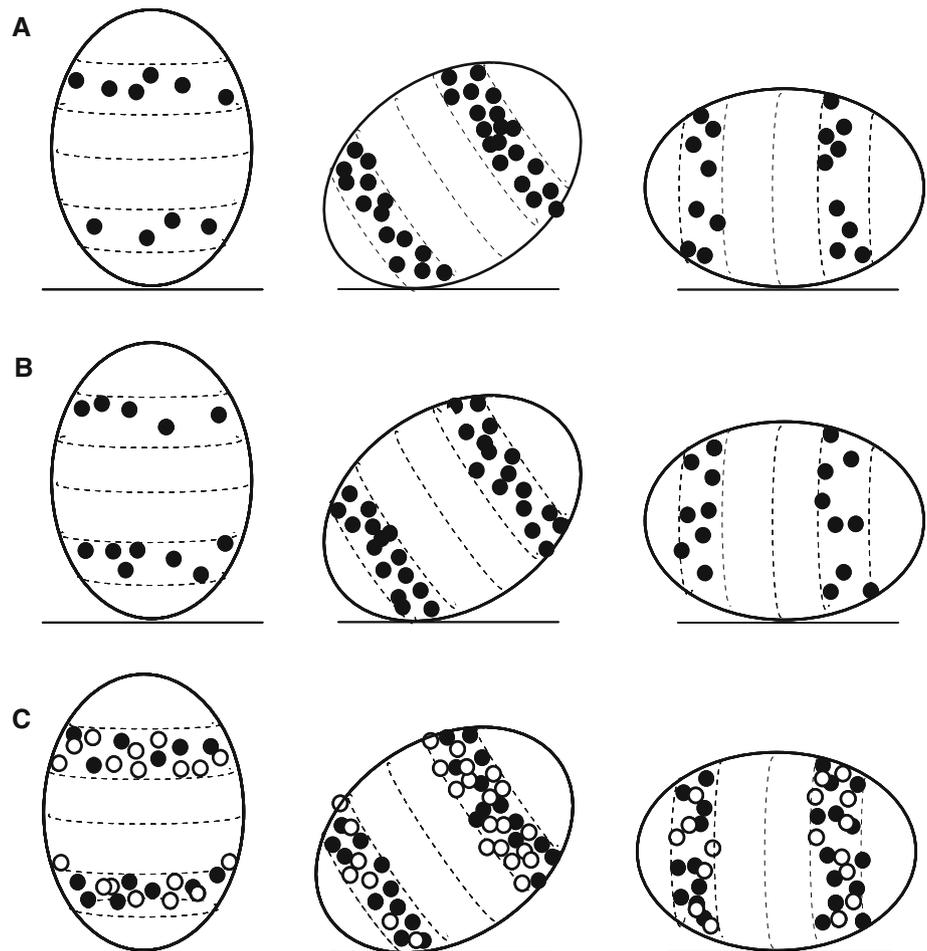
terminally, with the remaining papers reporting subterminal germination. The sites (terminal, subterminal and lateral) of germ tube emergence are important in the generic identification of powdery mildews on the basis of anamorphic characteristics (Cook and Braun 2009). In the present study, we therefore carefully checked the precise position of germ tube emergence in *O. neolycopersici* under a digital microscope and finally confirmed that all of these conidia initiated germ tubes subterminally, not terminally (see Fig. 2b). Hence, the present observation method effectively avoided misjudging the site of germ tube emergence in indistinct images.

Pseudochain conidia were easily released from conidiophores by blowing air at more than 1 m/s (Oichi et al. 2006). These conidia were fully mature and independent of each other (Oichi et al. 2006; Nonomura et al. 2009). In the present work, mature conidia on pseudochains were successfully transferred to tomato leaves or artificial surfaces with the air flow. Obviously, the landing of conidia on a surface was the first contact stimulus for initiating germination. We confirmed that these air-blown conidia had the same high germination rate as the conidia on the spore collector probe.

The most important finding in the present work was that the germ tube emergence sites of all of the conidia observed could be plotted on a definite latitudinal zone of the conidia, so that *O. neolycopersici* conidia possess a single predetermined zone for germ tube initiation, irrespective of their orientations after landing on the substrata. The interpretation that conidia produce germ tubes within the same zone is confirmed by our previous work (Nonomura et al. 2009), whereby germ tubes of KTP-01 conidia were shown to frequently elongate upward and fail to develop appressoria because their tips did not touch the leaf surface. In conventional light microscopic observations, in which infected plant leaves were fixed, decolored and stained in order to observe infecting powdery mildew conidia (Kashimoto et al. 2003), only conidia that successfully produced appressoria (i.e., germ tubes elongating downwards) remained on the leaf surface, since those with germ tubes elongating upwards (without appressoria) were carried away from the leaves during chemical treatments,

and were therefore missed in these observations. In this sense, the proposal of Cook and Braun (2009)—who actually proposed direct observations of conidia on an artificial hydrophobic surface to determine their germination pattern—is the correct method for establishing all patterns of germination in the tested conidia. Also, in the present study, we revealed that KTP-01 conidia show positive germination on artificial hydrophobic and hydrophilic surfaces as well as on host leaves.

Obviously, a predetermined germ tube emergence site for KTP-01 conidia seems to be less appropriate for the purposes of infection, because the germ tubes arise in different and often inappropriate directions on the host leaves (Nonomura et al. 2009). From this perspective, *O. neolycopersici* may be inferior to barley powdery mildew, where conidia exhibit rational postdetermination of germination (Wright et al. 2000). In our opinion, the manner of conidial germination in powdery mildews could be of evolutionary importance, especially in the context of establishing effective infection. Hence, we conclude that the present approach provides an experimental tool for analyzing this aspect.

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