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DOI

[10.1016/j.fgb.2008.06.002](https://doi.org/10.1016/j.fgb.2008.06.002)

Publication date

2008

Published in

Fungal Genetics and Biology

[Link to publication](#)

Citation for published version (APA):

van der Does, H. C., Duyvesteijn, R. G. E., Goltstein, P. M., van Schie, C. C. N., Manders, E. M. M., Cornelissen, B. J. C., & Rep, M. (2008). Expression of effector gene SIX1 of *Fusarium oxysporum* requires living plant cells. *Fungal Genetics and Biology*, 45(9), 1257-1264. <https://doi.org/10.1016/j.fgb.2008.06.002>

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Expression of effector gene *SIX1* of *Fusarium oxysporum* requires living plant cells

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ARTICLE INFO

Article history:

Received 5 March 2008

Accepted 10 June 2008

Available online 18 June 2008

Keywords:

Fusarium oxysporum

Tomato

Avirulence

Root colonization

Green fluorescent protein

Confocal microscopy

Two-photon microscopy

ABSTRACT

Fusarium oxysporum is an asexual, soil inhabiting fungus that comprises many different *formae speciales*, each pathogenic towards a different host plant. In absence of a suitable host all *F. oxysporum* isolates appear to have a very similar lifestyle, feeding on plant debris and colonizing the rhizosphere of living plants. Upon infection *F. oxysporum* switches from a saprophytic to an infectious lifestyle, which probably includes the reprogramming of gene expression. In this work we show that the expression of the known effector gene *SIX1* of *F. oxysporum* f. sp. *lycopersici* is strongly upregulated during colonization of the host plant. Using GFP (green fluorescent protein) as reporter, we show that induction of *SIX1* expression starts immediately upon penetration of the root cortex. Induction requires living plant cells, but is not host specific and does not depend on morphological features of roots, since plant cells in culture can also induce *SIX1* expression. Taken together, *F. oxysporum* seems to be able to distinguish between living and dead plant material, preventing unnecessary switches from a saprophytic to an infectious lifestyle.

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1. Introduction

Members of the *Fusarium oxysporum* species complex are asexual, soil inhabiting fungi that are generally known as efficient root colonizers. Even though all isolates of *F. oxysporum* appear to have a very similar lifestyle, notably feeding on plant debris and colonizing the rhizosphere of living plants, some are capable of entering and infecting living plant tissue, while others are not (Gordon and Martyn, 1997; Olivain and Alabouvette, 1999). This suggests the existence of isolate specific genes and the capacity to switch from a saprophytic to an infectious lifestyle, probably involving the reprogramming of gene expression.

To study the saprophytic/pathogenic lifestyle switch in pathogenic isolates of *F. oxysporum* we investigated the expression of the *SIX1* gene from *F. oxysporum* f. sp. *lycopersici* (Fol). Members of this *forma specialis* (f. sp.) can infect tomato plants and cause tomato wilt disease. The *SIX1* gene (for *Secreted In Xylem 1*) is found in all isolates of f. sp. *lycopersici* but not in any other *forma specialis* (pathogenic on other host plants such as melon, banana, tulip, etc.) (van der Does et al., 2008). The Six1 protein is an effector protein that is secreted into the xylem sap during colonization of xylem vessels and is required for full virulence of Fol (Rep et al., 2005a). In tomato plants carrying the *I-3* resistance gene *SIX1* can also trigger a recognition reaction that results in resistance of the plant (Rep et al., 2004).

Many effector genes that encode small, secreted proteins, like *SIX1*, are not or weakly expressed in axenic cultures, and are upregulated specifically during infection (Rep, 2005). Some of these genes, like *CgDN3* (*Colletotrichum gloeosporioides*), *AVR9* (*Cladosporium fulvum*) and *MPG1* (*Magnaporthe grisea*) are also upregulated during conditions of nitrogen starvation (Stephenson et al., 2000; Talbot et al., 1993; Van den Ackerveken et al., 1994). For Avr9 and Mpg1 it was shown that expression (partly) depends on a general nitrogen response factors (*NRF1* and *NPR1*, respectively) (Lau and Hamer, 1996; Perez-Garcia et al., 2001; Soanes et al., 2002). However, expression of other *in planta* induced genes does not respond to changes in nitrogen availability, for example *AVR4*, *ECP1* and *ECP2* (*C. fulvum*), *AvrLm1* (*Leptosphaeria maculans*), *MIG1* and the *MIG2* gene cluster (*Ustilago maydis*) (Basse et al., 2002, 2000; Gout et al., 2006; Van den Ackerveken et al., 1994; Wubben et al., 1994). Even for the genes that are expressed in an environment with limited nitrogen, it is not known which circumstances induce expression *in planta*, since there are indications that within the host plant, nitrogen is not limiting (Solomon and Oliver, 2001, 2002). In *F. oxysporum*, the genes expressed during nitrogen starvation partially overlap with the genes expressed during infection of host tissue (Divon et al., 2005), but no upregulation of (putative) effector genes was detected.

To study the circumstances that require the use of host specific effectors in *F. oxysporum* f. sp. *lycopersici* (Fol) and identify the cue from the host that induces the production of these proteins, we set out to investigate expression of *SIX1*. Using RT-PCR, confocal and two-photon microscopy, we show here that *SIX1* is expressed very

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early during infection, immediately after penetration of the root cortex. We also demonstrate that induced expression is highly dependent on the close proximity of living plant material.

2. Materials and methods

2.1. Expression studies: RNA isolations and PCR

Mycelium from *F. oxysporum* f. sp. *lycopersici* (Fol) isolate Fol007, described in Mes et al. (1999) was used to inoculate minimal growth medium (3% sucrose, 1% KNO₃ and 0.17% yeast nitrogen base without amino acids and ammonia). After 5 days of growth (25 °C, 175 rpm), microconidia were isolated and used to inoculate the test medium (25 ml with 5 × 10⁸ microconidia). Mycelium was harvested after 2 days at 25 °C, shaking at 175 rpm. For RT-PCR experiments, the mycelium was prepared and RNA isolations were performed as described in Rep et al. (2005b).

Roots and hypocotyls of tomato (*Solanum lycopersicum*) were sampled at 4, 8, 12 and 16 days after inoculation with Fol007 (tomato cultivar C32) or at 2, 4, 8 and 14 days after inoculation (tomato cultivar GCR 161), both susceptible interactions. At the first time point, material from 40 individual plants was pooled and at the subsequent time points 20 plants were pooled. Total RNA was isolated using TRIzol (Gibco), followed by an additional Lithium Chloride precipitation. The isolated RNA was used to make cDNA using Promega Rnasin (ribonuclease inhibitor) and Gibco Superscript II RNase H⁻ Reverse transcriptase, according to the instructions of the manufacturer (Gibco).

PCR reactions to check for the expression of *SIX1* and *FEM1* were performed on 2 µl cDNA in a total volume of 25 µl, using a forward primer on the gene of interest (p12-F1 [CCCCGAATTGAGGTGAAG] for *SIX1* and FemATG [ATGAAGTACTCTCGCTACC] for *FEM1*) and a reverse primer on the polyA tail. Primers were allowed to anneal at 55 °C, elongation was 1 min in 35 cycles. PCR products were separated on 1% agarose gels and visualized with ethidium bromide.

2.2. qPCR

Quantitative PCR (qPCR) was performed with Platinum SYBR Green qPCR from Invitrogen, using a 7500 Realtime PCR System from Applied Biosystems. RNA isolation and cDNA synthesis are described above. To quantify *SIX1* mRNA levels, we used two primer sets: *SIX1*-Q2-F [GCCTGGGACCTACGATTATT] with *SIX1*-Q2-R [AGTTGCGGATATGTGTTTGT] (product of 175 bp) and *SIX1*-Q3-F [GGGCGACTAGGAAAATCGAA] with *SIX1*-Q3-R [GCCACAGACAGGAAAAGTA] (product of 153 bp). To compare *SIX1* expression between axenic and *in planta* conditions, we also measured mRNA levels of the constitutively expressed *EF-1α* gene, also using two primer sets: FoTEF-Q1-F [CGGTAAGGTTCTTCAAGT] with FoTEF-Q1-R [TGACCGGGAGCGTCGATGA] (product of 138 bp) and FoTEF-Q2-F [CATCGGCCACGTCGACTCT] with FoTEF-Q2-R [AGAA CCCAGGCTACTTGAA] (product of 144 bp). All primers sets were first tested for generation of a PCR product of the expected size. The dissociation curves of the reaction products indicated absence of primer dimers. Each reaction was performed in duplicate, and for each gene the results of both primers sets were combined to calculate the average and standard deviation.

2.3. Plant inoculations

Fol007 and the tomato cultivar C32 (Kroon and Elgersma, 1993), that has general susceptibility to Fol, were used for the inoculations. Ten-day-old tomato seedlings were inoculated via the root

dip method (Mes et al., 1999; Wellman, 1939). Spores were isolated from 5-day-old potato dextrose broth (PDB) cultures (Difco) or 3- to 5-day-old cultures in minimal medium (1% KNO₃, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids or ammonia). After washing with water, the spores were diluted to 10⁷ spores/ml. The seedlings were dipped in a spore suspension for about 1 min and subsequently transferred to vermiculite (Agra-vermiculite, Eveleens, Aalsmeer, The Netherlands) and the plants were given extra nutrition (NPK 20:10:20 final concentration 1 g/l, Peters Professional, Heerlen, The Netherlands) three times a week. Seedlings inoculated in this way were used for imaging root slices under the confocal microscope (Fig. 4) and for RNA extraction of infected seedlings.

For the rest of the microscopic analyses (Figs. 3, 5 and 6 and Supplementary Fig. 2), 10-day-old tomato seedlings were taken carefully from the potting soil in which they were sown and the root system was washed in tap water. Clean plantlets were placed in Petri dishes, with the roots spread out on the bottom of the dish and the hypocotyl leaning to the vertical wall, with the leaves outside the Petri dish. The dish was filled with 25 ml water to which Fol spores were added to a final concentration of 10⁷ spores/ml. Holes in the lid allowed the Petri dish to be closed (to minimize evaporation) with the top parts of the plant sticking out.

2.4. Construction of GFP/RFP-expressing strains of Fol

The construct used for GFP-marking of Fol with *GFP* behind the constitutive *GPD* promoter is described elsewhere (Duyvesteijn et al., 2005). For construction of the *SIX1*-promoter-*GFP* construct the *SIX1* ORF, present in a NcoI-SpeI genomic fragment containing the *SIX1* gene in the pGEMT-easy vector, was first replaced by a XbaI restriction site in the following way: in pGEMT-easy containing the NcoI-SpeI fragment with *SIX1*, the entire *SIX1* open reading was deleted and replaced by an XbaI restriction site. This was done by PCR amplification of vector plus *SIX1* flanking sequences using primers P12-ATG-R (aaaatctagaTTTGTGCAAAGCTCAAATCC) and P12-TAA-F (aaaatctagaGTTGCGATCCAAAGCCCT) followed by XbaI-digestion of the linkers and religation. The *GFP* ORF was amplified by PCR (using Pfu polymerase), introducing XbaI restriction sites on both ends, which allowed cloning of *GFP* directly between the *SIX1* promoter and terminator in the above mentioned construct. Constructs were sequenced to check for the right orientation and rule out PCR mistakes. To facilitate homologous recombination of *GFP* into the *SIX1* locus in Fol, the 3' end of cassette was extended with the neighboring 1.7 kb SpeI-XbaI genomic DNA fragment. The correct orientation of this 1.7 kb extension was checked by restriction analyses. Finally, the *BLE* resistance gene (conferring resistance to zeocin) was introduced between the *SIX1* terminator (the region corresponding to the mRNA trailer) and the 3' flank for homologous recombination. For this, the *BLE* gene was retrieved from the pAN8.1 vector (Punt and van den Hondel, 1992) as an AgeI-XbaI fragment and cloned into the SgrAI-SpeI digested *SIX1* locus-*GFP* construct. HindIII and EcoRI were used to transfer the cassette to pPZP200, a binary vector for *Agrobacterium*-mediated transformation (ATMT) of *Fusarium* (Hajdukiewicz et al., 1994). Constructs were transformed to the *SIX1* knock out strain (described in Rep et al., 2004) in which the *SIX1* ORF had been replaced by a hygromycin resistance gene. After ATMT zeocin resistant transformants were checked for loss of hygromycin resistance (indicating homologous recombination at the *SIX1* locus), and of those correct insertion of the cassette was checked by PCR.

To make the construct with *RFP* under the control of the constitutive *GPD* promoter, the coding sequences of the hygromycin resistance protein (APH) and the monomeric Red fluorescent protein (mRFP) were fused. For this, the mRFP coding sequence was inserted in frame after the APH-encoding sequence (*hph*) in the

binary plasmid pPK2 (Covert et al., 2001). First, an *Apal* site was created before the stop codon of the *hph* gene in pPK2 by PCR amplification of the entire pPK2 plasmid with the primers HPH-End-*Apal*-R (AAAGGGCCCTTCCTTTGCCCTCGGACGA, *Apal* site underlined) and HPH-Fwd2-*Apal* (AAAGGGCCCTAGGGATCCACTTAACGTTAC, *Apal* site underlined), followed by digestion with *Apal*, ligation and transformation to *Escherichia coli*. This resulted in plasmid pPK2-HPH-*Apal*. Then, the *mRFP* coding sequence, kindly provided by Roger Tsien (Campbell et al., 2002), was amplified by PCR with primers *mRFP*-*Apal*-F (AAAGGGCCCATGGCCTCTCCGAGGACGT, *Apal* site underlined) and *mRFP*-*Apal*-R (AAAGGGCCCTTAGGCGCCGGTGGAGTGGC, *Apal* site underlined). The PCR fragment was digested with *Apal* and ligated into the *Apal* site of pPK2-HPH-*Apal*. The resulting plasmid was checked with restriction analyses for the correct orientation, sequenced to check for PCR mistakes and called pPK2-HPH-RFP. Transcription of the *hph*-RFP fusion in this plasmid is driven by the *Aspergillus nidulans* *gpdA* promoter and terminated by the *A. nidulans* *trpC* terminator (Roberts et al., 1989).

Agrobacterium-mediated *Fusarium* transformation of Fol was performed as described in (Takken et al., 2004).

2.5. Preparation of microscope slides

For preparation of slides for confocal microscopy presented in Fig. 4, infected plants were carefully taken out of the vermiculite and washed with water. Plant material was manually sliced into ~0.5 mm thick slices with a scalpel and the pieces were placed in a drop of water on glass slides. The cover glass was used to squash the material.

For two-photon microscopy, root-surface-confocal microscopy and stereo microscopy (Figs. 3, 5 and 6 and Supplementary Fig. 2), infection was performed in a Petri dish. For microscopic inspection with two-photon or confocal microscopy, plants were taken from the Petri dish and part of the root system was cut off for examination. The root was placed on a microscope slide in water. Adjacent to the root two coverslips had been glued to the microscope slide. A third coverslip was used to cover the root, leaning on the two adjacent coverslips, thus making a bridge under which the root was held in place without being squashed (the coverslips are 170 μ m thick). Images were taken immediately.

General inspection of *GFP* expression on roots (Fig. 6 and Supplementary Fig. 2) was done while the plantlets were still in the Petri dish, on the Leica MZ FLIII stereo microscope, magnifying from 10 \times 0.8 to 10 times.

2.6. Confocal microscopy

Microscopic analysis was performed with a confocal scanning laser microscope (LSM 510, Zeiss, Germany). Excitation was provided by an Ar-ion laser (488 nm) and a HeNe laser (543 nm). Fluorescence of *GFP* was detected at 505–550 nm and plant tissue autofluorescence at 650–700 nm. Images were scanned using Zeiss Plan-Neofluar 40 \times /1.3 oil or Plan-Neofluar 100 \times /1.3 oil objectives and a pinhole at 1 airy unit. For imaging the surface of tomato roots inoculated with a double-labeled Fol strain (*Psix1::GFP* and *Pgpd::RFP*) the Zeiss LSM510 confocal microscope was used with the same lasers (488 and 543 nm). Images were scanned in a multi track, *GFP* was recorded at 505–550 nm and *RFP* through a LP560 long pass filter, with a pinhole size of 2.0 airy units. All confocal pictures were analyzed with the Zeiss LSM510 software (v3.0 SP3).

2.7. Two-photon microscopy

Two-photon imaging was done on a Leica TCS SP5 microscope, coupled to a SpectraPhysics Mai Tai, Mode-locked Ti:Sapphire La-

ser at 850 nm. Standard green and red filters were used for the detection of *GFP* (green) and plant autofluorescence (red).

2.8. GFP expression in plant cell cultures

Microconidia (10^7) from a *SIX1*-promoter-*GFP* *GDP*-promoter-*RFP* Fol transformant were used to inoculate 1 ml of a 1-week-old plant cell culture, standard cell culture medium or cell culture filtrate, obtained by filtering medium from a 1-week-old cell culture through a 0.22 μ m filter (low protein binding durapore (PVDF), Millipore). Plant cell cultures (*Arabidopsis thaliana*, tomato and *Nicotiana benthamiana*) are described in (Testerink et al., 2004) and (Van Leeuwen et al., 2007).

GFP and *RFP* fluorescence was recorded after 24 h on a wide field fluorescence microscope (BX, Olympus, Japan) using Object Image image analysis.

3. Results

3.1. *SIX1* is strongly expressed in planta but not or very weakly in axenic cultures

The *SIX1* gene encodes a protein that is secreted by *F. oxysporum* f. sp. *lycopersici* into the xylem sap during colonization of tomato plants. To investigate whether expression of *SIX1* is regulated, RT-PCR reactions on *SIX1* were performed with cDNA derived from infected plants and from axenic Fol cultures. In axenic cultures, *SIX1* mRNA levels were lower than or comparable to the level of *FEM1*, a constitutively expressed gene coding for a structural cell wall protein, here used as a reference for the amount of fungal biomass (Schoffemeer et al., 2001). Fig. 1A shows *SIX1* expression in axenic cultures under different medium conditions. Two independent experiments are shown for each condition. Standard media used to grow *Fusarium* (potato dextrose broth [PDB, rich] and Czapek Dox [CDB, minimal]) did not result in high expression levels of *SIX1*, but on a minimal medium based on 3% sucrose and 1% KNO_3 the *SIX1* expression level was comparable to that of *FEM1*. Media designed to resemble plant and/or xylem sap conditions (Dixon and Pegg, 1972; Iwai et al., 2003; Joosten et al., 1990) did not induce *SIX1* expression.

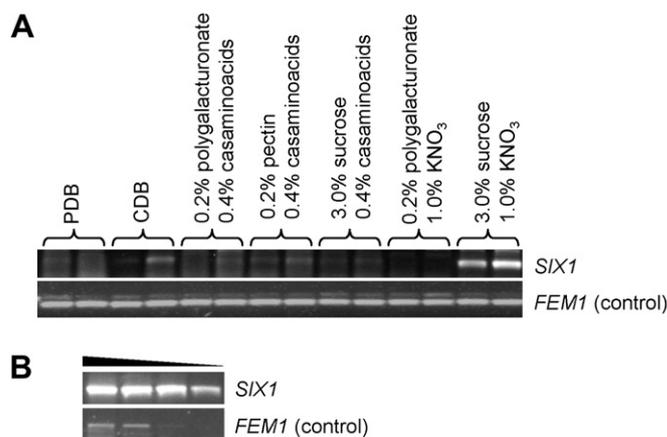


Fig. 1. *SIX1* expression is much higher *in planta* compared to expression in axenic cultures. Expression was visualized with RT-PCR on *SIX1* and *FEM1* (internal control for the amount of *Fusarium* derived cDNA). (A) cDNA extracted from Fol007 mycelium, grown axenically in the indicated medium for 2 days at 150 rpm, 25 °C. Each growth condition was tested in two separate experiments. Media were used as indicated. PDB: potato dextrose broth, CDB: Czapek Dox broth, all other media also contain 0.17% YNB (yeast nitrogen base without amino acids or ammonia) in addition to the indicated C- and N-source. (B) cDNA extracted from infected tomato seedlings, 11 days after inoculation. The first lane is an RT-PCR under standard conditions, in the subsequent lanes 10-, 100- and 1000-fold dilutions of template were used, respectively.

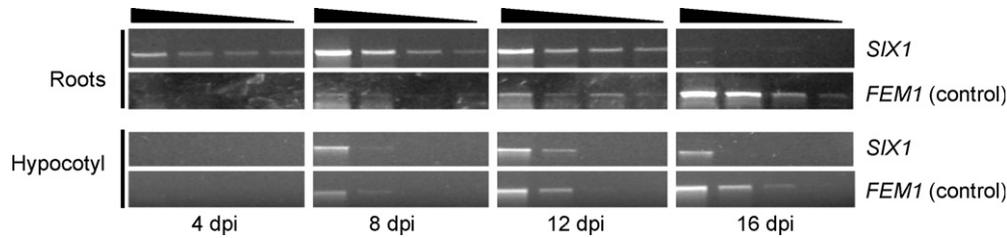


Fig. 2. Onset of *SIX1* expression during colonization of tomato roots. *SIX1* expression in roots is stronger, earlier and more transient compared to expression in hypocotyls. RT-PCR was performed on *SIX1* and *FEM1* (a control for the amount of Fol derived cDNA). RNA was extracted from infected tomato seedling roots or hypocotyls, 4, 8, 12 and 16 days after inoculation. Equal amounts of RNA were used to generate cDNA. Of each panel, representing a specific time point after inoculation, the first lane represents an RT-PCR under standard conditions and the subsequent lanes 10-, 100- and 1000-fold dilutions of template DNA.

In plants, on the other hand, *SIX1* expression greatly exceeded *FEM1* transcript levels (Fig. 1B). Using *FEM1* expression as an internal standard, *SIX1* expression *in planta* was significantly higher compared to axenic cultures. Based on these experiments, we concluded that the strong expression during infection cannot be attributed to the presence of some well known cell wall components (pectin or polygalacturonase) nor to alternative nitrogen sources. Starvation for carbon or nitrogen also failed to induce *SIX1* expression as did the use of xylem sap harvested from tomato plants as growth medium (data not shown).

3.2. During infection *SIX1* is expressed early, transiently and mainly in the roots

To determine at which stage of host colonization *SIX1* expression is induced, expression *in planta* was visualized in more detail in an RT-PCR time series of infected plants. Seedlings were har-

vested at 4, 8, 12 and 16 DPI (days post inoculation) and RNA extractions were done separately on the roots and hypocotyls. *SIX1* expression levels were compared to *FEM1* expression levels. As can be seen in Fig. 2 the amount of fungal mass, represented by the level of *FEM1* transcript, increased in time, in both roots and hypocotyls. *SIX1* expression starts very early and is predominant in the roots. It probably starts before day 4 and diminishes later. Expression in the hypocotyl was less strong, started later, and diminished later and only slightly.

To determine the differences in expression of *SIX1* between axenic cultures and different stages of host colonization more accurately, quantitative PCR (qPCR) was performed on cDNA from mycelium grown in PDB, CDB or MM (sucrose and KNO_3) and on cDNA from infected plants. We also included a 2 DPI time point in this experiment to obtain information on *SIX1* expression earlier than 4 days after inoculation. For this experiment, we used Fol *EF-1 α* as a constitutive control, as the primers designed on this gene were more reliable in the qPCR than *FEM1* primers. Assuming constitutive *EF-1 α* expression, *SIX1* expression *in planta* at 2, 4, 8 and

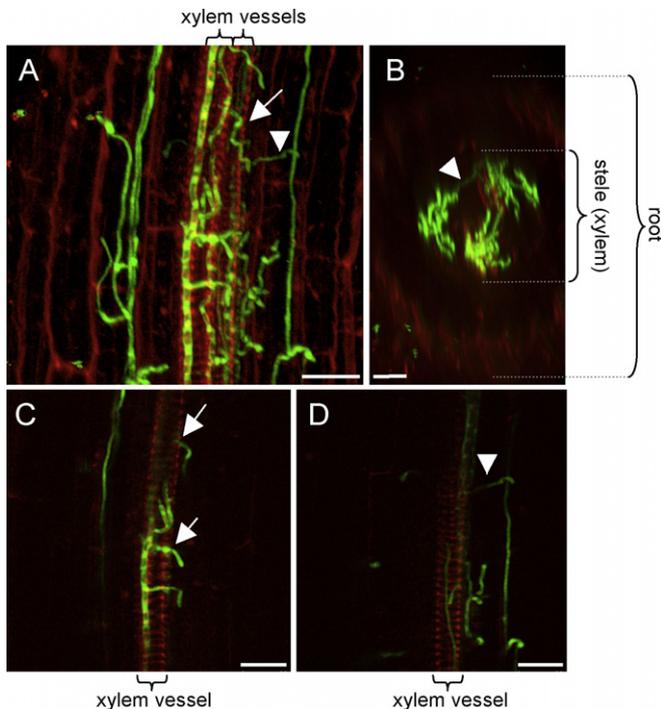


Fig. 3. Fol colonizing tomato xylem vessels, expressing *Psix1::GFP*. GFP fluorescence was visualized using two-photon imaging and is depicted in green. Tomato root autofluorescence is shown in red. The white bars are 25 μm in length. The image consists of 924 optical sections with a total depth of 280 μm . (A) A projection of the optical sections, showing colonization of the tomato xylem tissue in a longitudinal view through the root. (B) An cross-section of the same root calculated from the optical sections. (C) and (D) Two of the optical sections of the stack shown in (A).

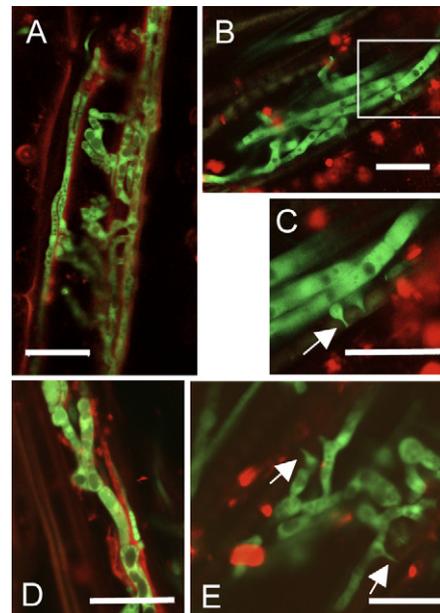


Fig. 4. Various types of hyphae in xylem vessels express *Psix1::GFP*. Similar hyphal shapes were observed in *Pgd::GFP* (constitutive) and *Psix1::GFP* (inducible) expressing strains. Confocal images show fluorescent Fol (GFP, green) colonizing the tomato xylem system (autofluorescence, red), all longitudinal sections. The white bars are 10 μm in length. (A) A xylem vessel with abnormally shaped Fol hyphae expressing *Pgd::GFP*. (B) Fol hyphae in a xylem vessel expressing *Pgd::GFP*. The hyphae make extensions that fill cavities in the cell wall of the xylem vessel elements (pit chambers). (C) Enlargement of a section of (B). (D) Xylem vessel with abnormally shaped Fol hyphae expressing *Psix1::GFP*. (E) Fol hyphae expressing *Psix1::GFP* making extensions.

14 DPI was, respectively, about 100, 200, 700 and 40 times higher than in MM confirming a strong increase of expression during infection (Supplementary Table 1). *SIX1* expression in PDB cultures was about 240 times lower than in MM and expression in CDB 2–3 times higher. The latter value is higher than apparent in the experiment shown in Fig. 1A, which might be explained by the different constitutive control that was used (*FEM1* versus *EF-1 α*).

3.3. *Psix1::GFP* is expressed in hyphae colonizing the xylem vessels

Because the Six1 protein was originally discovered in the xylem sap of infected plants, we expected the hyphae growing in xylem vessels to express *SIX1*. To test this, a strain carrying a *SIX1*-promoter-*GFP* fusion construct was made (*Psix1::GFP*). The reporter construct was designed in such a way that it was suitable for homologous recombination at the original *SIX1* locus, to avoid artificial expression due to position effects (Supplementary Fig. 1). Fol transformants harboring this construct (i.e. with the *SIX1* open reading frame replaced by *GFP*) are still pathogenic towards seedlings (as expected for a *SIX1* knockout strain (Rep et al., 2005a, 2004), data not shown), and were used to inoculate 10-day-old tomato seedlings. GFP fluorescence was imaged between 2 and 5 days after inoculation using two-photon microscopy (Fig. 3). With two-photon microscopy it is possible to image at greater depth compared to normal fluorescent microscopy (So et al., 2000), i.e. up to 300 μ m, the thickness of a small tomato root (Fig. 3B). This offered the possibility to image the Fol infection inside intact tomato roots. The Fol hyphae expressing *Psix1::GFP* grew in and along the xylem vessels. Fig. 3A shows a longitudinal view of an infected tomato root. In the center of the picture are several xylem vessels (vertical) with the typical ridged structure, heavily colonized. On the left and right side of the picture parenchyma cells are visible, not colonized by Fol. Fig. 3B is a cross-section

of the same location calculated from the stack of sections shown in Fig. 3A. It shows that colonization of Fol at this location is mainly localized to bundles of xylem vessels present in the center of this root, and surrounded by un-invaded parenchyma cells/root cortex cells. The outer layer of plant autofluorescence includes the root epidermis showing that almost the entire root was scanned. Hyphae sometimes penetrated the cell wall of a cell or vessel element (Fig. 3C, arrows) and sometimes grew along or around obstructions, as suggested by hyphae that have a step-shaped appearance (Fig. 3A, arrow). Fol is able to cross from one vessel bundle to another, as demonstrated by hyphae that connect two vessel bundles (Figs. 3A, B and D, arrowheads). All optical sections of this scan are available as a movie in the Supplementary Materials.

3.4. In xylem vessels Fol hyphae can have different shapes which all can express *Psix1::GFP*

To visualize the fungus in the xylem vessels in more detail, we sliced inoculated tomato roots (manually, \sim 0.5 mm thick) and viewed them with confocal microscopy (Fig. 4). For comparison, a Fol strain expressing GFP behind the constitutive GPD promoter (glucose 6-phosphate dehydrogenase from *A. nidulans* (Roberts et al., 1989)) was used. Hyphae of this *Pgpd::GFP* strain in xylem vessels sometimes appeared swollen or abnormally shaped (Fig. 4A), and can form extensions that fill cavities in the xylem vessel cell wall (pit chambers) (Figs. 4B and C, arrow). *Psix1::GFP*-expressing hyphae were observed in all the above situations: they were found to sometimes exhibit similar abnormal shapes, penetrate cells and make extensions to fill pit chambers (Figs. 4D and E, arrows). These results suggest that in the xylem the *SIX1* promoter is active in all hyphae, as no obvious differences in *GFP*-expressing hyphal structures were observed.

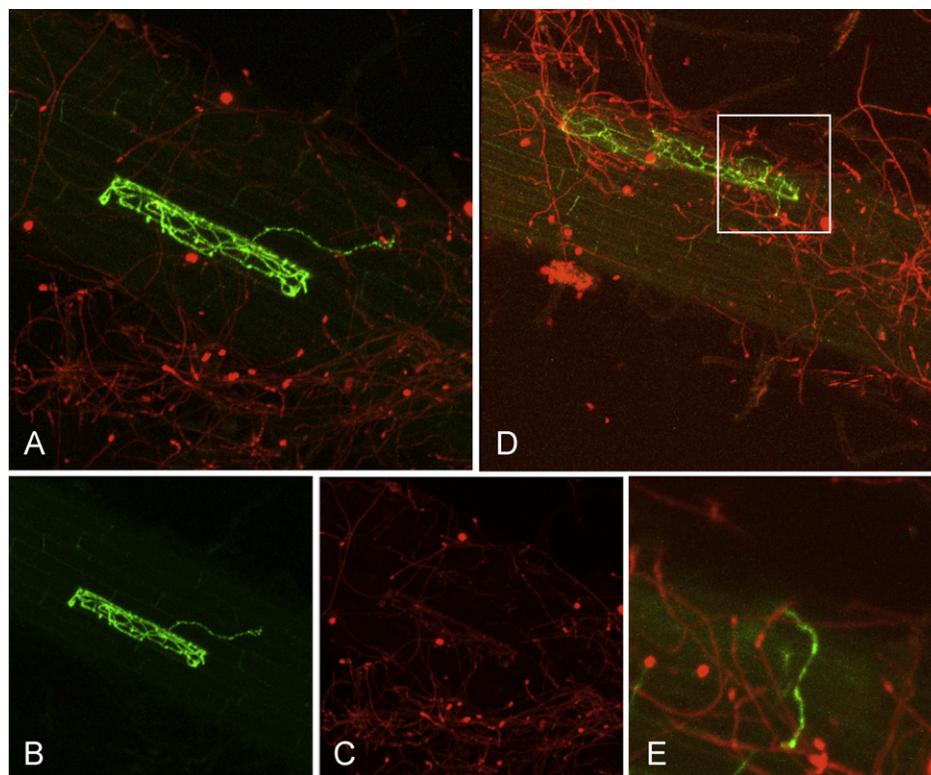


Fig. 5. Double fluorescent labeling of Fol reveals a sharp distinction between *SIX1*-expressing and *SIX1*-non-expressing hyphae. A Fol strain with *Psix1::GFP* (GFP fluorescence shown in green) and constitutive *RFP* (*Pgpd::RFP*, RFP fluorescence shown in red) colonizing a tomato root. (A–C) and (D and E) show two independent penetration events. (B) and (C) show separate GFP and RFP fluorescence, respectively, both combined in (A). (E) An enlargement of a section of (D) (scanned separately).

3.5. *Psix1::GFP* expression starts immediately upon penetration of the root

To pinpoint the moment of initial induction of *SIX1* expression, we visualized the transition of a non-*SIX1*-expressing hypha to a *SIX1*-expressing hypha. To do this, the *Psix1::GFP* strain was transformed with *RFP* (encoding red fluorescent protein) behind the constitutive *GPD* promoter (*Pgpd::RFP*) so that hyphae that do not express *Psix1::GFP* could be visualized using *RFP* fluorescence. Tomato roots were inoculated with this double-labeled *Fol* and the surface was inspected with confocal microscopy.

Fol was found to form a mycelial mass around the tomato root and the hyphae on the outside of the root did not express *Psix1::GFP*, only *Pgpd::RFP* (Fig. 5). *Psix1::GFP*, on the other hand, appeared to be expressed after (or shortly before) the moment *Fol* actually enters the root. We observed *Psix1::GFP* expression at distinct locations along the root (roughly 20 'infection patches' per root system). At these entry points, GFP was present in the hyphae inside one or a few cells of the outer root tissue and in one or two hyphae outside the root connected to hyphae inside (Fig. 5). Apparently, the *SIX1* promoter is activated immediately upon penetration of the plant root epidermis. The optical sections of these scans are available as movies in the [Supplementary Materials](#).

3.6. *Psix1::GFP* is not expressed on dead plant material

Remarkably, *Psix1::GFP* expression, but not root colonization, was lost when the tomato seedlings were killed before inoculation. Killing of the seedlings was done either by boiling, submersion in 70% ethanol, snap freezing, removal of all non-root tissue (including hypocotyl, cotyledons, leaves and meristem) or BASTA treatment. BASTA treatment needed to be performed 1 day prior to inoculation, the other methods could be applied just before inoculation and still result in loss of *SIX1* expression. Also, ground or chopped seedling roots did not induce *Psix1::GFP* expression when provided in agar plates. Similarly, root exudate could not trigger expression of *Psix1::GFP* (data not shown). This indicates that the cue that induces *SIX1* expression is only present in or around living tomato roots and not in their lifeless components or exudates. When the seedlings were put in the dark 1 day prior to inoculation and during infection, the number of infection patches was somewhat reduced, but still significant ([Supplementary Fig. 2](#)). This suggests that the ability to photosynthesize is not the main prerequisite for *SIX1* expression.

3.7. On non-host plants *Psix1::GFP* expression is rare or absent

To see if the cue that induces *SIX1* expression is specific for tomato, expression of *Psix1::GFP* was also tested on non-host plants. On all non-host seedlings that were tested (*Arabidopsis thaliana*, *Zea mays* and *Nicotiana benthamiana*) *Fol* was able to colonize the root surface (visualized with the *Pgpd::GFP* strain [constitutive], data not shown). None of these plants developed *Fusarium* wilt disease, confirming that these are indeed non-host plants for *Fol*. On *N. benthamiana* and *Z. mays* only very occasionally *Psix1::GFP* expression was observed in single, short hyphae (on *N. benthamiana* on average once per root system), but not on *A. thaliana* (Fig. 6). Possibly, the *Psix1::GFP* expression on *N. benthamiana* and *Z. mays* was the result of rare instances of limited entry of root tissue. However, in this experiment we could not determine whether the general absence of expression of *Psix1::GFP* on non-host plants was caused by the inability of the tissue to induce *SIX1* expression or that it was a result of the inability of *Fol* to enter these (non-host) roots and reach the place where induction normally would occur.

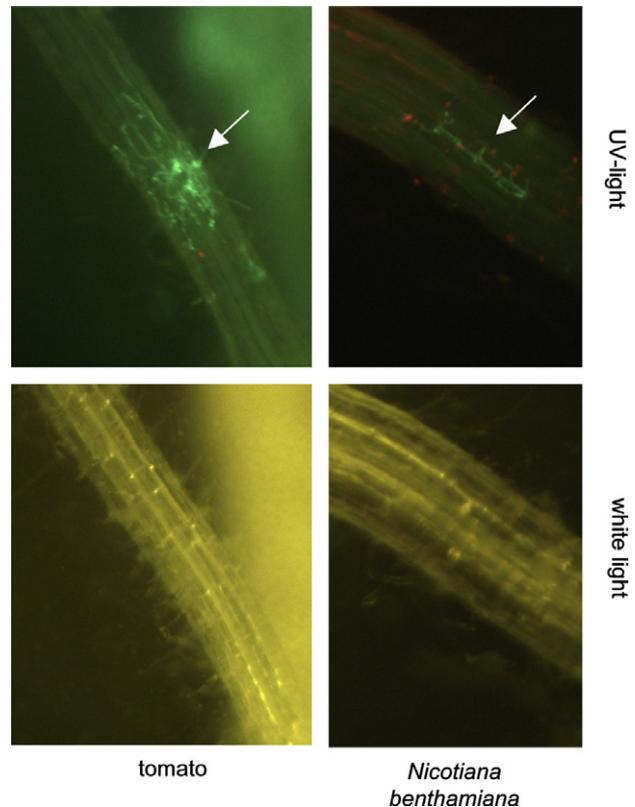


Fig. 6. *SIX1* expression is occasionally induced in non-host plants. Tomato or *Nicotiana* seedlings inoculated with a *Fol* strain that expresses GFP under control of the *SIX1* promoter (*Psix1GFP*), 2 days after inoculation. The white arrow points to the place where *Fol-Psix1GFP* enters the root and expresses GFP. In tomato seedlings infection would progress if the experiment would be prolonged, whereas in *N. benthamiana* seedlings this picture shows the maximum of GFP-expressing hyphae that we observed.

3.8. Plant cell cultures can induce *Psix1::GFP* expression

To better distinguish between these possibilities we investigated whether cell cultures could be used as a system to monitor the induction of *SIX1* expression without the need for actual infection. We found that cell cultures of tomato were indeed able to induce *SIX1* expression. When a conidial suspension of *Fol* was added to tomato MSK8 cell cultures, after 24 h a fraction (an estimated 5–20% of the hyphae) expressed *Psix1::GFP* (Fig. 7). Tobacco BY2 cell cultures had the same effect (Fig. 7), but an *A. thaliana* cell culture did not induce expression (data not shown). Freezing or boiling the plant cells before inoculation prevented the induction of expression (data not shown). When observed under the microscope, it seemed that the hyphae expressing *Psix1::GFP* did not necessarily need to be in physical contact with the plant cells, nor did we observe penetration of the plants cells. However, when only the medium of the MSK8 tomato cell cultures was used (after removal of the MSK8 cells) expression was reduced to almost background levels when compared to the situation where the cells were present (Fig. 7, panels culture filtrate). This shows that the presence of plant cells (either host [MSK8] or non-host [BY2]) is required for *Psix1::GFP* expression.

4. Discussion

In this work we show that expression of the *SIX1* gene is very high at the earliest stages of infection, and very low or absent in fungi growing on the root surface or in artificial medium (axenic cultures). None of the culture conditions or tomato extracts tested

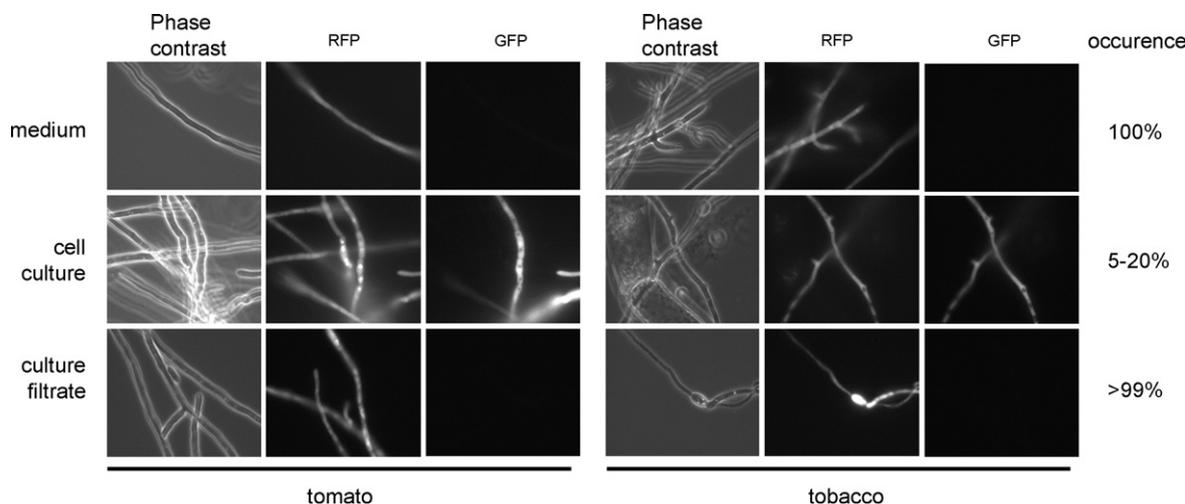


Fig. 7. *Fol* expresses *SIX1* (*Psix1::GFP*) in plant cell cultures. A double-labeled *Fol* strain (with *Psix1::GFP* and *Pgpd::RFP* [constitutive]) 24 h after addition to plant cell cultures. Left hand panels: tomato MSK8 cell cultures and controls; right hand panels: tobacco BY2 cell cultures and controls. Pictures were taken in phase contrast or under standard conditions to show GFP or RFP fluorescence. The medium used to maintain the tomato or tobacco cell cultures (row 'medium') did not induce *Psix1::GFP* expression. The tomato and tobacco cell cultures themselves induced *Psix1::GFP* expression in approximately 5–20% of the hyphae (row 'cell culture') but when the medium in which tomato or tobacco cells had grown was used (row 'culture filtrate') *Psix1::GFP* expression was reduced to an estimated <1% of the hyphae.

was able to induce *SIX1* expression to the levels found during infection. Even tomato seedlings that were completely intact, but dead, failed to induce expression. This apparently excludes nutritional signals as a host cue for induction of *SIX1* expression, but instead hints towards a signal that is transient and requires the continuous presence of living plant cells. Perhaps this elusive cue is a substance that quickly degrades or evaporates or that is in another way linked to the presence of living material.

SIX1 expression is induced in tomato cell cultures, showing that specific structural features or specific root cells are not required for induction of *SIX1* expression. A tobacco cell culture (BY2) was capable of inducing *SIX1* expression to a similar level as a tomato (MSK8) cell culture, showing that the cue is not host specific. This suggests that the rare *Psix1::GFP* expression that we observed on non-host plants may be attributed to occasional and limited penetration of the outer root (failed infection attempts), rather than the absence of the proper signals in these plants.

It is not clear what the biological relevance is of the low, but detectable, *SIX1* expression observed in CDB and minimal medium with sucrose and KNO_3 or how to interpret the observation that only 5–20% of hyphae express *SIX1* when growing in tomato cell cultures. While *in planta* all hyphae penetrating the root seem to strongly express *SIX1*, in these two circumstances only a fraction of the *SIX1*-expressing capacity is triggered. Curiously, penetration of plant cells does not seem to occur in cell cultures, whereas penetration of the tissue is a requirement for expression in plants. The slight *SIX1* expression in sucrose/ KNO_3 medium seems to be caused by all hyphae very weakly expressing *SIX1*, with some having somewhat higher expression (*Psix1::GFP* expression, data not shown), whereas the expression in cell cultures is very strong in a fraction of the hyphae and absent in the rest of the mycelium. This suggests that the regulation of *SIX1* expression might be mediated by different repressing and/or activating pathways, which only work together optimally *in planta*.

The *SIX1* promoter does not contain any known motifs. We did notice that *SIX1* and the other known *SIX* genes (*SIX2*, *SIX3* and *SIX4*, all encoding small proteins secreted in xylem during infection) share some common characteristics in the promoter (Houterman et al., 2007). In or close to each of the promoters a transposon (miniature impala) is present and each contains a variant of the motif 'TCGGCAGTTW'. The function of these features is still unclear. For other plant induced genes, like *MIG1* and *LtmM*, serial

deletions in the promoter region provided information on the different effects of subregions on expression levels (Basse et al., 2000; May et al., 2008). *LtmM* expression was also shown to vary depending on the type of infected tissue (May et al., 2008).

Host signals for induction of virulence genes that have been identified previously comprise hydrophobic surfaces or ridged surfaces that can influence the formation of infection structures (Beckerman and Ebbole, 1996; Collins et al., 2001; Kolattukudy et al., 1995; Lee and Dean, 1993), as well as relatively stable chemical substances. In some cases topographical and chemical signals act synergistically (Collins et al., 2001; Soanes et al., 2002) and for some genes only a chemical signal is sufficient. Some examples of the latter are cell wall components that induce expression of cell wall degrading enzymes (Huertas-Gonzalez et al., 1999; Ruiz-Roldan et al., 1999; Sexton et al., 2000), phytoalexins that induce the enzymes that can detoxify them (e.g. pisatin demethylase 1) (Straney and VanEtten, 1994), and the amino acids homoserine and asparagine, found in high levels in pea seedling hypocotyls and capable of inducing expression of *pelD* (Yang et al., 2005).

The apparent inability to induce full *SIX1* expression with anything but living material points to a type of host signal that has not been described before. Translated to the natural situation, our findings show that *F. oxysporum* is highly effective in distinguishing dead (plant) material from living plants. For a fungus like *F. oxysporum* that can live on dead plant material as well as colonize living plants, this is a trait that could be very useful to prevent unnecessary switches from saprophytic to combat mode.

Acknowledgments

The authors gratefully acknowledge the late Wijnand Takkenberg for assistance with microscopy, Bastiaan Bargmann, Christa Testerink, Bas van Schooten, Joop Vermeer, Ringo van Wijk and Wilfried Jonkers for cell culture maintenance, Roger Tsien for kindly providing the *mRFP* sequence and Harold Lemereis, Ludek Tikovsky and Thijs Hendrix for plant and greenhouse management.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.06.002.

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