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An Avirulent Tomato Powdery Mildew Isolate Induces Localized Acquired Resistance to a Virulent Isolate in a Spatiotemporal Manner

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Hypersensitive response (HR) of plant cells to the attack of pathogens induces resistance to subsequent attacks by a broad spectrum of pathogens, leading to acquired resistance. In this study, we characterized the localized acquired resistance (LAR) in the epidermal cells of tomato. First, we report the discovery of a new isolate of tomato powdery mildew occurring in Japan, KTP-02, which has a different virulence spectrum compared with the previously-characterized isolate, KTP-01. Using these two isolates, we investigated LAR phenomenon in the epidermal cells of tomato plants carrying the *Ol-4* resistance gene. *Ol-4* encodes a nucleotide-binding site leucine-rich repeat protein that triggers HR in the epidermal cells in response to KTP-01 but not KTP-02. We mounted a single conidium of KTP-01 on a single tomato epidermal cell and then monitored the progress of HR in that cell by live microscopy. Once HR occurred in that cell, we mounted a single conidium of KTP-02 on cells adjacent to or at one-cell distance from the first challenged cells, in different time points. With a digital microscope, we consecutively tracked the progress of HR (i.e., induction of LAR) in those cells. Results showed that, in tomato plants carrying the *Ol-4* gene, HR to KTP-01 results in induction of HR in the adjacent epidermal cells challenged with KTP-02. Our results show that LAR can be triggered only in adjacent cell layer and lasts 24 to 48 h after HR occurred in the first cell. We did not observe the reverse phenomenon, induced susceptibility to KTP-01 by KTP-02. Altogether, we report an advanced technique for investigating LAR phenomena, and provide data on spatiotemporal characteristics of LAR in tomato epidermal cells.

Acquired resistance in plants has been reported in two forms: localized acquired resistance (LAR) (Ross 1961a; Yarwood 1960) and systemic acquired resistance (SAR) (Ross 1961b). It is known that those pathogens that cause hypersensitive response (HR) on plant tissues induce resistance to subsequent attacks by a broad spectrum of pathogens in distant tissues, leading to SAR induction (Durrant and Dong 2004), or in cellular layers sur-

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rounding the HR, resulting in LAR induction (Costet et al. 1999). Recent evidence suggests that HR is not necessary for the induction of acquired resistance but the events leading to HR probably are required (Hammerschmidt 2009).

It is not clear yet whether LAR and SAR are two facets of the same basic mechanism or whether two separate mechanisms are involved in these two phenomena (Ross 1961b; Ryals et al. 1994). Comparison of the gene expression pattern in LAR and SAR tissues showed that the expression of defense response genes, including *pathogenesis-related* (*PR*) genes, is much higher in LAR than that in SAR tissues (Cordelier et al. 2003). Also, activity of *O*-methyltransferases, enzymes of the phenylpropanoid pathway that is one of the important pathways for defense responses (Dixon 2001), increased in LAR but not in SAR (Cordelier et al. 2003). These comparisons suggest that, even if LAR and SAR are based on the same mechanism, the amplitude of the induced resistance is higher in LAR. Contrasted with SAR, systemic induced susceptibility (SIS) has been also reported; virulent strains of the bacterial phytopathogen *Pseudomonas syringae* induced systemic susceptibility to secondary *P. syringae* infections in the host plant *Arabidopsis thaliana* (Cui et al. 2005).

Oidium neolycopersici is a highly polyphagous fungus, causing powdery white spots on the adaxial tomato leaf surface (Jones et al. 2001). Since the first report in 1986 in the Netherlands, the presence of different *O. neolycopersici* races has been reported in different parts of the world (Bai et al. 2005; Ivic et al. 2009; Yolageldi et al. 2008). The *O. neolycopersici* isolates identified in the Czech Republic and in Japan, *O. neolycopersici* Cz and KTP-01, respectively, showed virulence spectra different from other isolates (Bai et al. 2005).

Tomato response to *O. neolycopersici* is conditioned by several resistance genes, including *Ol-1* and *Ol-4* (Bai et al. 2003). It has been reported that HR is the main resistance mechanism of tomato to *O. neolycopersici* (Huang et al. 1998). Particularly, two different forms of HR have been observed in tomato response to *O. neolycopersici*. Single-cell HR (Bai et al. 2005; Huang et al. 1998), also defined as fast HR (Li et al. 2007), happens in the presence of *Ol-4*. The *Ol-4* gene is derived from *Solanum peruvianum* LA2172 and is homologous to the *Mi-1* gene encoding a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein (Seifi et al. 2011). The resistance conferred by *Ol-4* is complete and race-specific, because near-isogenic line (NIL)-Ol-4 is immune to all tested *O. neolycopersici* isolates except *O. neolycopersici* Cz (Bai et al. 2005). On the other hand, multiple-cell HR (Bai et al. 2005; Huang et al. 1998), also described as slow HR (Li et al. 2007), occurs in

tomato plants carrying the *Ol-1* gene. The *Ol-1* gene was identified from the wild tomato species *S. habrochaites* G1.1560 (Huang et al. 2000) and mapped on the long arm of chromosome 6 of tomato (Bai et al. 2005). Resistance mediated by *Ol-1* is incomplete and race-nonspecific, because NIL-Ol-1 showed variable levels of resistance to all different *O. neolycopersici* isolates thatwere tested (Bai et al. 2005). HR is the hallmark of resistance (*R*)-gene-mediated resistance to biotrophic pathogens (Nimchuk et al. 2003). *Ol-1* is likely not an *R* gene (Seifi 2011) and the form of cell death in NIL-Ol-1 resembles autophagic programmed cell death (Love et al. 2008).

In this study, we report the discovery of a new *O. neolycopersici* isolate from Japan, KTP-02. We show that KTP-02 resembles the previously reported isolate, KTP-01, for some mycological characteristics but differs from KTP-01 in its virulence spectrum. Subsequently, we describe the LAR phenomenon at the single-cell level in tomato epidermal cells by using the different responses of NIL-Ol-4 to both KTP-01 and KTP-02.

RESULTS

KTP-02 is a new *O. neolycopersici* isolate that suppresses HR associated with the *Ol-4* function.

KTP-01 is a Japanese isolate that is virulent on the universally susceptible *S. lycopersicum* 'Moneymaker' (MM) tomato as well as on resistant 'Grace' that was bred in The Netherlands (Kashimoto et al. 2003). In many disease tests with KTP-01 in Japan in 2005 and 2006, NIL-Ol-4 (carrying the *Ol-4* gene in MM background) and *S. peruvianum* LA2172 showed complete resistance to this isolate by triggering HR in all cells invaded by the fungus (Fig. 1A and C). HR occurred at a single-cell level, preventing any further elongation of the secondary hyphae. In contrast, delayed cell death occurred in both NIL-Ol-1 (carrying the *Ol-1* gene in MM background) and *S. habrochaites* G1.1560 (Fig. 1B and D), leading to incomplete resistance. During the maintenance of *S. peruvianum* LA2172 in Japan in 2009, we discovered the occurrence of an *O. neolycopersici* isolate capable of infecting this species by producing abundant progeny conidia. We isolated and maintained it via single conidium as a new isolate, KTP-02. To confirm the virulence of KTP-02 to the *Ol-4* gene, we compared the response of NIL-Ol-4 with both KTP-01 and KTP-02 isolates (Supplementary Table S1). MM was susceptible to both KTP-01 and KTP-02 isolates. NIL-Ol-4 was completely resistant to KTP-01 by triggering HR. In contrast, NIL-Ol-4 was as susceptible as MM in response to KTP-02 and no HR was observed in cells attacked by KTP-02.

To characterize KTP-02, we compared it with KTP-01 based on the internal transcribed spacer (ITS) sequence of the ribosomal DNA (rDNA) and morphological characteristics. ITS has been used successfully for phylogenetic studies of tomato powdery mildews (Kiss et al. 2001). The ITS sequence analysis revealed that KTP-02 belongs to *O. neolycopersici* species because the ITS sequence was identical to that of KTP-01 and other *O. neolycopersici* isolates (Fig. 2). Morphologically, the size of the conidium, conidiophore, and foot cells and the arrangement of conidia on conidiophores were the same in KTP-01 and KTP-02 (Table 1). Further, we compared the germination rate of conidia of KTP-01 and KTP-02 under different temperature and light-intensity conditions. No difference was found under tested conditions in the germination rate between these two isolates. It is worth mentioning that the optimum temperature for conidial germination was 25°C. Overall, there is no effect of light intensity on the germination rate in either of these isolates, although KTP-02 conidia germinated faster under low light intensity (50 lx) at the 2-h time point (Supplementary Fig. S1).

KTP-01 inoculation on single epidermal cells of NIL-Ol-4 induces HR to KTP-02 in the adjacent cells.

Having two *O. neolycopersici* isolates with different virulence spectra, we studied the acquired resistance possibly induced by avirulent strain (KTP-01) against the virulent one (KTP-02) in single-cell level in NIL-Ol-4, by using a digital microscope. In principle, a conidium of KTP-01 was mounted on a single epidermal cell of NIL-Ol-4. This cell underwent HR at 18 h after inoculation (Fig. 3). Then a conidium of KTP-02 was mounted on a single cell adjacent to the first cell in different time points after occurrence of HR in the first cell. If there is LAR induced by KTP-01, we expected to observe complete HR to KTP-02 in the adjacent cells. Results showed that in NIL-Ol-4, HR was induced by KTP-02 in cells adjacent to the cell where HR occurred in response to the challenge of KTP-01 (Fig. 4, and the supplementary video).

This induced LAR was observed at 0.5, 12, and 24 h but not at 48 h after occurrence of HR in the first cell (18 h after mounting KTP-01 conidium). Also, we tested the possibility of induction of resistance in distal cells. We repeated the previous experiment and, this time, we mounted KTP-02 conidia on one-cell-distant cells in relation to the cell thatwas challenged with KTP-01 conidium. No HR was observed in one-cell-distant cells challenged with KTP-02 (Fig. 3B), suggesting that there is no induced resistance in those cells.

KTP-02 inoculation does not induce susceptibility to KTP-01.

In order to test the possibility of SIS induction in tomato epidermal cells in response to *O. neolycopersici*, we performed another experiment. We first mounted KTP-02 conidia on a single epidermal cell of NIL-Ol-4. Then, in a time series (0.5,
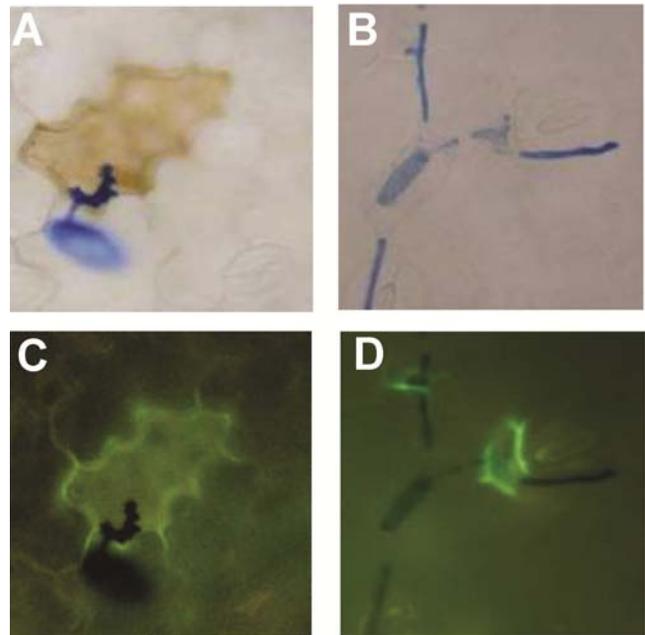
Fig. 1. Death of cells attacked by *Oidium neolycopersici* KTP-01. **A,** Tomato epidermal cell, which was attacked by infection pegs of an appressorium of KTP-01 (stained in blue) on a leaf of *Solanum peruvianum* LA2172, committed hypersensitive response (HR). HR occurred at a single-cell level and prevented any further elongation of the secondary hyphae. **B,** Tomato epidermal cells of *S. habrochaites* G1.1560 responded to the attack of KTP-01 by triggering delayed cell death (DCD). Compared with HR, DCD developed slowly and allowed the fungus to produce secondary hyphae and the penetration of neighboring cells. **C** and **D,** The same as A and B, respectively, but under the fluorescent microscope. Samples for microscopy were taken 10 days after inoculation.

12, 24, and 48 h after appressorial formation of KTP-02), we mounted KTP-01 conidia on cells adjacent to the first challenged cell. If there was an SIS phenomenon induced by KTP-02, we expected to observe susceptibility to KTP-01 in the adjacent cells (i.e., no HR). However, in this experiment, all the cells challenged with KTP-01 underwent HR, implying that they were resistant to KTP-01. Therefore, there was no induction of susceptibility to avirulent strain KTP-01 by the virulent one, KTP-02.

DISCUSSION

Here, we report a new isolate of *O. neolycopersici*, KTP-02, occurring in Japan. The morphological and physiological characteristics of this isolate are similar to those of KTP-01. However, the virulence spectrum of KTP-02 is different from KTP-01 because complete resistance mediated by *Ol-4* is effective to KTP-01 but not to KTP-02. The *O. neolycopersici* Cz isolate also showed a similar virulence spectrum (Bai et al. 2005), which may suggest that KTP-02 and *O. neolycopersici* Cz belong to the same race. *Ol-4*, which encodes an NBS-LRR protein, triggers HR in response to KTP-01 but not to KTP-02. In *R*-gene-mediated resistance to biotrophic pathogens, HR occurs as the result of resistance and avirulence recognition (Nimchuk et al. 2003). The fact that no HR occurred in NIL-

Ol-4 cells invaded by KTP-02 suggests that *Ol-4* cannot recognize the effectors of KTP-02. It would be interesting to investigate the differences in the effectors of KTP-02 and KTP-01.

In the next step, we investigated the LAR phenomenon in epidermal cells of tomato. We showed that challenging the epidermal cells of NIL-Ol-4 with KTP-01 induces resistance to KTP-02 in one cell layer adjacent to the challenged cell (Fig. 4) and within 48 h after observing HR in that cell. This fact might imply that some molecules were probably acting as the mobile signals triggering the acquired resistance, similar to signal molecules triggering SAR. It is shown that the signal complex of SAR consists of molecules such as azelaic acid in combination with lipid-derived molecules or lipopeptides (Parker 2009). Symplastic movement of such molecules would be determined by permeability of plasmodesmata of the neighboring cells. Plasmodesmatal permeability could change rapidly; for instance, even slight increases in cytoplasmic calcium concentration cause closure of plasmodesmata in a few seconds (Holdaway-Clarke et al. 2000; Tucker and Boss 1996). It has been reported that wounding results in callose deposition in plasmodesmata, plugging the neck region of plasmodesmata (Hughes and Gunning 1980). Such changes in plasmodesmatal permeability and, therefore, the symplastic movement of signal molecules could be the reason why LAR mediated by challenging the epidermal cells with KTP-01 lasts
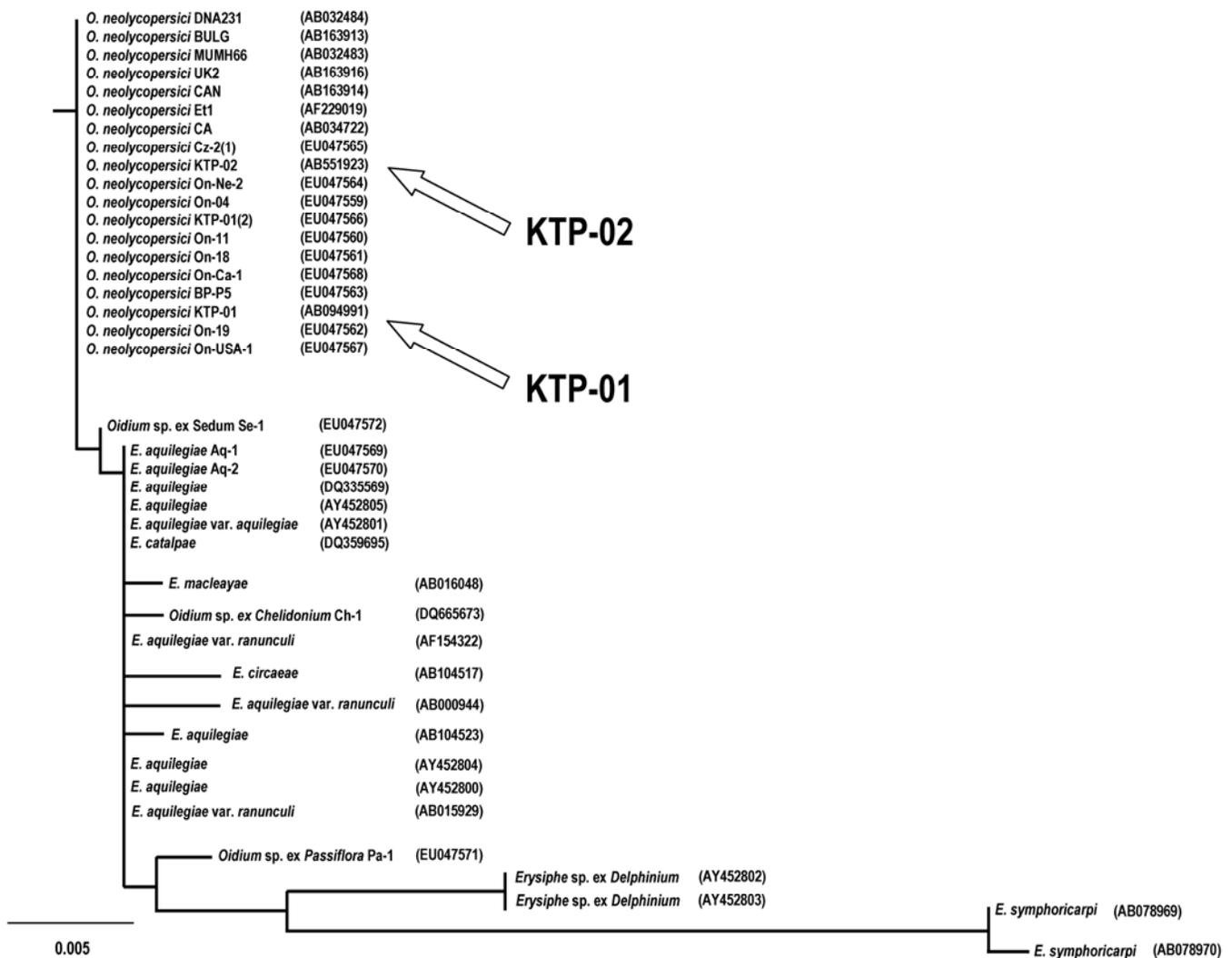

Fig. 2. Phylogenetic tree of different powdery mildew species based on the internal transcribed spacer sequence. The newly discovered powdery mildew isolate, KTP-02, belongs to the *Oidium neolycopersici* clade.

between 24 and 48 h after HR occurrence in the first challenged cell (Fig. 3).

Because there was an example of induced susceptibility in contrast to induced resistance (Cui et al. 2005), we tested the possibility of this phenomenon in our pathosystem. We first challenged a single epidermal cell with KTP-02 and then evaluated the response of adjacent cells to KTP-01. Our results showed that there is no SIS phenomenon in our pathosystem, suggesting that SIS is not a common phenomenon in plant–pathogen interactions. SIS reported previously in the *P. syringae*–*Arabidopsis* pathosystem is probably a consequence of the production of the phytotoxin coronatine, a jasmonic acid mimic, leading to a mutually antagonistic interaction between the salicylic acid and jasmonic acid signaling pathways (Cui et al. 2005).

In summary, we reported here the discovery of a new Japanese isolate of *O. neolycopersici* and described the spatiotemporal kinetic of LAR in tomato epidermal cells. To fully understand the spatiotemporal kinetic of LAR and its limitation on cells adjacent to the infection site, additional molecular analyses of the HR induction by means of appropriated markers are necessary. At this moment, the limiting factor of this molecular analysis is the lack of method to isolate intracellular contents of a single tomato epidermal cell. Currently, we are working on micropipette-manipulation techniques (Fujita et al. 2004) and hope in the near future to monitor the expression of molecular markers for HR in intracellular contents of a single epidermal cell attacked by a fungal appressorium. Further, by performing single-cell transcript and protein profiling, our single-cell studies will help to unravel mobile signals leading to LAR.

MATERIALS AND METHODS

Plant material.

NIL harboring the *Ol-1* and *Ol-4* genes (Bai et al. 2005) in the genetic background of *S. lycopersicum* MM were used. *S. habrochaites* G1.1560 (donor of *Ol-1*) and *S. peruvianum* LA2172 (donor of *Ol-4*) were also included.

Fungal material.

Two Japanese isolates (KTP-01 and KTP-02) of *O. neolycopersici* were used in the present study. KTP-01 (Kashimoto et al. 2003) is a laboratory stock and used routinely in Japan to test resistance or susceptibility of tomato plants (Matsuda et al. 2005). KTP-02 was isolated from the diseased leaves of *S. peruvianum* LA2172 (resistant to KTP-01) and identified as *O. neolycopersici* on the basis of its molecular and morphological characteristics in the present study.

KTP-01 and KTP-02 were maintained by inoculating their conidia into cotyledons of 10-day-old seedlings of MM and NIL-Ol-4, respectively. The conidia were collected from co-

Table 1. Comparison of morphological characteristics between KTP-01 and KTP-02

Characteristic	KTP-01	KTP-02	Significance
Conidium (μm)	$31.8 \pm 2.5 \times 17.7 \pm 1.5$	$31.2 \pm 4.2 \times 17.1 \pm 1.6$	None
Conidiophore (μm)	96.9 ± 13.9	107.2 ± 12.8	None
Foot cell (μm)	37.7 ± 8.6	40.3 ± 7.3	None
Conidia on conidiophore	Singly, pseudochains of maximum of four conidia	Singly, pseudochains of maximum of four conidia	…
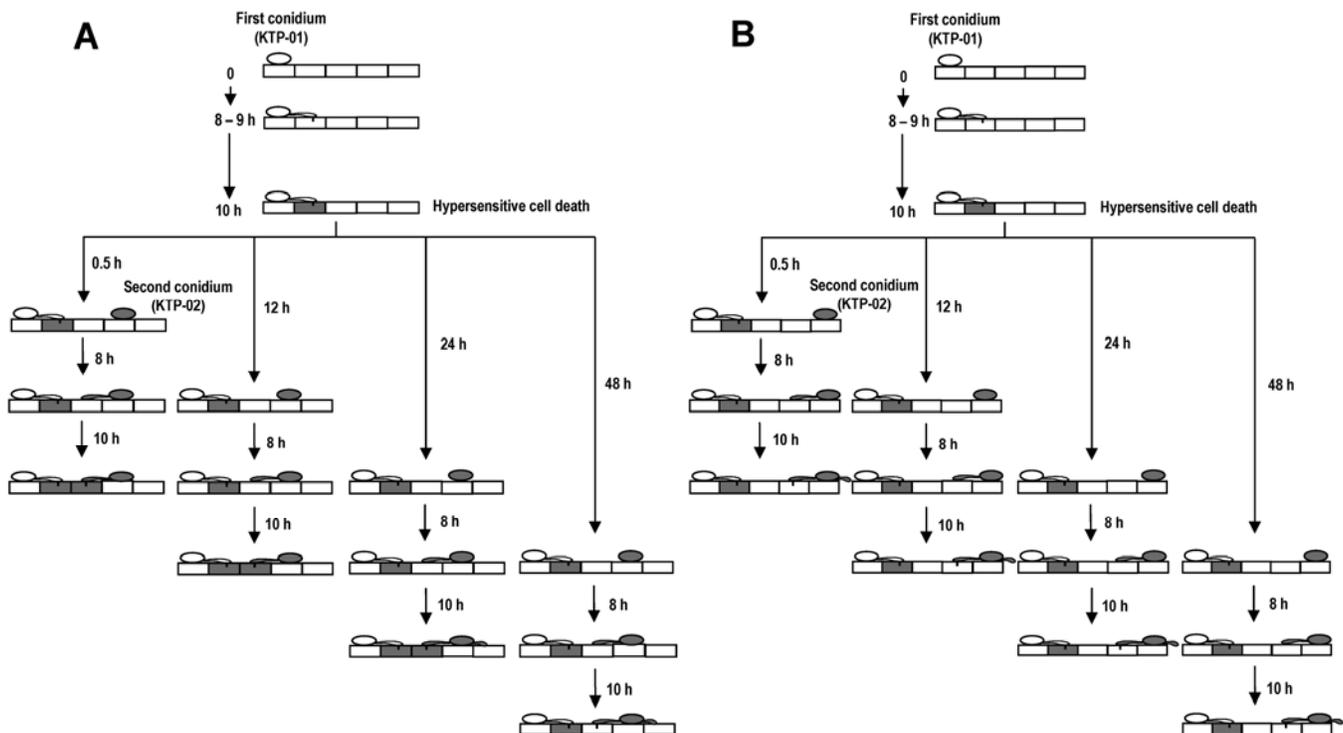
Fig. 3. Localized acquired resistance in epidermal cells of the tomato near-isogenic line Ol-4. Conidium of KTP-01 was mounted on a single epidermal cell. This cell underwent hypersensitive response (HR) (gray colored cells) in response to KTP-01 after 18 h. Upon occurrence of HR in this cell, a conidium of KTP-02 was mounted **A,** on a single cell adjacent to the first cell or **B,** in a one-cell-distant cell in a time series of 0.5, 12, 24, and 48 h after occurrence of HR in the first cell. For each time point, 15 conidia were mounted, and then the occurrence of HR in the second cell was monitored. In the adjacent cell (A), the percentage of the cells that showed HR was 74, 67, 40, and 0% at the 0.5-, 12-, 24-, and 48-h time points, respectively. However, in one-cell-distant cells (B), no occurrence of HR was observed.

nidiophores on infected leaves and transferred to cotyledons (one conidium per cotyledon) using a glass needle installed with a micromanipulator and a microscope KH-2700 (Nonomura et al. 2009). Inoculated cotyledonal seedlings were placed for 2 weeks in different growth chambers (25 ± 1°C under continuous light of 4,000 lx).

Disease test.

To prepare abundant conidia used for inoculation, KTP-01 and KTP-02 conidia on the infected cotyledons were collected using an electrostatic spore collection probe (Nonomura et al. 2009), suspended in water, and sprayed onto leaves of MM and NIL-Ol-4, respectively. KTP-01-inoculated MM plants were isolated in a spore-exclusion box (1.5 by 1 by 2 m³) whose roof and lateral faces were furnished with an electrostatic screen (Matsuda et al. 2006) in a temperature-controlled greenhouse (25 ± 3°C) in order to prevent air-carried conidia of KTP-02 from contaminating KTP-01. Successful prevention was confirmed by showing no appearance of KTP-02 colonies on leaves of LA2172 plants that were placed in the box as an indicator. KTP-02-inoculated NIL-Ol-4 plants were placed in a different temperature-controlled greenhouse (25 ± 3°C).

Inoculation was conducted according to the method described before (Bai et al. 2005); conidia (2 × 10⁴ conidia/ml) of KTP-01 and KTP-02 were collected from infected leaves of MM and NIL-Ol-4, respectively, and suspended in water to produce inoculum. The inoculum was evenly sprayed on the 4-week-old plants. Plants were evaluated for their resistance or susceptibility by disease index (DI). Plants showing no sporulation were scored as resistant (DI = 0). Plants with a high level of sporulation, comparable with that on the susceptible control MM plants, were considered to be susceptible (DI = 3).

Sequencing of the rDNA ITS region.

To determine the rDNA ITS sequence of isolate KTP-02, conidia were collected from inoculated tomato leaves and DNA was extracted using a Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was car-
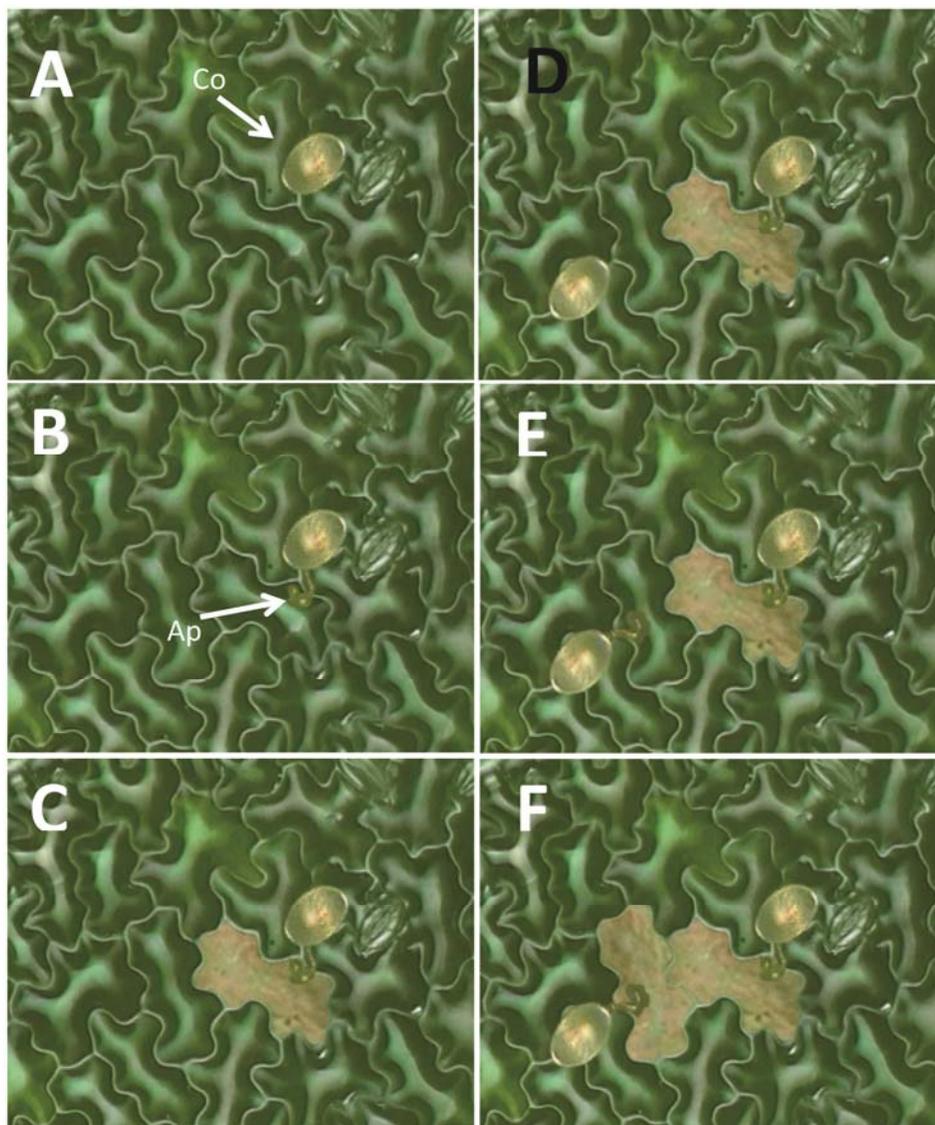
Fig. 4. Consecutive digital micrographs illustrating the induction of localized acquired resistance in tomato epidermal cells. **A,** A conidium (Co) of *Oidium neolycopersici* isolate KTP-01 was mounted on a single epidermal cell of near-isogenic line Ol-4 carrying the *Ol-4* resistance gene. **B,** The conidium germinated and produced an appressorium (Ap, approximately 8 to 9 h after mounting the conidium) for penetration of the epidermal cell. **C,** Hypersensitive response (HR) occurred in the epidermal cell (starting from approximately 18 h after mounting the conidium) as the result of the function of the *Ol-4* gene. **D,** Upon occurrence of HR, a conidium of *O. neolycopersici* isolate KTP-02 was mounted on the adjacent epidermal cell. **E,** The conidium germinated and produced appressorium for penetration. **F,** HR occurred in the epidermal cell inoculated with KTP-02.

ried out in an iCycler (Bio-Rad, Hercules, CA, U.S.A.) using the ITS1F and ITS4 fungal-specific primers designed by Gardes and Bruns (1993) and White and associates (1990), respectively. PCR was performed according to the protocols described by Szentiványi and associates (2005). The nucleotide sequence of KTP-02 was deposited at the DNA Data Bank of Japan (DDBJ) under the accession number AB551923.

Phylogenetic analysis.

The ITS sequences of different powdery mildews were obtained from the DDBJ database, which were combined with KTP-02 in the phylogenetic analysis. The sequences were initially aligned using CLUSTALW. The FIGTREE v1.3.1 software was used to create distance matrices and then to infer dendrograms using neighbor-joining analysis. The strength of groupings was estimated using bootstrap analysis (Felsenstein 1985) with 1,000 replicates.

Single-cell monitoring
of induced resistance or susceptibility.

Conidia were collected from conidiophores formed on MM and NIL-Ol-4 cotyledons using a probe of an electrostatic spore collector and were transferred to particular sites of test leaves. In principle, a conidium of KTP-01 was mounted on a single epidermal cell of NIL-Ol-4. This cell underwent HR in response to KTP-01 at 18 h after inoculation of the KTP-01 conidium. Upon occurrence of HR in this cell, a conidium of KTP-02 was mounted on a single cell adjacent to or with one-cell distance from the first cell in a time series of 0.5, 12, 24, and 48 h after occurrence of HR in the first cell. For each time point, 15 conidia were mounted. By digital microscopic observation, the dead epidermal cells were detectable without any chemical treatments or staining because these cells collapsed and contrasted with the surrounding noncollapsed cells (Nonomura et al. 2010).

Microscopic observation.

To compare two different forms of cell death, HR and delayed cell death, Japanese isolate KTP-01 (Kashimoto et al. 2003) was applied on *S. habrochaites* G1.1560 and *S. peruvianum* LA2172. Inoculation was conducted by collecting conidia on conidiophores with an electrostatic spore collector and suspending them in water containing Mixpower (10^{-5} diluted) to produce inoculum (Nonomura et al. 2009). Tested leaves (third and fourth true leaves) of 1-month-old plants were spray inoculated by the method described above. At 2 and 3 days postinoculation, five leaflets of the third and fourth true leaves were harvested from five individual plants per genotype. To observe cytological responses in inoculated tomato leaves, the sample leaves were detached from the plants, decolored in a boiling alcoholic lactophenol solution for 1 to 2 min, and stained with aniline blue, according to a previous method (Sameshima et al. 2004). The leaves were observed under an Olympus light and fluorescence microscope (B excitation, B absorption filter, and O-515 barrier filter). More than 10 conidia per leaflet were counted.

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