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Differential colonization and defence responses of resistant and susceptible melon lines infected by *Fusarium oxysporum* race 1·2

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A green fluorescent protein (GFP)-expressing strain of *Fusarium oxysporum* f.sp. *melonis* race 1·2 (FOM 1·2-GFP) was used to visualize infection of a susceptible melon cultivar, Ein Dor (ED). At 1–2 days post-inoculation (d.p.i.), the fungus grew on the root epidermis and adhered to epidermal cell borders. By day 4, the mycelium crossed the cortex and endodermis through narrow pores in cell walls and reached xylem vessels, where it sporulated and produced secondary hyphae that grew upwards. Colonization dynamics of ED seedlings were compared with those of a genetically resistant line, BIZ. FOM 1·2-GFP colonized the resistant plant's vascular system, but the incidence of seedling infection was lower than in ED, suggesting stronger defence responses in BIZ expressed at the pre-xylem stage of infection. Infection of the vascular system of BIZ was slower: at 11 d.p.i., FOM 1·2-GFP only colonized the lower hypocotyl sections of BIZ, whilst the upper hypocotyls of ED were already infected, indicating that BIZ also restricted FOM 1·2 movement in the xylem. The expression patterns of three defence genes were compared between the resistant and susceptible genotypes using real-time PCR. Transcript levels of phenylalanine ammonia lyase (PAL), chitinase (CHI) and hydroperoxide lyase (HPL) were induced to a greater extent in BIZ than in ED. A constitutive two- to fourfold difference between BIZ and ED in the basal levels of all three transcripts was also apparent. Both the constitutive and inducible defence responses could contribute to reduced vascular colonization of the resistant genotype.

Keywords: chitinase, *Cucumis melo*, *Fusarium oxysporum* f.sp. *melonis*, GFP, HPL (hydroperoxide lyase), PAL (phenylalanine ammonia lyase)

Introduction

Fusarium wilt disease of melon (*Cucumis melo*) is caused by the soilborne fungus *Fusarium oxysporum* f.sp. *melonis* (FOM). It is one of the most important diseases of melon worldwide and one of the least controllable, because once the soil is infested, the pathogen can persist in the soil by colonizing non-susceptible hosts and by producing durable chlamydospores (Schippers & van Eck, 1981). Four physiological races (0, 1, 2 and 1·2) of the pathogen have been identified, according to their reactions with differential melon genotypes. Resistance to races 1 and 2 is conferred by single dominant genes, *Fom-2* and *Fom-1*, respectively (Joobeur *et al*., 2004; Brotman *et al*., 2005). Both genes also confer resistance to race 0. Partial resistance to race 1·2 in melon cv. Isabelle was shown to be polygenic (Perchepied *et al*., 2005), whilst in another melon genotype, breeding line BIZ, two recessive genes were shown to confer full resistance (Herman & Perl-Treves, 2007).

Fusarium oxysporum infects the root system, wherein it progresses through the epidermis, cortex and endodermal tissues and penetrates the xylem vessels through the pits. From this stage on, the fungus uses the xylem for upward movement and establishment throughout the plant (Bishop & Cooper, 1983). Whilst in the xylem, the mycelium sporulates, and microconidia are carried upwards by the xylem stream. At vessel ends, conidia germinate and the secondary mycelium penetrates the next vessel. Plant infection by *F. oxysporum* is therefore a complex process that comprises several stages of host–pathogen interaction: recognition of the host roots and adsorption; penetration of hyphae through the different root tissues; penetration and progression in the xylem; and adaptation to the internal plant environment. To be successful, the fungus must overcome different plant

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defence responses at each stage (Beckman, 1987; Beckman & Roberts, 1995; Di Pietro *et al*., 2003; Michielse & Rep, 2009). During the final stage of infection, the fungus secretes lytic enzymes and toxins that lead to disease symptoms, including necrotic lesions, chlorosis and wilting.

The interaction of *F. oxysporum* with different model hosts, such as tomato (Beckman, 1987; Beckman & Roberts, 1995; Recorbet *et al*., 2003), and more recently *Arabidopsis* (Berrocal-Lobo & Molina, 2008; Michielse & Rep, 2009), has been extensively studied. The plant's response to *F. oxysporum* consists of a complex array of anatomical and biochemical responses. PR proteins, reactive oxygen species and phytoalexins are expressed locally and systemically (Beckman, 1987). Ethylene and auxin have been implicated in mediating vascular gel formation (Beckman, 1987; Beckman & Roberts, 1995). In *Arabidopsis*, ethylene, ABA, salicylic acid and jasmonic acid (JA) defence pathways all contributed to defence against *F. oxysporum* (Berrocal-Lobo & Molina, 2008). The multitude and complexity of responses suggest that different species could express distinct responses. The factors that allow successful invasion and eventual wilting in the susceptible host, and the effective barriers and biochemical defences that are mounted in a genetically resistant host remain to be determined. In several crop species, resistant varieties have been identified and genetically characterized, but the defence mechanisms that confer resistance remain elusive. Melon is a valuable vegetable crop in which the genetics of resistance to FOM have been studied and the trait represents a fundamental target in melon breeding (Burger *et al*., 2003). Understanding the physiological and molecular interactions that render a melon plant resistant to this devastating pathogen is of scientific and practical importance.

In this study, a green fluorescent protein (GFP)-expressing strain of FOM race 1·2 was used to visualize the stages of fungal invasion of a susceptible melon cultivar, Ein Dor (ED), an open-pollinated Ananas-type variety susceptible to all FOM races. The dynamics of colonization of ED were then compared with those of BIZ, a breeding line resistant to all four races of FOM. Finally, the expression patterns of a few defence-related genes were compared between resistant and susceptible seedlings of the above genotypes.

Materials and methods

Plant material

The melon genotypes (ED and BIZ) were provided by Zeraim Gedera Ltd., Israel. Seedlings were grown in a growth chamber at 25–28°C with a 16-h photoperiod.

Plasmid construct

Plasmid pSK1019 was kindly provided by Dr Seogchan Kang (Pennsylvania State University, USA). The plasmid contains the *eGFP* gene as a 1·6-kb fragment under the Ch-GPD (glyceraldehyde 3-phosphate dehydrogenase) promoter, cloned between the *Eco*RI and *Hind*III sites of a pBHt2 vector (Mullins *et al*., 2001). The construct was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation (1·5 Kv, 200 ohms, 50 μF).

Fungal transformation

A FOM race 1·2 isolate (FOM 1·2), collected in Israel (Cohen *et al*., 1989), was maintained on potato dextrose agar (PDA; Difco Laboratories) at 25°C. A conidial suspension was obtained by adding sterile water to the cultured plates, mixing the suspension and filtering it through a four-layered gauze pad. Transformation of FOM was carried out as previously described by Mullins *et al*. (2001) with the following modifications: 200 μL mixed bacterial cells and conidia (10^6 conidia per mL) were plated directly onto 12 mL co-cultivation medium, following incubation at 25°C for 48 h. Selection medium (10 mL) containing hygromycin B (100 μg mL^{-1}) and cefotaxime (100 μg mL^{-1}) was overlaid on each plate and incubated at room temperature for 5–7 days. Transformants that developed under selection were isolated and maintained on PDA plates supplemented with hygromycin B (50 μg mL^{-1}). The colony with the highest GFP expression was chosen for further work.

Inoculum preparation

A fungal suspension, started from an agar-plate culture, was grown in an Ehrlenmeyer flask with 250 mL liquid medium (0·5% PDA and 0·5% yeast extract; Difco Laboratories) at 25°C on a rotary shaker at 180 r.p.m., for 5 days. The concentration of conidia in the suspension culture was adjusted to 10^6 conidia per mL for plant inoculation with sterile distilled water (or other concentrations as stated).

Plant inoculation

Seeds of melon genotypes ED and BIZ were germinated in sterilized potting mixture (three parts peat, one part perlite) in 250-mL autoclaved pots, at 24–27°C. After emergence of the first true leaf, plants were carefully removed from the pot and gently swirled in tap water to wash away soil particles. Roots were trimmed to 5 cm in length and immersed in a freshly prepared spore suspension (10^6 conidia per mL) for 3 min. The inoculated seedlings were re-planted, four plants per pot, into a new pot with similar potting mixture. Control plants were similarly trimmed and replanted but not inoculated. Ten-day-old seedlings germinated on sterile Whatman filter paper in Petri dishes were used to monitor the plant-fungus interaction during the first three days after inoculation. After emergence of the first true leaf, a conidial suspension (~200 μL, 10^5 conidia per mL) was pipetted onto the exposed roots.

Microscopic examination

Observations were performed at 1, 2, 3, 4, 6, 11 and 13 days post-inoculation (d.p.i.). Inoculated and control plants were carefully taken out of the pot or Petri dish and gently rinsed in tap water to wash away soil particles and unattached fungus. Cross sections and longitudinal sections of tap root, crown and hypocotyl were hand-sectioned (20–30 μm) and each placed directly on a glass slide in a water drop with a cover slip. Microscopic examinations were performed using an Olympus IX81 confocal scanning laser microscope, with an excitation of 488 nm by Argon laser and detection of the emitted light at 505–525 nm. Transition light images were acquired by differential interference contrast. Images were taken using a Plan Apox60 water immersion lens with numerical aperture of 1·0. In some of the sections, plant cell walls were counter-stained with a drop of propidium iodide (10 $\mu g\,mL^{-1}$ in water). In the experiment quantifying colonization, three time points were selected: 4 d.p.i., when entry to root xylem has just commenced, 6 d.p.i., and 11 d.p.i., when wilt symptoms appeared in the susceptible plants. Ten seedlings of each genotype were inoculated at the cotyledon stage and examined at each time point. In each seedling, several hand sections were prepared from the middle of five segments: the main root, the crown, lower hypocotyl (0·5 cm below soil level), middle hypocotyl (the middle 0·5 cm) and upper hypocotyl (0·5 cm below the cotyledons), and observed under the confocal microscope. At this stage the stem above the cotyledon had not yet elongated. A seedling was scored as colonized at a given tissue section when fluorescent mycelium (regardless of the amount) was recorded in the section examined. Colonization rate was expressed as the percentage of seedlings in the examined section that contained GFP-expressing mycelium.

RNA extraction and gene-expression analysis by real-time PCR

Before inoculation, a day-0 control consisting of intact BIZ and ED seedlings was sampled to account for basal differences between genotypes. Seedlings were then inoculated as described, and hypocotyls were sampled and rapidly frozen at 1 and 3 d.p.i. Non-inoculated controls were similarly trimmed and replanted. Another control group was left intact to verify that inoculation was successful. Two replicates consisting of 3–6 hypocotyls were collected in two separate experiments. Total RNA from seedling hypocotyls was extracted using the Tri-reagent buffer (Molecular Research Center Inc.). RNA was treated with DNAase and further purified using RNeasy Mini columns (Qiagen). Total RNA (2 μg) was subjected to first-strand synthesis using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's procedure, using oligo(dT) as a primer. Primers used for real-time PCR experiments were as in Yedidia *et al*. (2003) for the *hydroperoxide lyase* (*HPL*) and *phenylalanine ammonia lyase-1* (*PAL*) genes, and as in Shoresh *et al*. (2005) for the *chitinase-1* (*CHI*) gene. Actin was used as an endogenous reference gene in the same samples (forward primer: CGTGCTGGATTCTG GTGATGG, reverse primer: CGTGC-TGGATTCTGG TGATGG, based on GenBank Accession No. gi: 2865172). All primers were designed to match cucumber sequences but worked with high efficiency in melon, and amplicons were sequence-verified and shown to consist of a single, homologous PCR product. PCR optimization was carried out to obtain efficient amplification of the selected genes and clean melting curves. PCR was carried out in a 20-μL reactions containing 1X SYBR Green PCR Master Mix (PE Applied Biosystems), 500 nM primers (for each forward and reverse primer) and 0·8 μL of the reverse transcription reaction. Quantitative analysis was performed using the GeneAmp® 7300 Sequence Detection System (PE Applied Biosystems) with PCR conditions of 95°C for 15 s and 60°C for 1 min, for 40 cycles. The absence of primer-dimer formation was examined in no-template controls. Each sample was examined in triplicate, using relative quantification analysis. This method normalizes the expression of the specific gene vs. the reference gene, using the formula RQ (relative quantity of transcript) = $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{\text{specific gene}} - CT_{\text{reference gene}}$ of the given sample, and $\Delta\Delta CT = \Delta CT$ – arbitrary constant (the highest ΔCT in the sample set; for further elaboration, see PE Applied Biosystems Sequence Detector User Bulletin no. 2). The CT (threshold cycle) value is defined as the PCR cycle number that crosses an 'above background signal' threshold defined by the machine.

Statistical analysis

Standard errors of the percentage of colonized seedlings were computed as $100 \times [b \times (1-b)/n]^{0.5}$, where b is the proportion of a given class, and n the total number of plants (Downie & Heath, 2007). The differences between the proportions of colonized seedlings of ED vs. BIZ melons at each tissue × time-point combination were tested by the binomial test using SPSS.15 software. The differences between transcript RQ values for ED and BIZ at each treatment × time point were tested by the *t*-test for equality of means using SPSS.15 software.

Results

Initial interaction between FOM 1·2 and susceptible seedlings: 1–3 d.p.i.

The GFP-expressing FOM 1·2 strain (designated FOM 1·2-GFP) fluoresced brightly and was readily visible on the surface of melon roots as well as within the xylem vessels, even at a low (×100) magnification (Fig. 1). No loss of fluorescence or photo-bleaching was detected during scanning laser microscope analyses. No differences were observed in morphology or growth characteristics of PDA cultures between the FOM 1·2-GFP and the non-transformed FOM 1·2 strain, and the two were

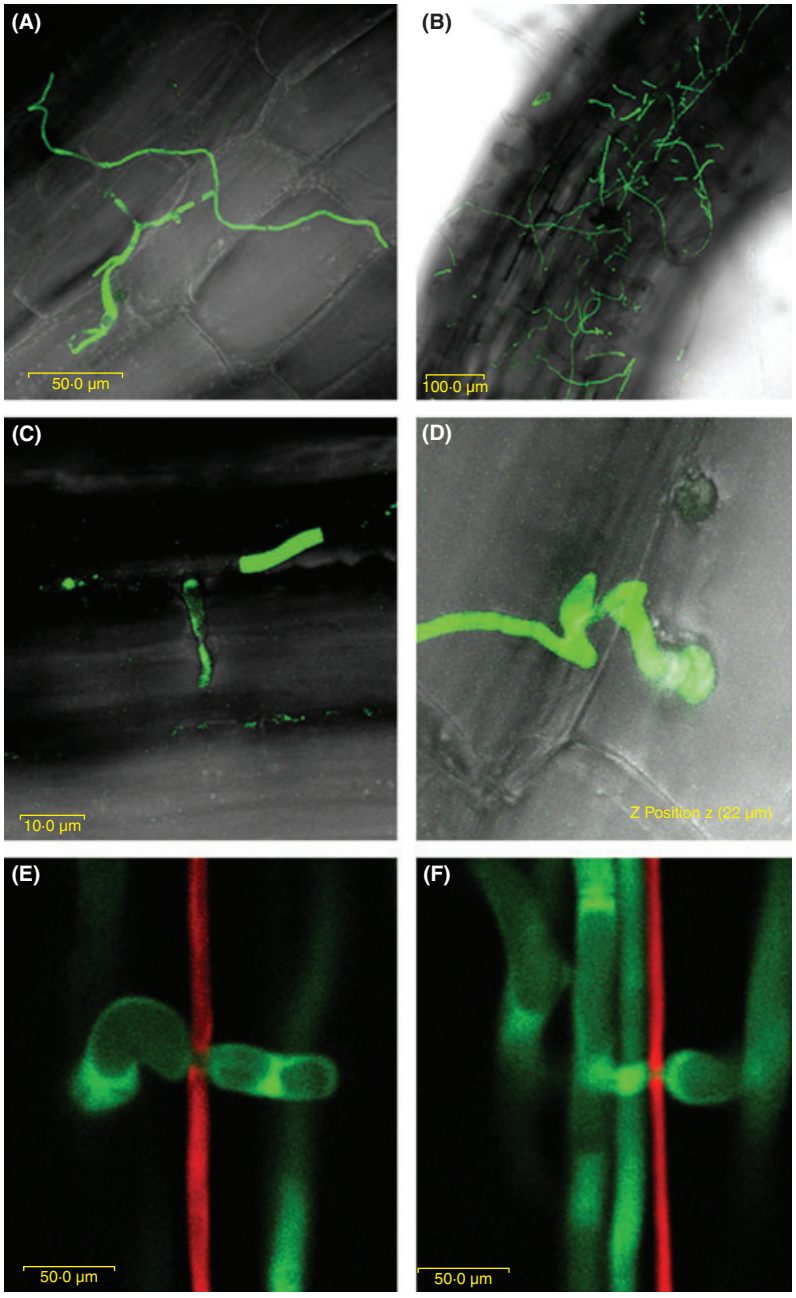

Figure 1 Early stages of infection (2–3 d.p.i.) of susceptible melon cv. Ein Dor seedlings by *Fusarium oxysporum* f.sp. *melonis* race 1·2 expressing a green fluorescent protein (GFP) reporter gene (FOM 1·2-GFP). (A) FOM 1·2-GFP hyphae growing over the epidermis at 1 d.p.i. (B) Hyphal growth over the root and loose attachment to root hairs on the primary root at 2 d.p.i. (C) Hyphae attached and growing along the borders between root epidermal cells at 3 d.p.i. (D) Hypha (seen from above, growing from left to right) penetrating an epidermal cell at 3 d.p.i. (E) Penetration of a root cell by a single germinating microconidium. (F) Penetration of a root cell by a hypha. Transition light images were acquired by differential interference contrast. Panels C and D represent superpositions of Z-stack series that focus deeper into the tissue in 1-µm steps. In panels E and F, sections were counter-stained with propidium iodide.

indistinguishable with respect to their pathogenicity on melon hosts.

The compatible interaction between FOM 1·2-GFP and the susceptible host genotype, ED, was studied using confocal scanning laser microscopy. Ten seedlings, germinated in Petri dishes, were sampled at each time point (1, 2 and 3 d.p.i.). During these 3 days all seedlings appeared healthy, without any visible disease symptoms.

At 1 d.p.i., hyphae were loosely attached to the epidermal surface (Fig. 1A) on the main root at the initial application sites. At 2 d.p.i., hyphae spread along the main root and were more closely attached to root hairs (Fig. 1B). At this stage, no plant cell penetration was observed. At 3 d.p.i., hyphae were attached to the central root, at what appeared as random sites along the entire length of the roots. Individual hyphae were clearly attached and grew along the borders between epidermal cells (Fig. 1C) towards penetration sites. No particular penetration structures were seen, but Z-stack series of images at 3 d.p.i. (Fig. 1C and D) demonstrated that hyphae had penetrated into the epidermal tissues of primary and lateral roots. Figure 1D shows a hypha penetrating an epidermal cell layer and making its way deeper, via a small aperture that it appears to cross by constricting

its diameter. Cellular penetration by germinating microconidia (Fig. 1E) and by mature hyphae (Fig. 1F) was clearly observed. It could not be determined whether the penetrating hyphae progressed through the cortex intracellularly, or within the intercellular spaces.

Vascular colonization: 4–11 d.p.i.

Petri-dish-grown seedlings proved inadequate to follow the interaction with FOM 1·2-GFP over a longer period, so a switch was made to soil-grown seedlings coupled with a standard inoculation protocol. At 4 d.p.i., the plants looked healthy and symptom-free. However, the tap root was densely coated by mycelium (Fig. 2A) and sites of penetration into epidermal cells were observed (as in Fig. 1E, F). No preference of the fungus for the root tips, either intact or wounded, was noticed. At this point, mycelium was rarely observed in xylem vessels. All seedlings still appeared healthy at 6 d.p.i., but the fungus had entered the xylem vessels and began to move upwards. The root and the crown section were colonized in all seedlings, and the hypocotyl of some seedlings was colonized as well (Fig. 2B), whereas the upper hypocotyl was still uncolonized.

Wilt symptoms began to appear at 8 d.p.i., and, by 11 d.p.i., prominent symptoms such as chlorosis and seedling collapse were visible. At this point, the mycelium was distributed in the xylem vessels of the root and hypocotyl, including the upper hypocotyl (Fig. 2C). At 14 d.p.i., all seedlings were dead.

Observations of numerous longitudinal and cross sections of infected hypocotyls revealed that once the vascular system was penetrated by hyphae, subsequent growth was restricted to the xylem and hyphae were seen growing along tracheal elements (Fig. 2D). In some cases, hyphae appeared to grow out of an infected xylem vessel into neighbouring parenchyma tissue (Fig. 2E; in this figure, a hypha appears to grow from one vessel to its neighbour). Nevertheless, stem cross sections showed that hyphae were limited to xylem vessels, even at 13 d.p.i., when the seedling was densely populated by the fungus (Fig. 2F). Sporulation occured within the xylem, with the resulting microconidia germinating and producing secondary hyphae (Fig. 2G).

Comparison of fungal colonization in susceptible vs. resistant melon genotypes

After obtaining a qualitative description of FOM 1·2-GFP infection in susceptible ED seedlings, the dynamics of colonization were compared between ED and a FOM 1·2-resistant genotype, BIZ. The proportion of total seedlings sectioned which were infected with fluorescent mycelium was recorded (Fig. 3).

At 4 d.p.i., the fungus was clearly visible in the xylem vessels of the tap root of 40% of the ED samples and only 10% of the BIZ samples (Fig. 3, difference significant at $P = 0{\cdot}013$). At 6 d.p.i., plants remained symptom-free, but the fungus had spread in the roots of both genotypes and entered the crown region of 40% of BIZ seedlings and 100% of ED seedlings ($P < 0{\cdot}001$). In ED, but not BIZ, it had already reached the lower hypocotyl (50% of seedlings, $P < 0{\cdot}001$).

By 11 d.p.i., disease symptoms such as browning of the lower hypocotyl and cotyledon wilting were frequent in ED, whilst BIZ seedlings showed no symptoms. The main root, crown and lower hypocotyl of all ED and BIZ seedlings were colonized, whereas in the middle hypocotyls, 30% infection was found in BIZ, compared to 100% in ED (Fig. 3; $P < 0{\cdot}001$). No mycelium was observed in the upper hypocotyl of BIZ, whereas 70% of ED samples were infected there ($P < 0{\cdot}001$). Additional seedlings from the same experiment were left intact for further observations. ED seedlings wilted and died 14 days after inoculation, whereas BIZ plants remained symptomless (except for occasional chlorosis) and were visibly healthy even at 21 d.p.i. The experiment was repeated, and similar patterns of fungal progression were observed (not shown).

Defence gene expression in response to FOM inoculation

To study the molecular basis of the resistance of BIZ to FOM 1·2, and associate it to the observed colonization patterns, a number of defence-related transcripts were monitored to determine whether constitutive, pre-infection differences in transcript levels existed between the two genotypes, and whether the two differed in their early responses to FOM 1·2 infection.

Sets of hypocotyl samples were harvested before inoculation and at 1 and 3 d.p.i. and analysed by quantitative real-time PCR. Similar patterns of expression were observed in both biological replicates.

Transcript levels of PAL in intact seedlings were fourfold higher in resistant BIZ hypocotyls than in susceptible ED ones (Fig. 4). At 1 d.p.i., mock-inoculated ED seedlings showed a threefold PAL induction compared to intact seedlings, probably as a result of root wounding; at 3 d.p.i. the response levelled-off. In BIZ control seedlings, a wounding response was not apparent at 1 d.p.i., but reached fivefold induction at 3 d.p.i. In both genotypes, the response to FOM 1·2-GFP was much higher than that to wounding alone. However, the extent of induction and its temporal pattern differed between the two genotypes. At 1 d.p.i., the relative quantity (RQ) in ED was 44 (eightfold PAL induction over the respective control) and 66 (25-fold induction) in BIZ. At 3 d.p.i., the RQ of PAL decreased by about half in ED (the wound response of control seedlings also showed the same pattern); in BIZ, transcript levels continued to increase, reaching an RQ value of ~170 (25 times the level of intact seedlings and five times that of the respective wounded control).

The expression patterns of chitinase (CHI) were even more striking. A constitutive difference of 3·5-fold between BIZ and ED was apparent on day 0 (Fig. 4). A significant wound response was detected in ED at 1 d.p.i. (RQ = 9, nine times greater than the day-0 control).

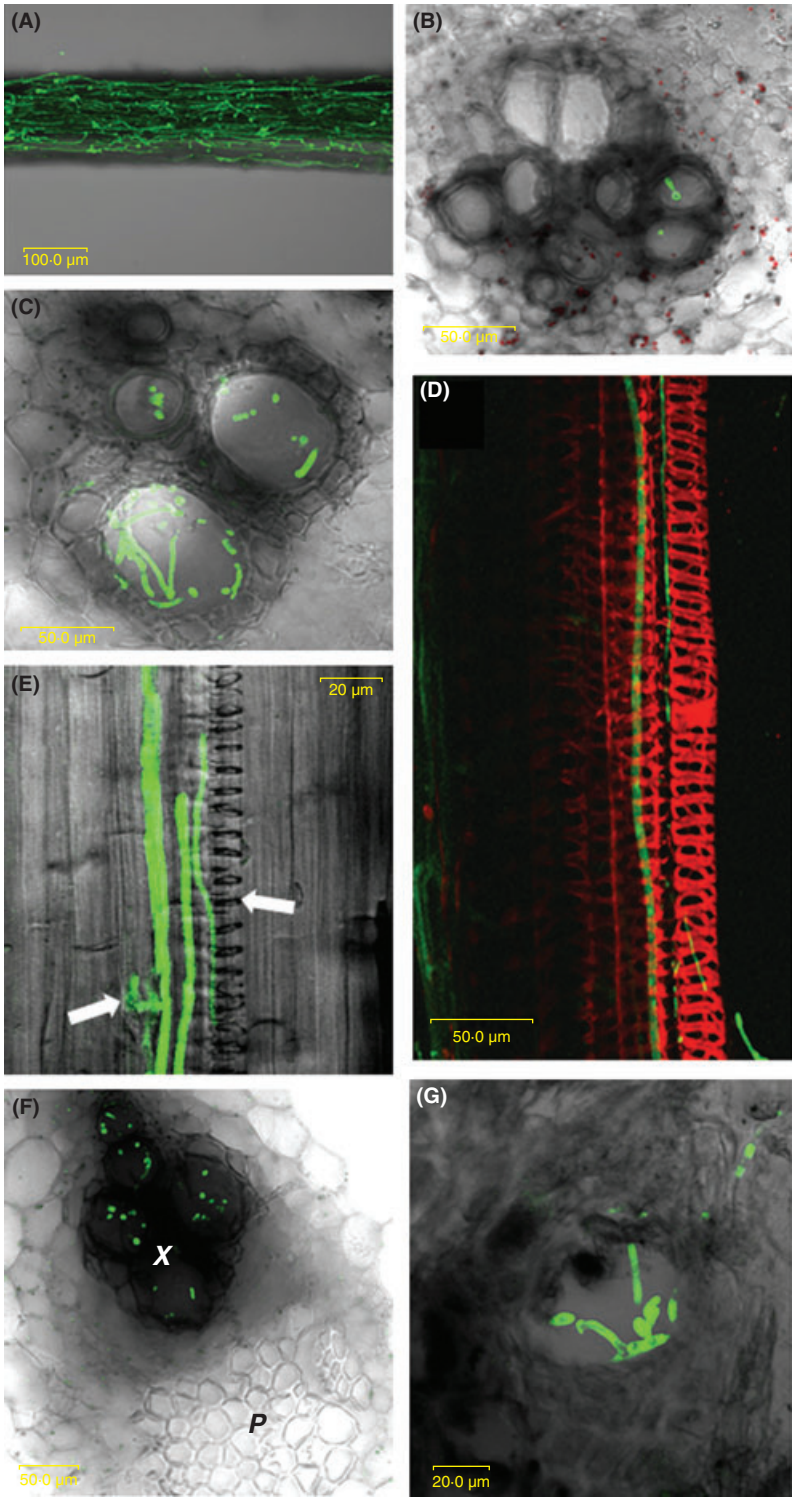

Figure 2 Advanced infection (4–13 d.p.i.) of susceptible melon cv. Ein Dor seedlings by *Fusarium oxysporum* f.sp. *melonis* (FOM 1·2-GFP). Images were acquired as in Fig. 1. (A) Mycelium densely covering the main root at 4 d.p.i. (B) Cross section of lower hypocotyl, with only a few vessels populated by fungal mycelium, at 6 d.p.i. (C) Cross section of upper hypocotyl, with heavily populated xylem vessels, at 11 d.p.i. (D) Hypha growing upwards, confined in a xylem vessel: longitudinal section, counterstained with propidium iodide. (E) Longitudinal section of root segment at 6 d.p.i. Lower arrow indicates site where mycelium appears to grow in tissue adjacent to infected xylem vessels. Upper arrow indicates apparent passage of hypha from one vessel to another. (F) Cross section of an upper hypocotyl segment at 13 d.p.i. The mycelium is restricted to xylem vessels (X), whereas nearby phloem vessels (P) are not colonized. (G) Cross section of an upper hypocotyl segment: conidium germinating in the xylem to produce secondary mycelium.

A more moderate increase occurred in BIZ (from RQ = 3·5 to 5·6). CHI transcript levels increased further in the control treatment at 3 d.p.i. (RQ = 69 and 80 in ED and BIZ, respectively), indicating that the wound response of the two genotypes was similar. However, the response to FOM 1·2 differed between genotypes: in ED, FOM-induced CHI transcripts were lower than in the respective wound control at both time points. In BIZ, on the other hand, the RQ value following FOM induction was 22 at 1 d.p.i. (four times the wounded control) and 518 at 3 d.p.i., representing a sixfold induction over the wounded control, and 145-fold over the day-0 control.

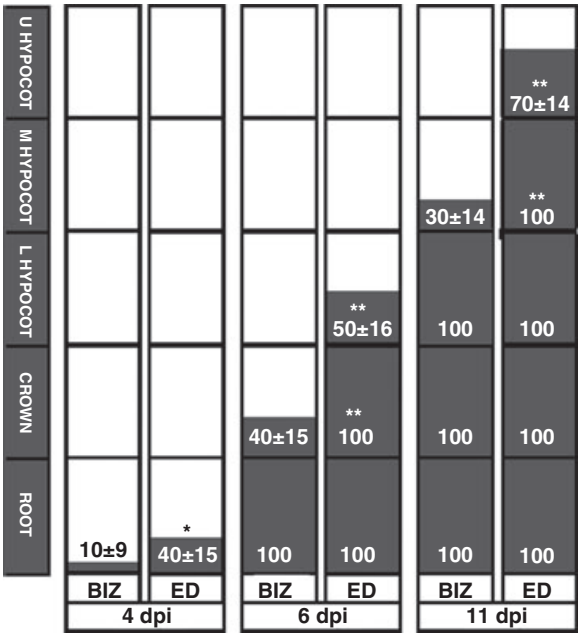

Figure 3 Colonization rate of susceptible melon cv. Ein Dor (ED) seedlings by *Fusarium oxysporum* f.sp. *melonis*, compared to that of resistant (BIZ) seedlings. One-week-old seedlings were inoculated with a suspension of 10^6 conidia of FOM 1·2-GFP per mL. At 4, 6 and 11 d.p.i., 10 seedlings of each genotype were sectioned, and a few cross sections from each of the indicated regions (main root, crown, lower-, middle- and upper-hypocotyl) were viewed under the confocal laser microscope to monitor green fluorescent protein (GFP)-expressing mycelium in the xylem vessels. The proportion of sections harbouring the fungus was calculated, along with the respective standard errors. The differences in colonization proportions between ED and BIZ for each sample pair were tested for significance using the binomial test, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$.

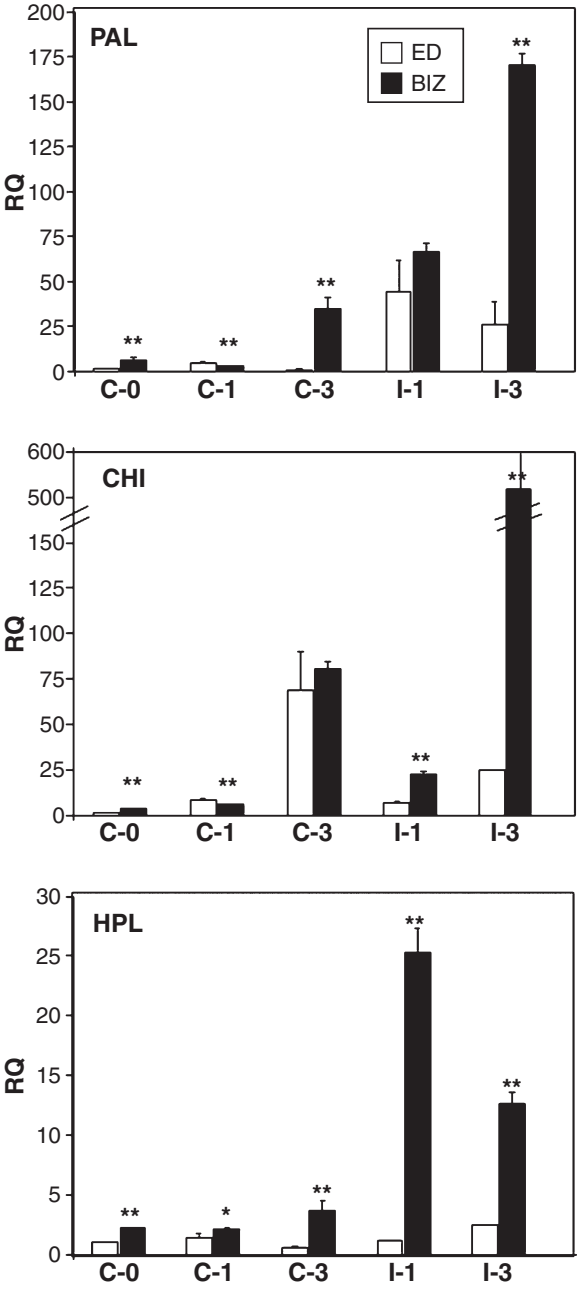

Figure 4 Changes in defence gene transcripts following inoculation of melon hypocotyls with *Fusarium oxysporum* f.sp. *melonis* (FOM 1·2-GFP). Real-time PCR analysis was performed on 1-week-old seedlings of the BIZ (resistant) and ED (susceptible) genotypes. C-0, untreated seedlings; C-1 and C-3, mechanical wounding after 1 and 3 days, respectively; I-1, I-3, seedlings wounded and infected with FOM 1·2-GFP and sampled at 1 and 3 d.p.i., respectively. RQ, relative quantity of transcript, calculated on the basis of an actin gene standard, as the average of triplicate PCR reactions with standard deviation. The lowest sample was given a value of 1 and the rest are expressed relative to that sample. PAL, phenylalanine ammonia lyase (top); CHI, chitinase (middle); HPL, hydroperoxide lyase (bottom). The differences between RQ values of each ED–BIZ pair of samples were tested using the *t*-test, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}005$.

Hydroperoxide lyase (HPL) also had a distinct expression pattern. Similar to CHI and PAL, the basal transcript levels were twofold higher in BIZ than in ED (Fig. 4). Induction by wounding alone was less than twofold, but a detectable difference between BIZ and ED was observed in all sample pairs. At 1 d.p.i., ED did not respond to FOM 1·2, and at 3 d.p.i. a twofold increase in RQ (from 1·2 to 2·4) was detected. The response of BIZ was more rapid: at 1 d.p.i. RQ was 25 (11 times the wounded control and 21 times ED). Two days later, the level of HPL in BIZ began to decrease, but was still significantly greater than in ED.

Discussion

In this study, a GFP-marked strain of FOM was used to follow colonization of root and stem tissues in ED, a susceptible melon variety. At 3 d.p.i., attachment had occurred along the root epidermis and by day 4 the fungus was found in the root xylem; this implies that penetration of the epidermis, cortex and the xylem took place between days 2 and 4. By day 11, the xylem was heavily

populated with mycelium, and conidia were produced and germinated within the vessels. Secondary spore formation in the xylem was reported by Mace & Veech (1971) and Beckman *et al*. (1972), whilst passage from one vessel to an adjacent one via pit pairs was documented by Stromberg & Corden (1980). In the present study, *in vivo* GFP imaging allowed these events to be clearly documented. Sporulation and germination of secondary mycelium is considered crucial for rapid upward colonization, since mycelial growth is probably too slow to account for the fast colonization rates observed (Beckman & Roberts, 1995). Conidia can be carried rapidly by the transpiration stream but are trapped at the ends of vessels; to progress further, they must germinate, and the new hyphae have to penetrate the adjacent vessels. Lateral movement within the xylem is also required in case the plant successfully occludes a vessel.

These findings can be related to other studies and current models of the stages of interaction between *F. oxysporum* and its hosts (Beckman, 1987; Beckman & Roberts, 1995). The fungus and its host were shown to influence each other's growth even before attachment (Steinberg *et al*., 1999) by an as-yet-unknown mechanism. Such early responses are in agreement with the strong induction of defence genes observed here at 1 d.p.i.. Lagopodi *et al*. (2002) used a *F. oxysporum* f.sp. *radicis-lycopersici* GFP strain to monitor invasion of a susceptible tomato cultivar. The temporal patterns reported in that study are similar those observed here: attachment at 1–3 d.p.i., followed by penetration at 4 d.p.i. along epidermal cell borders. Olivain & Alabouvette (1999) focused on early interactions between tomato and pathogenic vs. non-pathogenic GUS-expressing *F. oxysporum* strains, and observed penetration events already occurring at 1 d.p.i. The present study did not find visibly differentiated penetration structures, but observed the mycelium forcing itself through narrow openings that were apparently digested in cell walls. Other studies reported typically thinner penetration hyphae (Rodriguez-Galvez & Mendgen, 1995) passing through pores produced by lysing host walls.

The earliest responses of the plant during 'determinative phase I' of the infection, before the fungus reaches the xylem, were described by Rodriguez-Galvez & Mendgen (1995) using TEM analysis of susceptible cotton roots infected by *F. oxysporum* f.sp. *vasinfectum*. They reported wall apposition, lignification and suberization, with prominent papillae forming at attempted penetration sites. More detailed imaging by time-lapse confocal microscopy (Czymmek *et al*., 2007) showed *Arabidopsis* cell collapse and whole root retraction during early *Fusarium* infection; cell rupture and changes in tissue autofluorescence were apparent even before the responding cell layer was invaded. Olivain *et al*. (2003) also focused on the earliest responses of flax seedlings to pathogenic and non-pathogenic *F. oxysporum* strains, consisting of cell-wall appositions and HR-like events, H_2O_2 burst, and apoplast alkalinization. There were clear differences between the responses to pathogenic and non-pathogenic strains, the latter eliciting stronger responses. This suggests that already at the pre-vascular stage, a resistant genotype could elicit a stronger response. In the present study, colonization rates of BIZ appeared lower than those of ED as early as 4 d.p.i., when xylem penetration had barely begun. Together with the early defence-gene induction detected at 1 and 3 d.p.i., it seems that resistant melon plants respond more efficiently already at the pre-vascular stage.

The vast majority of studies focused, however, on 'determinative phase II' of the interaction that occurs in the xylem, and suggested that the main resistance responses occur within or along the vessels. In the study by Gao *et al*. (1995), mycelium outgrowth from specific vessels was monitored microscopically. This allowed accurate scoring of the ratio of infected vessels and the computation of a 'colonization quotient' to measure the rate of fungal progress. Another possible way to quantify colonization involves quantitative PCR of fungal DNA (Schena *et al*., 2004). In the present study, the amount of mycelium seen in infected root and hypocotyl sections was notably lower in BIZ than in ED. Infected vessels were not counted, but tissue sections were scored as infected by, or devoid of, GFP-expressing hyphae. Using this tool, it was found that the dynamics of fungal spread were different in BIZ and ED (Fig. 3). Penetration and colonization of the taproot and secondary roots also occurred in BIZ, but a quantitative difference in colonization rate was apparent by 4 d.p.i. Later, at 6 and 11 d.p.i., the difference between the two genotypes became more prominent, with a second line of defence probably being expressed, which reduced pathogen movement within the xylem and did so more efficiently in BIZ. Thus, resistant plants are not immune from the pathogen, but are able to quantitatively inhibit its progression by expressing an efficient defence response. In this experiment, the method of artificial inoculation included root trimming and immersion in a concentrated spore suspension to obtain highly uniform, reproducible infection. BIZ plants were kept as long as 35 d.p.i. and, despite colonization, developed no symptoms. It would be interesting to extend this study and follow colonization patterns in mature plants, and to use natural infection regimes with intact roots, to determine whether colonization rates of BIZ would be even lower than in the present study.

To explore the possible molecular basis of resistance in BIZ, the expression of three representative defence genes was monitored by real-time PCR analysis. To determine whether the two genotypes differed in their defence capability, transcript levels of the selected genes were compared in the hypocotyls of resistant and susceptible seedlings before infection, and at 1 and 3 d.p.i.

Hydroperoxide lyase (HPL) is part of the octadecanoic pathway. It cleaves fatty acid hydroperoxides formed by lipoxygenases, and produces omega-oxo acids and volatile C6- and C9-aldehydes and alcohols (Noordermeer *et al*., 2001). These molecules have been associated with plant defence response to pathogens and wounding; some HPL products are signal molecules that induce

production of phytoalexins (Zeringue, 1992). A 12-fold induction of HPL at 1 d.p.i. in BIZ (and no induction in ED) may indicate involvement of the JA pathway in response to FOM.

Chitinases (CHI) are major hydrolytic enzymes that plants secrete in response to pathogen attack. Benhamou *et al*. (1990) reported rapid induction of CHI and glucanase proteins in *Fusarium*-resistant tomatoes upon infection. Transgenic tobacco plants with constitutively higher CHI levels were more resistant than non-transformed plants to *Cercospora nicotianae* and *Rhizoctonia solani* (Broglie *et al*., 1991; Zhu *et al*., 1994). In the present study, CHI was strongly induced in BIZ (~145-fold), but in ED induction was moderate. The response of BIZ to FOM 1·2 is highly specific, superimposed on the response to wounding alone; the latter response did not differ between ED and BIZ.

Phenylalanine ammonia lyase (PAL) is the regulatory enzyme catalysing the first step of the phenylpropanoid pathway. It converts phenylalanine to cinnamic acid, then the pathway diverges between production of salicylic acid and lignin precursors. Mauch-Mani & Slusarenko (1996) evaluated the importance of PAL in the defence against *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*) in *Arabidopsis*. The *PAL* gene was locally induced during an incompatible interaction, and inhibiting PAL activity rendered the resistant plant more susceptible. In the present study, PAL was induced 25-fold at 3 d.p.i. in BIZ plants; in ED, maximal induction (eightfold) occurred at 1 d.p.i. and later decreased. The transcript levels of three additional genes, anionic peroxidase (melon EST MU5298, Cucurbit Genomics Database, http://www.icugi.org/), quinone hydroperoxide lyase (melon EST MU6448) and enolase (melon EST MU4003), were also analysed in the same samples (data not shown). Their transcript levels were similar in BIZ and ED hypocotyls and did not show significant responses to FOM 1·2 inoculation or wounding. This further emphasizes the specificity of the responses of PAL, CHI and HPL.

Maher *et al*. (1994) transformed tobacco plants with bean *PAL-2* and obtained plants with constitutively lower PAL activity that were more susceptible to *Cercospora nicotianae*, implying that constitutive PAL levels were responsible for resistance in the wild type. It has been proposed that constitutive differences in defence enzymes, chitinases and β-1,3-glucanases, were responsible for broad, quantitative resistance of tomato against *Alternaria solani* (Lawrence *et al*., 2000). In the present study, a pre-formed, two- to threefold difference in the levels of PAL, HPL and CHI transcripts between ED and BIZ was repeatedly measured. If these differences underlie a similar advantage in enzymatic activity, BIZ could be better prepared, or primed, against fungal attack at the very initial stages of infection. Thus, both constitutive and induced differences in defence gene expression were detected between the resistant and the susceptible melon genotypes. The differential expression of these genes could indicate their mechanistic importance in the defence against FOM. At this point, however, it is not clear whether the three defence genes are indeed crucial for mounting an effective defence, or merely represent 'markers' of the response. A transgenic approach or mutant analysis of these genes could further understanding of their involvement in defence.

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References

Beckman CH, 1987. *The Nature of Wilt Diseases of Plants*. St Paul, MN, USA: APS Press.

Beckman CH, Roberts EM, 1995. On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. *Advances in Botanical Research* **21**, 35–77.

Beckman CH, Elgersma DM, Machardy WE, 1972. Localization of fusarial infections in vascular tissue of single-dominant-gene resistant tomatoes. *Phytopathology* **62**, 1256–60.

Benhamou N, Joosten M, Dewit P, 1990. Subcellular localization of chitinase and of its potential substrate in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Plant Physiology* **92**, 1108–20.

Berrocal-Lobo M, Molina A, 2008. *Arabidopsis* defense response against *Fusarium oxysporum*. *Trends in Plant Science* **13**, 145–50.

Bishop CD, Cooper RM, 1983. An ultrastructural study of vascular colonization in three vascular wilt diseases. 1. Colonization of susceptible cultivars. *Physiological Plant Pathology* **23**, 323–43.

Broglie K, Chet I, Holliday M, *et al*., 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 1194–7.

Brotman Y, Kovalski I, Dogimont C *et al*., 2005. Molecular markers linked to papaya ring spot virus resistance and *Fusarium* race 2 resistance in melon. *Theoretical and Applied Genetics* **110**, 337–45.

Burger Y, Katzir N, Tzuri G *et al*., 2003. Variation in the response of melon genotypes to *Fusarium oxysporum* f.sp. *melonis* race 1 determined by inoculation tests and molecular markers. *Plant Pathology* **52**, 204–11.

Cohen R, Katan T, Katan J, Cohen R, 1989. Occurrence of *Fusarium oxysporum* f.sp. *melonis* race 1,2 on muskmelon in Israel. *Phytoparasitica* **17**, 319–22.

Czymmek KJ, Fogg M, Powell DH, Sweigard J, Park SY, Kang S, 2007. *In vivo* time-lapse documentation using confocal and multi-photon microscopy reveals the mechanisms of invasion into the *Arabidopsis* root vascular system by *Fusarium oxysporum*. *Fungal Genetics and Biology* **44**, 1011–23.

Di Pietro A, Madrid MP, Caracuel Z, Delgado-Jarana J, Roncero MIG, 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Molecular Plant Pathology* **4**, 315–25.

Downie NM, Heath RH, 2007. *Basic Statistical Methods*. New York, USA: HarperCollins.

Gao H, Beckman CH, Mueller WC, 1995. The rate of vascular colonization as a measure of the genotypic interaction between various cultivars of tomato and various formae or races of *Fusarium oxysporum*. *Physiological and Molecular Plant Pathology* **46**, 29–43.

Herman R, Perl-Treves R, 2007. Characterization and inheritance of a new source of resistance to *Fusarium oxysporum* f.sp. *melonis* race 1.2 in *Cucumis melo*. *Plant Disease* **91**, 1180–6.

Joobeur T, King JJ, Nolin SJ, Thomas CE, Dean RA, 2004. The fusarium wilt resistance locus Fom-2 of melon contains a single resistance gene with complex features. *The Plant Journal* **39**, 283–97.

Lagopodi AL, Ram AFJ, Lamers GEM *et al.*, 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Molecular Plant-Microbe Interactions* **15**, 172–9.

Lawrence CB, Singh NP, Qiu JS, Gardner RG, Tuzun S, 2000. Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. *Physiological and Molecular Plant Pathology* **57**, 211–20.

Mace ME, Veech JA, 1971. Fusarium wilt of susceptible and resistant tomato isolines – host colonization. *Phytopathology* **61**, 834–40.

Maher EA, Bate NJ, Ni WT, Elkind Y, Dixon RA, Lamb CJ, 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proceedings of the National Academy of Sciences, USA* **91**, 7802–6.

Mauch-Mani B, Slusarenko AJ, 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *The Plant Cell* **8**, 203–12.

Michielse CB, Rep M, 2009. Pathogen profile update: *Fusarium oxysporum*. *Molecular Plant Pathology* **10**, 311–24.

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S, 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* **91**, 173–80.

Noordermeer MA, Veldink GA, Vliegenthart JFG, 2001. Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. *ChemBioChem* **2**, 494–504.

Olivain C, Alabouvette C, 1999. Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. *New Phytologist* **141**, 497–510.

Olivain C, Trouvelot S, Binet MN, Cordier C, Pugin A, Alabouvette C, 2003. Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogenic strains of *Fusarium oxysporum*. *Applied and Environmental Microbiology* **69**, 5453–62.

Perchepied L, Dogimont C, Pitrat M, 2005. Strain-specific and recessive QTLs involved in the control of partial resistance to *Fusarium oxysporum* f. sp. *melonis* race 1.2 in a recombinant inbred line population of melon. *Theoretical and Applied Genetics* **111**, 65–74.

Recorbet G, Steinberg C, Olivain C *et al.*, 2003. Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. *New Phytologist* **159**, 73–92.

Rodriguez-Galvez E, Mendgen K, 1995. The infection process of *Fusarium oxysporum* in cotton root tips. *Protoplasma* **189**, 61–72.

Schena L, Nigro F, Ippolito A, Gallitelli D, 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* **110**, 893–908.

Schippers B, van Eck WH, 1981. Formation and survival of chlamydospores in *Fusarium*. In: Nelson PE, Tousson TA, Cook RJ, eds. Fusarium: *Disease, Biology, and Taxonomy*. University Park, PA, USA: Penn State University Press, 250–60.

Shoresh M, Yedidia I, Chet I, 2005. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology* **95**, 76–84.

Steinberg C, Whipps JM, Wood D, Fenlon J, Alabouvette C, 1999. Mycelial development of *Fusarium oxysporum* in the vicinity of tomato roots. *Mycological Research* **103**, 769–78.

Stromberg EL, Corden ME, 1980. Scanning electron-microscopy of *Fusarium oxysporum* f.sp. *lycopersici* in xylem vessels of wilt-resistant and susceptible tomato plants. *Canadian Journal of Botany* **58**, 2360–6.

Yedidia I, Shoresh M, Kerem Z, Benhamou N, Kapulnik Y, Chet I, 2003. Concomitant induction of systemic resistance to *Pseudomonas spingae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Applied and Environmental Microbiology* **69**, 7343–53.

Zeringue HJ, 1992. Effects of c-6-c-10 alkenals and alkanals on eliciting a defense response in the developing cotton boll. *Phytochemistry* **31**, 2305–8.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ, 1994. Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. *Bio/Technology* **12**, 807–12.