The *Fusarium oxysporum* Effector Six6 Contributes to Virulence and Suppresses I-2-Mediated Cell Death

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Plant pathogens secrete effectors to manipulate their host and facilitate colonization. *Fusarium oxysporum* f. sp. *lycopersici* is the causal agent of Fusarium wilt disease in tomato. Upon infection, *F. oxysporum* f. sp. *lycopersici* secretes numerous small proteins into the xylem sap (Six proteins). Most Six proteins are unique to *F. oxysporum*, but Six6 is an exception; a homolog is also present in two *Colletotrichum* spp. *SIX6* expression was found to require living host cells and a knockout of *SIX6* in *F. oxysporum* f. sp. *lycopersici* compromised virulence, classifying it as a genuine effector. Heterologous expression of *SIX6* did not affect growth of *Agrobacterium tumefaciens* in *Nicotiana benthamiana* leaves or susceptibility of *Arabidopsis thaliana* toward *Verticillium dahliae*, *Pseudomonas syringae*, or *F. oxysporum*, suggesting a specific function for *F. oxysporum* f. sp. *lycopersici* Six6 in the *F. oxysporum* f. sp. *lycopersici*–tomato pathosystem. Remarkably, Six6 was found to specifically suppress I-2-mediated cell death (I2CD) upon transient expression in *N. benthamiana*, whereas it did not compromise the activity of other cell-death-inducing genes. Still, this I2CD suppressing activity of Six6 does not allow the fungus to overcome I-2 resistance in tomato, suggesting that I-2-mediated resistance is independent from cell death.

Plants are challenged by a wide variety of microbes, including viruses, bacteria, fungi, and oomycetes (Agrios 1997). Thanks to their multilayered immune system, plants recognize and defeat most potential invaders (Jones and Dangl 2006). However, pathogenic microbes evolved various mechanisms that suppress plant immunity, resulting in disease (Win et al. 2012). Mostly, this disease-causing ability is mediated by secreted virulence factors—called effectors—that alter host-cell function or otherwise promote host colonization (Hogenhout et al. 2009). Therefore, identification and characterization of effectors provide insights into the mechanisms underlying susceptibility to pathogens.

Pathogenic strains of the fungus *Fusarium oxysporum* invade roots of a broad variety of plant species and cause wilt disease through colonization of the xylem vessels (Tjamos and Beckman 1989). Each strain can only infect one or a few closely

related hosts, and strains infecting the same host are grouped into the same host-specific form or forma specialis (f. sp.) (Armstrong and Armstrong 1981; Katan 1999; Katan and Di Primo 1999). Thus far, most effectors have been identified from *F. oxysporum* strains infecting tomato and belonging to f. sp. *lycopersici*. The first *F. oxysporum* f. sp. *lycopersici* effectors that have been identified are proteins encoded by avirulence (AVR) genes; namely *Avr1*, *Avr2*, and *Avr3* (Houterman et al. 2008, 2009; Rep et al. 2004). Avr proteins trigger host resistance upon recognition by corresponding resistance (R) proteins in plants. Hence, *F. oxysporum* f. sp. *lycopersici* is divided into three races named after the corresponding R gene that recognizes the Avr protein. Functional analyses revealed that *Avr2* and *Avr3* trigger resistance in tomato plants carrying the R genes *I-2* and *I-3*, respectively (Rep et al. 2004; Houterman et al. 2009). In addition to triggering resistance, both proteins are required for full pathogenicity on susceptible tomato lines (Houterman et al. 2009; Rep et al. 2005), classifying them as genuine effectors. *F. oxysporum* f. sp. *lycopersici* effector *Avr1*, recognized by *I* and *I-1*, does not contribute to virulence on susceptible cultivars but suppresses *I-2*- and *I-3*-mediated resistance (Houterman et al. 2008). All three Avr proteins were originally identified in the xylem sap of *F. oxysporum* f. sp. *lycopersici*-infected tomato plants (Houterman et al. 2007). In addition to the Avr proteins, 11 other secreted-in-xylem (Six) proteins have been identified thus far; all are relatively small (<300 amino acids [aa]), generally cysteine rich, and produced with a signal peptide for secretion (Houterman et al. 2007; Lievens et al. 2009; Ma et al. 2010; Schmidt et al. 2013). Six proteins generally do not have sequence homology to proteins with known functions (Rep 2005), prohibiting reliable prediction and assignment of specific functions to these proteins. Expression of at least some *SIX* genes (e.g., *AVR3*, *SIX2*, *AVR2*, and *SIX5*) is regulated by the transcription factor Sge1; a knockout of the gene encoding this regulator impairs pathogenicity of the fungus and abolishes *SIX* gene expression (Michielse et al. 2009). Analysis of the *F. oxysporum* f. sp. *lycopersici* genome revealed that most *SIX* genes are localized on chromosome 14 (Ma et al. 2010; Schmidt et al. 2013). This lineage-specific chromosome is required for infection of tomato and can be shuttled via horizontal transfer from *F. oxysporum* f. sp. *lycopersici* to a nonpathogenic strain, converting this strain into a tomato pathogen (Ma et al. 2010). This highlights the importance of the *SIX* gene-carrying chromosome for the ability of the fungus to cause disease. Until now, *Avr2* and *Six5* have exclusively been found in *F. oxysporum* f. sp. *lycopersici*, suggestive of a tomato-specific function for these two effectors. For other Six proteins, such as *Avr1*, *Six6*, *Six7*, *Six8*, and *Six9*, close homologs are present in other formae speciales (Chakrabarti et al. 2011; Lievens et al. 2009; Thatcher et al.

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2012), implying a more generic function for these proteins. Among these, Six6 is of special interest because homologs are also present in two other fungal plant pathogens, *Colletotrichum orbiculare* and *C. higginsianum* (Gan et al. 2013; Kleemann et al. 2012).

Here, we describe the functional characterization of *F. oxysporum* f. sp. *lycopersici* Six6 and show that it is a genuine effector protein important for pathogenicity.

RESULTS

F. oxysporum f. sp. *lycopersici* Six6 homologs are present in several formae speciales of *F. oxysporum* and in two *Colletotrichum* spp.

Upon infection, *F. oxysporum* f. sp. *lycopersici* secretes Six6 into the xylem sap of tomato (Lievens et al. 2009). The Six6 precursor is encoded by a gene spanning 648 bp (GenBank accession ACY39286.1). SignalP 4.0 (Petersen et al. 2011) predicts a 16-aa signal peptide yielding a mature protein of 199 aa. Six6 contains seven cysteines and has a predicted molecular weight of 22 kDa (ProtParam) (Wilkins et al. 1999). National Center for Biotechnology Information BLAST searches did not reveal hits to proteins with known functions (data not shown) but uncovered the presence of Six6 in other *F. oxysporum* formae speciales. In total, six Six6 homologs were identified in *F. oxysporum* ff. spp. *melonis* (ACY39292.1),

niveum (ADO60274.1), *radicis-cucumerinum* (ACY39291.1), *passiflorae* (ADO60275.1), *vasinfectum* (ADO60273.1), and *cubense* (Chakrabarti et al. 2011; Lievens et al. 2009; Schmidt et al. 2013; our unpublished observations), respectively. The amino acid sequences of *F. oxysporum* ff. spp. *melonis*, *niveum*, and *radicis-cucumerinum* Six6 are identical while the others diverge (Fig. 1). *F. oxysporum* f. sp. *passiflorae* Six6 shows the highest similarity with *F. oxysporum* f. sp. *lycopersici* Six6, having 93% sequence identity at the protein level. Six6 of *F. oxysporum* ff. spp. *melonis* and *vasinfectum* share 90 and 89% sequence conservation, respectively, with *F. oxysporum* f. sp. *lycopersici* Six6. The most distantly related Six6 is that of *F. oxysporum* f. sp. *cubense*, having 60% identity. The number of nonconservative amino acid substitutions is overall very low (e.g., between *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *melonis* Six6, there is only one nonconservative change, Asn to Leu).

Outside the *F. oxysporum* species complex, Six6 homologs were found in *C. higginsianum*, called ChEC36, and in *C. orbiculare*. ChEC36 is 9 aa longer than the Six6 homologs from *F. oxysporum* and has 38% protein sequence identity with *F. oxysporum* f. sp. *lycopersici* Six6. The Six6-like *C. orbiculare* protein is 223 aa long and shares 57% identity with *F. oxysporum* f. sp. *lycopersici* Six6. Alignment of the six Six6 homologs (Fig. 1) shows that the N-terminal half of the mature protein is less conserved than the C-terminal half (10 versus 33%). In the C-terminal half, three conserved motifs (ExCVx

	Signal peptide -----															
Fol Six6	MKLALIASIL	AAGCVAGPLA	QTE-SESADV	AEHTINYIDI	APEEFEPKKA	-NLSSLVSRD	TLPVSTCPAG									
Fop Six6	MKLALIASIL	AAGCVAGPLA	QTE-SESADV	AEHTINYIDI	APEEFEPKKA	-NLSSLVSRD	TLPDHTCPAG									
Fom*Six6	MKLALIASIL	AAGCVAGPLA	QTE-PESADV	AEHTINYIDI	APEEFEPKKA	-DLSSLVSRD	TLPVTNCPAG									
Fov Six6	MKLALIASIL	AAGCVAGPLA	QTE-SESADV	AEHTINYIDI	APEEFEPKKA	-NLSSLVSRD	TLPP-TCPRY									
Foc Six6	MKVALVISIF	IASCIASPLD	PAKTPSPPG	AEHTLNVDI	TPTGPEFGNV	NGSSALISRD	TLPHTACPAG									
ChEC36	-MFSLLVXIL	PVSCVVIPLS	QVEDSATKLL	LDVAPVELDV	ALAVHEPLNT	TSLAIIGPRD	ARTNPPCPPG									
Co Six6	MKELTVVAIL	AASCAAPLS	EVDGS-GNSI	PTVELSPVDG	PPAELEPLNA	-NLTSLVARD	TGPDRTCPAG									
71																
Fol Six6	QKYDRSVCYK	ADKIRSFCAV	NPRSNREKIT	DTPCQPREIC	VQRNLSNGKS	FAKCIPIVDL	VEWKTSAAGN									
Fop Six6	QTYDRSVCYK	ANTIRSFCAV	NPRSNREKIT	NTPCKPQEIC	VQRRLSNGKS	FAKCIPIVDL	VEWKTSAAGN									
Fom*Six6	QTYDRSVCYK	ANTIRSFCAV	NPRSNREKIT	DTPCKPQEIC	VQRSLNNGKS	YAKCIPVVDL	VQWKTSPNGN									
Fov Six6	TTYDESICIT	AGVVRSSCVF	TADPGVRRGV	NANCNKNEIC	VERNLSNGKR	YAKCIPVVDL	VEWKTSAAGN									
Foc Six6	QTYDRSVCYK	SHTIRSFCAV	NPRSNREQIT	DTPCNSGEVC	VQRRLSSGKS	YAKCLPVRDL	VSWRTDPDGD									
ChEC36	QVFDRSICYT	SGIISRYCNT	VPQSPNGDRK	EVFCANNEVC	VQRLLSNGKP	FADQCQLISSL	IQRWTDPIGS									
Co Six6	QTYDRSVCYK	YNTIRRFCAV	NPRSNREDIK	NTPCRADIEIC	VQRNLSNGKS	FAQCIELVKL	IRWKTG-AGN									
141																
Fol Six6	KEGCTTTSVN	PAGYHHLGTI	VYDINKNPIE	VDKISYFGEP	GNVNEGIGGS	TSYFSSDNFQ	FSKSRYMKS									
Fop Six6	KEGCTTTSVN	PAGYHHLGTI	VYDINKNPIE	VDKISYFGEP	GNVNEGIGGS	TSYFSSDNFQ	FSKSRYMKS									
Fom*Six6	KEGCTTTSVN	PAGYHHLGTI	VYDVNKNPIE	VDKISYFGEP	GNVNEGIGGS	TSYFSSDLFH	FSKSRFMKS									
Fov Six6	KEGCTTTSVN	PAGNHHLGTI	VYDVNKNPIQ	VDKISYFGEP	GNVDEGIGGS	TSYFSSNLFH	FSKSRYMKS									
Foc Six6	KEGCTTVEAN	PIGYHSLATM	IYDINNPNPIQ	VDKIRYLGEF	GDANEGIGGS	VSNFSSDRFR	FTGSNYMKGK									
ChEC36	KEGCTTASSK	RGKTHAMGTI	VYDINEHPIQ	VGKIKYFGEP	GDVPKGTSGT	TNNFASDSD	FDNGHFMRIC									
Co Six6	AEGCTSVSVK	SGGYHSLGTI	LYDTNSNPIQ	VDKIRYLGEF	GDVNEGIGGG	SSYFNSDFYN	FGGGHSMKAC									
211																
Fol Six6	DMRTFMT---	-----														
Fop Six6	DMRKFTMT---	-----														
Fom*Six6	GMHTFMT---	-----														
Fov Six6	DMRTFMT---	-----														
Foc Six6	DIEFIYY---	-----														
ChEC36	ITSMGNGNLN	AFSWL---														
Co Six6	VFTGGYGNLN	AFSWVI---														

Fig. 1. Six6 homologs carry conserved motifs in their C-terminal part. Alignment of *Fusarium oxysporum* f. sp. *lycopersici* Six6 and Six6 homologs from *F. oxysporum* and two *Colletotrichum* spp. The signal peptide sequence predicted by Signal P is depicted, conserved amino acids are highlighted, and conserved motifs are underlined. Fol = *F. oxysporum* f. sp. *lycopersici*, Fop = *F. oxysporum* f. sp. *passiflorae*, Fom = *F. oxysporum* f. sp. *melonis*, Fov = *F. oxysporum* f. sp. *vasinfectum*, Foc = *F. oxysporum* f. sp. *cubense*, ChEC36 = *Colletotrichum higginsianum*, and Co = *C. orbiculare*. Fom*: because Six6 sequences of *F. oxysporum* ff. spp. *melonis*, *niveum*, and *radicis-cucumerinum* are identical, only the *F. oxysporum* f. sp. *melonis* sequence is shown.

RxLSxGK, GxxEGCT, and TxxYDxNxxPIxVxKIxYxGEpG) can be discerned.

In summary, Six6 is relatively widespread and well conserved within the *F. oxysporum* complex. Furthermore, homologs are present in *C. higginsianum* and in *C. orbiculare*.

SIX6 is expressed during early stages of *F. oxysporum* f. sp. *lycopersici* infection.

Six6 was originally identified in xylem sap of 7-week-old tomato plants 2.5 weeks after infection with *F. oxysporum* f. sp. *lycopersici* (Houterman et al. 2007; Lievens et al. 2009). To determine whether *SIX6* is also expressed during earlier stages of infection, RNA was collected from *F. oxysporum* f. sp. *lycopersici*-infected tomato seedlings 4, 8, and 12 days postinoculation (dpi). Expression of *SIX6* was monitored using reverse-transcriptase polymerase chain reaction (RT-PCR) and a *SIX6* transcript could be detected at all selected time points in infected plants but not in mock-inoculated controls (Fig. 2A). Expression of *SIX6* was not detected in *F. oxysporum* f. sp. *lycopersici* mycelium when grown in synthetic medium but the transcript was found when grown in the presence of suspension-cultured tomato cells (Fig. 2B). In conclusion, *SIX6* is expressed at early and late stages of host infection and its expression requires the presence of living host cells.

***SIX6* knockouts of *F. oxysporum* f. sp. *lycopersici* show reduced virulence.**

To determine the role of Six6 in virulence, *SIX6* knockout mutants were generated in *F. oxysporum* f. sp. *lycopersici* by replacing *SIX6* with a hygromycin resistance cassette. PCR-based screening of 92 hygromycin-resistant transformants identified 23 transformants that had undergone an in locus recombination resulting in deletion of the *SIX6* gene.

Six independent *SIX6* knockout strains were selected for further analysis. To assess a possible contribution of *SIX6* to virulence, 10-day-old susceptible tomato seedlings were inoculated with either i) the *SIX6* knockout strains, ii) two inde-

pendent ectopic transformants not having a *SIX6* gene deletion, iii) wild-type *F. oxysporum* f. sp. *lycopersici* (Fol007, race 2) or iv) water, to serve as a mock control. Three weeks after inoculation, plant weight and disease index were measured. The disease index (Rep et al. 2004) was scored on a scale from 0 to 4,

Fig. 2. *SIX6* is expressed during early and late stages of infection and its expression is triggered by living host cells. Reverse-transcriptase polymerase chain reaction analysis of *SIX6* or *FEM1* expression using RNA isolated from **A**, roots of tomato seedlings, which were either mock or *Fusarium oxysporum* f. sp. *lycopersici* inoculated and harvested 4, 8, and 12 days after inoculation; or **B**, *F. oxysporum* f. sp. *lycopersici* cultivated in either synthetic (NO₃) medium or in the presence of suspension cultured tomato (MSK8) cells. Water was included as a negative control (-), while genomic DNA of *F. oxysporum* f. sp. *lycopersici* (+) and its *FEM1* gene were used as positive controls.

Fig. 3. *Fusarium oxysporum* f. sp. *lycopersici* *SIX6* knockout mutants are reduced in virulence. Susceptible tomato seedlings were inoculated with wild-type *F. oxysporum* f. sp. *lycopersici* (Fol007), strains in which *SIX6* was deleted (Δ *SIX6* numbers 1 to 6), or a knockout strain (Δ *SIX6*-3) in which *SIX6* was reintroduced (Δ *SIX6*-3 + *SIX6*-1 to *SIX6*-5). As a control, mock-inoculated plants were used (mock), and two transformants (ect), in which the *SIX6* deletion construct was integrated ectopically. **A**, Average plant weight of 20 plants is plotted against the average disease index of the same plants. Deletion of *SIX6* impairs pathogenicity as shown by the increased plant weight compared with infection with the wild type or the two ectopic transformants. **B**, Two of the five independent knockout strains carrying the *SIX6* complementation construct display a similar average disease index and plant weight as the *F. oxysporum* f. sp. *lycopersici* wild type. Error bars represent the standard error. Clustering is based on an analysis of variance (Fisher's protected least significant difference significant at 95%) using either the disease index (solid line) or plant weight (dashed line).

with 0 having no symptoms (no wilting, no brown vessels) and 4 being either dead or small and wilted (Supplementary Fig. S1 shows representative examples of the disease classes). The disease index plotted against the plant weight of the *F. oxysporum* f. sp. *lycopersici* bioassay in a dot plot is shown in Figure 3A. The different treatments are clustered based on significant differences in diseases index (solid lines) or plant weight (dotted lines). Significant differences between the groups were determined in an analysis of variance (ANOVA) using Fisher's protected least significant difference (PLSD) at 95%, resulting in three distinct classes for the disease index and five for weight (Supplementary Table S1). All *SIX6* knockout strains exhibited a significant increase in plant weight compared with the wild-type Fol007 or the ectopic controls (Fig. 3A), indicating a reduction in fungal virulence. Surprisingly, however, a significant reduction in disease index was only observed for two of the six *SIX6* knockout strains (Δ *SIX6*-1 and Δ *SIX6*-3 knockouts).

To ascertain that the reduced virulence of the mutants was indeed caused by loss of *SIX6*, mutant Δ *SIX6*-3 was transformed with a *SIX6* complementation construct. PCR confirmed the presence of an ectopically inserted construct in five transformants and virulence of these strains was assessed using bioassays. One transformant (Δ *SIX6*-3 + *SIX6*-4) showed fewer disease symptoms than the *SIX6*-3 knockout. This impairment in virulence was possibly due to a mutation caused by the ectopic insertion of the *SIX6* construct or an unlinked mutation due to the transformation procedure. Statistical analysis (ANOVA with PLSD at 95%) showed that two transformants (Δ *SIX6*-3 + *SIX6*-1 and Δ *SIX6*-3 + *SIX6*-3) cluster together with the *SIX6* knockout with regard to the disease index and plant weight, indicating no complementation of virulence (Fig. 3B). However, in the two other *SIX6* transformants (Δ *SIX6*-3 + *SIX6*-2 and Δ *SIX6*-3 + *SIX6*-5), pathogenicity was fully restored. These two complementants cluster with the wild-type Fol007 and have both a high disease index and a strong reduction in plant weight, confirming full restoration of virulence (Fig. 3B). Hence, the reduction in virulence of the *SIX6* knockout can be functionally complemented using an ectopically expressed *SIX6* gene.

To analyze the expression of *SIX6* in the *SIX6*-3 knockout and the derived complementants, RT-PCR was performed on RNA isolated from roots of infected tomato seedlings (Fig. 4). To assess the presence of fungal RNA, confirming successful *F. oxysporum* f. sp. *lycopersici* infection, *FEM1* was used as a positive control. An *FEM1* transcript was detected in cDNA from all inoculations except for the mock control. Notably, the intensity of the *FEM1* bands varied for the respective comple-

mentation lines and, in general, the strains exhibiting the strongest increase in plant weight (Δ *SIX6*-3 and Δ *SIX6*-3 + *SIX6*-4) showed the lowest amount of *FEM1* amplification product, indicative for a lower abundance of the fungus in these infected plants. Next, expression of *SIX6* was analyzed, and a *SIX6* amplification product was found for wild-type Fol007 and all *SIX6* complementants (Fig. 4) but not for the Δ *SIX6*-3 strain, further confirming that it is a genuine knockout. Because the control without reverse transcriptase (-RTase) did not show a *SIX6* amplification product, it can be excluded that the bands originate from contaminating genomic DNA. In conclusion, because deletion of *SIX6* impairs *F. oxysporum* f. sp. *lycopersici* pathogenicity, which can be restored by ectopic complementation, *SIX6* can be considered a genuine *F. oxysporum* f. sp. *lycopersici* effector gene.

Six6 does not enhance virulence of *Agrobacterium tumefaciens* in *Nicotiana benthamiana* or alter susceptibility to *Verticillium dahliae* or *F. oxysporum* in *Arabidopsis thaliana*.

To test whether the virulence-promoting activity of Six6 extends to pathogens other than *F. oxysporum* f. sp. *lycopersici*, its ability to enhance virulence of a bacterial pathogen was assessed. The disarmed *Agrobacterium tumefaciens* strain GV3101 was transformed either with a binary vector encoding solely a tandem affinity purification (TAPi) tag, or with the same vector carrying a C-terminal TAPi-tagged *SIX6*. The sequence encoding the endogenous signal peptide of Six6 was removed (dSP*SIX6*) to enforce a cytosolic localization of the protein. As a positive control, the *Pseudomonas syringae* effector gene *AvrPto* was used. When *AvrPto* was expressed in *Nicotiana benthamiana* leaves using *A. tumefaciens*, its bacterial growth was increased (Hann and Rathjen 2007).

When grown in liquid culture, no growth differences were found between *A. tumefaciens* carrying the empty TAPi binary vector or vectors containing *SIX6* or *AvrPto* (data not shown), showing that the mere presence of these transgenes did not affect bacterial growth. In planta growth of the transformed bacteria was monitored at two time-points after infiltration in *N. benthamiana* leaves (Fig. 5A). *A. tumefaciens* containing the empty TAPi vector typically grew 1 log CFU cm⁻² in 4 days. Expression of *SIX6* did not alter growth of *A. tumefaciens* in planta; the *SIX6* transformants grew at the same rate and to the same titer as the empty vector control (1 log CFU cm⁻²/2 days) (Fig. 5A). The positive control, *A. tumefaciens* carrying *AvrPto*, grew both faster and to higher titers, >2 log CFU cm⁻²/2 days. The lack of an Six6-induced phenotype is not due to the lack of *SIX6* expression because the protein

Fig. 4. *SIX6*-complemented *SIX6* *Fusarium oxysporum* f. sp. *lycopersici* knockouts express *SIX6* during plant infection. Reverse-transcriptase polymerase chain reaction analysis of *SIX6* and *FEM1* using RNA isolated from roots of 10-day-old seedlings inoculated with Fol007, the Δ *SIX6*-3 knockout-mutant, or one of the five knockout strains complemented with *SIX6*. Mock-inoculated seedlings and water were included as a negative control (-) while genomic DNA of *F. oxysporum* f. sp. *lycopersici* (+) and its *FEM1* gene were used as positive controls.

could readily be detected on a Western blot probed with a PAP antibody recognizing the TAPi tag fused to Six6 (Fig. 6B). Hence, Six6 does not promote growth of *A. tumefaciens* in *N. benthamiana*.

To test whether Six6 alters susceptibility to other plant pathogens, stably transformed *Arabidopsis thaliana* Col-0 plants were generated expressing *SIX6*. Expression of the *SIX6* gene was

driven by the 35S promoter and, to aid detection of the Six6 protein, it was fused to a TAPi tag. Two independent transgenic lines (Six6-1 and Six6-2) containing a single insertion of the 35S::dSPSIX6-TAPi construct were selected for analysis. The *SIX6*-overexpressing lines did not exhibit morphological aberrations or show a phenotype distinct from nontransformed *Arabidopsis* when grown under standard conditions (Fig. 5B,

Fig. 5. *SIX6* does not alter growth of *Agrobacterium tumefaciens* in *Nicotiana benthamiana* or susceptibility to *Verticillium dahliae*, *Fusarium oxysporum*, or *Pseudomonas syringae*. **A**, In planta growth of *A. tumefaciens* carrying *SIX6*, a positive control (*AvrPto*), or an empty vector (tandem affinity purification [TAPi]). Samples were taken 0, 2, or 4 days after *N. benthamiana* leaf infiltration (dpi). Bacterial growth of the empty vector control and *SIX6* did not differ, whereas the bacterial titer was significantly higher in the *AvrPto* control. Bars indicate standard deviation of three replicates; * indicates significant difference between TAPi and *AvrPto* in a student *t* test (2 dpi, $P = 0.0006$; 4 dpi, $P = 0.02$). **B**, **D**, and **E**, Bioassays were performed on Col-0 and two independent Col-0 35S::dSPSIX6-TAPi (Six6-1 and Six6-2) transformants. **B**, Photographs showing disease development (chlorosis and slightly reduced stature) of three representative plants 23 days after mock (upper rows) or *V. dahliae* inoculation (lower rows). **C**, Western blot showing the presence of the TAPi-tagged Six6 protein in *Arabidopsis thaliana* leaf protein extracts of the Six6 transformants. Detection was done using the PAP antibody. M = protein marker. **D**, Disease index scored at 9 and 15 days after *F. oxysporum* inoculation. Bars indicate standard deviation of 24 replicates. **E**, Bacterial growth of *P. syringae* 3 dpi. Bars indicate standard deviation of eight replicates; * indicates significant difference between the wild-type Col-0 and the Six6-2 transformant in a student *t* test ($P = 0.02$).

compare top panels). Accumulation of the Six6 protein in the two transformants was confirmed by Western blotting (Fig. 5C). Six6 is present in many *F. oxysporum* strains (Fig. 1) but not in the *Arabidopsis*-infecting strain Fo5176 (Thatcher et al. 2012). Hence, this strain was used to assess a potential change in disease susceptibility to *F. oxysporum* in *Arabidopsis* due to expression of *SIX6*. Thirteen-day-old seedlings were infected with Fo5176 and disease index was scored on a scale from 0 to 5, in which 0 means that no disease symptoms developed and 5 that plants are dead (Supplementary Table S2) at either 9 or 15 dpi (Fig. 5D). No significant difference ($P > 0.05$) between disease symptoms on wild-type Col-0 and Col-0 35S::dSPSIX6-TAPi was observed, leading us to conclude that Six6 does not alter susceptibility of the plant to this strain of *F. oxysporum*.

To assess a potential contribution of Six6 to susceptibility of *Arabidopsis* toward another wilt-causing fungus, bioassays were performed using *Verticillium dahliae*. Twenty 1-day-old wild-type plants and the two *SIX6*-expressing *A. thaliana* lines were inoculated with *V. dahliae* spores and disease symptoms were scored 23 dpi (Fig. 5B). The amount of chlorosis was scored (discussed below) as a measure to quantify disease symptoms.

Disease symptoms were relatively weak and Six6 did not enhance or reduce symptom development on *A. thaliana* (Fig. 5B).

Finally, in planta growth of the plant-pathogenic bacterium *P. syringae* DC3000 was analyzed. Twenty 2-day-old *A. thaliana* plants were syringe infiltrated with *P. syringae* and leaf discs were collected from the infiltrated area at 3 dpi. A small but significant, decrease in bacterial growth was observed (<1 log CFU cm⁻²) in the 35S::dSPSIX6-TAPi line Six6-2 compared with the untransformed line but no difference was observed in line Six6-1 (Fig. 5E).

In summary, transient *SIX6* expression does not increase susceptibility of *N. benthamiana* to *Agrobacterium tumefaciens*, nor does *SIX6* expression in *Arabidopsis thaliana* enhance susceptibility to Fo5176 or *V. dahliae*. Furthermore, susceptibility of *A. thaliana* toward *P. syringae* was not consistently affected by expression of *SIX6*.

Six6 suppresses I-2-mediated cell death.

Many effectors enhance virulence of a pathogen by suppressing cell death, a response that often accompanies plant defense (Guo et al. 2009; Stassen and Van den Ackerveken

Fig. 6. Six6 suppresses I-2-mediated cell death and ion leakage. Co-expression of *AVR1*, *SIX6*, and *SIX8* with *AVR2* and *I-2*. **A**, Pictures of *Nicotiana benthamiana* leaves taken 48 h after infiltration (hpi) (left panel) and visualization of cell death with trypan blue staining (right panel). Lower circle: agroinfiltration of *I-2* and *AVR2*, upper circle: agroinfiltration of *AVR1*, *SIX6*, and *SIX8* respectively. Effect on *I-2*-mediated cell death was scored in the overlap of the circles. **B**, Accumulation of Avr1, Six6, and Six8 shown using a Western blot probed with the PAP antibody (all Six proteins carry a tandem affinity purification [TAPi] tag). Arrows indicate the expected position of the Avr1-, Six6-, and Six8-TAPi fusion proteins. Ponceau S staining shows equal loading; M = protein marker. Conductivity of *N. benthamiana* leaf discs sampled from infiltrated leaf areas co-expressing **C**, *I-2*, *AVR2*, and *AVR1* or **D**, *I-2*, *AVR2*, and *SIX6*. Leaf discs were collected at 26 hpi and conductivity was monitored at 0, 2, and 8 h after harvesting and compared with that of *I-2*-, *AVR2*-, and *Avr2*^{V→M}-expressing regions present on the same leaf. Bars represent standard deviation of five replicates; * indicates a significant difference between the *Avr2*^{V→M} control and *AVR1* ($P = 0.04$) or *SIX6* ($P = 0.002$) in a student *t* test

2011). To test whether Six6 can interfere with cell death, the *SIX6* gene was co-expressed with a set of cell-death-inducing genes using agroinfiltration in *N. benthamiana* leaves. Because many R proteins can trigger cell death, representative members from different *R* gene families were selected. Four distinct solanaceae *R* genes (*Rx*, *Pto*, *Cf4*, and *I-2*) that confer resistance to a viral, a bacterial and two fungal pathogens, respectively, were selected for analysis (Table 1). Co-expression of these *R* genes with their corresponding *AVR* genes triggers a cell death reaction generally referred to as a hypersensitive response (HR). As a positive control for a cell death suppression assay, we tested whether *F. oxysporum* f. sp. *lycopersici* Avr1 can be used. We reported before that *F. oxysporum* f. sp. *lycopersici* Avr1 suppresses *I-2*-mediated resistance of tomato (Houterman et al. 2008). To test whether Avr1 can suppress *I-2*-mediated cell death (I2CD), the *I-2* gene was co-expressed with an *AVR1* construct that lacks its signal peptide and prodomain (dPDAVR1) to assure that both proteins co-localize in the cytosol. The suppressing effect of Avr1 on I2CD is compared with the effect of Six8, a *F. oxysporum* f. sp. *lycopersici* Six protein with a size similar to Avr1 and Six6, in Figure 6A. In contrast to Six8, Avr1 was found to suppress cell death and reduce necrosis of the overlapping area. To better visualize the contrast between the living green cells and the dead brown cells, the dead cells were stained with trypan blue. The region where *AVR1*- and *I-2*/*AVR2*-expression overlaps showed a slightly reduced blue staining compared with the Six8 control (Fig. 6A, right panel). This is not due to a lack of expression because Western Blot analysis shows accumulation of Six8 and the other two effectors (Fig. 6B). Therefore, in addition to its suppressive effect on *I-2*-mediated resistance, Avr1 also suppresses I2CD, which makes it a proper positive control for a cell-death-suppression assay.

Notably, *SIX6* infiltration reduced I2CD to an even larger extent than Avr1, as exemplified by the reduced trypan blue staining (Fig. 6A, lower panel). Because the *SIX6* construct does not carry the sequence encoding the signal peptide (dSPSIX6), this implies that the Six6-mediated suppression of the I2CD occurs inside the plant cell. Its cell-death-suppressive effect was exclusively observed for *I-2* because *Rx*-, *Pto*-, and *Cf4*-mediated HR were unaffected by Six6 (Table 1). Next, we analyzed whether Six6 can also suppress programmed cell death (PCD) induced by non-*R* genes. *INF1*, encoding a *Phytophthora infestans* elicitor, and *Bax*, encoding a mammalian protein inducing apoptotic cell death (APC), were co-agroinfiltrated with *SIX6*. No cell-death suppression was observed in either case (Table 1) (data not shown). In summary, Six6, like Avr1, suppresses cell death triggered by the R protein *I-2*. Interestingly, this effect seems to be *I-2* specific because cell death induced by other genes was unaffected by *SIX6* co-expression.

Six6 suppresses ion leakage accompanying I2CD.

Visual scoring of cell death is semiquantitative. To measure cell death more quantitatively, ion leakage was measured, because it is known to accompany cell death (Coll et al. 2011).

SIX6, *AVR1*, or *SIX8* were co-expressed with *AVR2* and *I-2* using agroinfiltration in *N. benthamiana*. At 26 h postinfiltration (hpi), leaf discs were collected from the infiltrated area. These discs were transferred to water and conductivity of the solution was recorded for up to 12 h. For the first 10 h, the conductivity curves were highly reproducible but, at later time points, variation increased (data not shown). Therefore, two representative time points, 2 and 8 h after sampling, were selected, and these are depicted in Figure 6C and D. As negative control, an Avr2 variant (Avr2^{V→M}) (Houterman et al. 2009) was used that is not recognized by *I-2*. Co-expression of Avr2^{V→M} with *I-2* and *AVR2* resulted in an identical conductivity curve as co-expression of *SIX8*, validating its use as a negative control (Supplementary Fig. S2).

At 8 h after sampling, the *AVR1*-infiltrated leaf discs showed a significantly ($P = 0.04$) lower conductivity than the Avr2^{V→M} control, which is in line with its ability to suppress I2CD (Fig. 6C). Conductivity in the *SIX6*-infiltrated discs was also significantly reduced at this time point compared with the Avr2^{V→M} control ($P = 0.002$) (Fig. 6D). Hence, cell-death suppression by Avr1 and Six6 is accompanied by a significant suppression of I2CD-mediated ion leakage.

Six6-GFP is present in cell cytoplasm and nucleus.

Determining the subcellular localization of a protein can provide insight into the processes it affects. Hence, the *SIX6* gene without the sequence encoding the signal peptide (dSPSIX6) was fused to green fluorescent protein (*GFP*) and expressed in *N. benthamiana* leaves by agroinfiltration. As a reference, *GFP* alone was expressed using the same vector. The integrity of the Six6-GFP fusion after agroexpression was confirmed by Western blot analysis (Fig. 7C). At 2 dpi, leaves were harvested and fluorescence was visualized using confocal microscopy. In epidermal cells expressing solely GFP, green fluorescence was observed at equal intensities in the cytoplasm and nucleus (Fig. 7B). Expression of dSPSIX6-GFP also resulted in green fluorescence in both the cytoplasm and the nucleus; however, in this case, fluorescence appeared stronger in the nucleus than in the cytoplasm, suggesting a nuclear preference for the Six6-GFP fusion protein (Fig. 7A, ×2 magnification).

In conclusion, upon agroinfiltration, Six6-GFP localizes in both the nucleus and in the cytoplasm, suggesting that it is freely diffusible and is not tethered to a membrane or localized in a specific organelle.

DISCUSSION

F. oxysporum f. sp. *lycopersici* effectors differ in their distribution within the *F. oxysporum* species complex. Whereas some are highly *F. oxysporum* f. sp. *lycopersici* specific, others are also found in other formae speciales. Six6 is not only present in other formae speciales of *F. oxysporum* but two homologs are also present in *C. higginsianum* and *C. orbiculare*. The Six6 homologs in the *F. oxysporum* complex are more related to *F. oxysporum* f. sp. *lycopersici* Six6 than the ones from

Table 1. Overview of cell death-inducing proteins tested in the Six6 suppression assay

Cell death-triggering protein	Donor organism	Protein class ^a	Conferring resistance to	Recognition of	Type of cell death ^b	Suppression by Six6
I2	Tomato	CNL	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	Avr2	HR	Yes
Rx	Potato	CNL	Potato virus X	CP	HR	No
Pto	Potato	CNL	<i>Pseudomonas syringae</i>	AvrPto	HR	No
Cf4	Tomato	RLP	<i>Cladosporium fulvum</i>	Avr4	HR	No
Inf1	<i>Phytophthora infestans</i>	Elicitor	...	...	PCD	No
Bax	Mouse	Elicitor	...	...	CD	No

^a CNL = coiled-coil nucleotide-binding leucine-rich repeat protein and RLP = receptor-like protein.

^b HR = hypersensitive response, PCD = programmed cell death, and ACD = apoptotic cell death.

C. higginsianum and *C. orbiculare*. This might reflect independent evolution of the gene in the genera *Colletotrichum* and *Fusarium* after speciation. However, because Six6 is not found in other *Fusarium* or *Colletotrichum* spp. or in other genera, another possibility is that *SIX6* was exchanged between *Colletotrichum* spp. and *F. oxysporum* after speciation by horizontal gene transfer and then evolved at different rates within the respective fungal species. If so, transfer did not occur recently because their Six6 sequences have considerably diverged. Despite the relatively low conservation of Six6 between the two genera, three conserved C-terminal motifs can be discerned. These motifs might represent regions with conserved structural

or biochemical functions and are good targets for future structural and functional analysis.

SIX6 is expressed in planta during infection and is essential for full virulence of *F. oxysporum* f. sp. *lycopersici*. Rather surprisingly, deletion of *SIX6* merely affected plant weight but not the disease index of infected tomato plants. Clearly, the *SIX6* knockout still has the capacity to cause severe vessel browning when evaluated 3 weeks after infection. The high residual plant weight suggests that the infection process is slower, allowing the plant to continue to grow during early stages of infection. This interpretation is supported by the observation that plant weight is highest for plants inoculated with strains

Fig. 7. Six6-green fluorescent protein (GFP) is localized in the cytoplasm and cell nucleus in *Nicotiana benthamiana* leaves upon agroinfiltration. Confocal image of leaves agroinfiltrated with **A**, dSPSIX6-GFP (left and right at a x2 magnification) or **B**, GFP alone. Fluorescence in the leaf epidermal cells was determined 24 h after infiltration (hpi) with confocal microscopy using an excitation wavelength of 488 nm and a x40 magnification. Each panel consists of a picture taken with a 650-nm-long path filter (upper left) for plant autofluorescence; and a 520- to 550-nm-band path filter (upper right) for GFP, transmitted light (lower left), and a stack of all three pictures (lower right). Arrows indicate different cell organelles (Cp = cytoplasm, N = nucleus, and Ch = chloroplast). **C**, Correct size of the fusion proteins was confirmed by Western blotting of leaf protein extracts using an anti-GFP antibody. Ponceau S staining as loading control. M = protein marker.

$\Delta SIX6-3$ and $\Delta SIX6-3 + SIX6-4$, that have a relatively low amount of fungal biomass based on their *FEM1* expression (Fig. 4). Remarkably, all *F. oxysporum* f. sp. *lycopersici* effector mutants analyzed thus far (*AVR1*, *AVR2*, *AVR3*, and *SIX6*) are compromised in virulence, which implies limited redundancy among *F. oxysporum* f. sp. *lycopersici* effectors. This notion corresponds with the relatively small number of candidate effector genes encoded on the pathogenicity chromosome ($n = 16$) and the limited number of candidate effector proteins found in the xylem sap ($n = 14$) (Houterman et al. 2007; Lievens et al. 2009; Ma et al. 2010; Schmidt et al. 2013).

Although Six6 is a virulence factor for *F. oxysporum* f. sp. *lycopersici*, transient expression of *SIX6* did not affect growth of the bacterium *Agrobacterium tumefaciens*. Also, stable expression of *SIX6* in *Arabidopsis thaliana* did not alter its susceptibility to the plant-pathogenic bacterium *P. syringae* or to the fungal pathogens *V. dahliae* and *F. oxysporum*. This result could be explained by the presumptive Six6 host target being i) not present in this plant species, ii) not present in the cytosol, or iii) not required for susceptibility to the aforementioned pathogens. Alternatively, iv) these pathogens might carry effectors whose activity is similar to that of Six6, concealing the effect of the expression of this gene. Because no Six6 homologs are present in Fo5176, *P. syringae*, or *V. dahliae*, such effectors would then not be related at the sequence level. We favor the fourth option because we found Six6 to be conserved in *F. oxysporum* strains infecting diverse hosts such as melon, banana, cotton, cucumber, watermelon, and passion fruit. The gene is also present in *C. higginsianum* that infects *A. thaliana*, and *C. orbiculare*, which causes disease in cucumber and melon, making alternative i unlikely. An argument to reject ii comes from our suppression assays, in which cytosolic Six6 was found to suppress I2CD in *N. benthamiana*. Hence, at least the Six6 target involved in I2CD disturbance must be present intracellularly in *N. benthamiana*. Interestingly, suppression of I2CD seems to be a specific feature of the Six6-target, because no suppression of cell death was found when *SIX6* was co-expressed with other *R-AVR* gene pairs, *INFL1*, or the cell-death-inducing protein Bax (Table 1). This finding is surprising because, in agroinfiltration assays, cell-death-suppressing effectors typically act more broadly, as exemplified by AvrPtoB, HopPtoE, and HopPtoF from *P. syringae*, suppressing cell death accompanying both HR and APC (Abramovitch et al. 2006; Jamir et al. 2004). Also, *Phytophthora infestans* and *P. sojae* effectors suppress various types of cell death such as APC, PCD, and HR mediated by different R proteins (Dou et al. 2008; Gilroy et al. 2011; Kelley et al. 2010; Wang et al. 2011). In addition to effectors from bacteria and oomycetes, SPRYSEC-19, an effector of the nematode *Globodera rostochiensis*, also suppresses cell death mediated by several coiled-coil nucleotide-binding leucine-rich repeat immune receptors (Postma et al. 2012). To our knowledge, only the effector CgDN3 from *C. orbiculare* suppresses specifically only one form of cell death: the one mediated by the secreted protein NIS1 (Yoshino et al. 2012). Possibly, Six6 is a weak suppressor, and I2CD may be relatively easy to suppress because I-2-mediated resistance to the AVR2-carrying *F. oxysporum* f. sp. *lycopersici* strain typically does not involve HR (Beckmann 2000). The I2CD observed in *N. benthamiana* might be an anomaly due to overexpression of *I2-AVR2* in this heterologous system, resulting in a cell death response, which may be easier to suppress than that induced by *R* genes that do rely on HR for their function (Thomma et al. 2011).

With the host target of Six6 remaining unidentified, we can only speculate as to how Six6 contributes to virulence of *F. oxysporum* f. sp. *lycopersici* on susceptible plants. The localization of GFP-tagged Six6 in the nucleus and cytoplasm of *N.*

benthamiana leaves upon transient expression suggests that the effector target could be localized in either or both of these sub-cellular compartments. It has been shown that I-2 activation requires Avr2 recognition in the nucleus (Ma et al. 2013) and nuclear-localized Six6 might interfere with this event, thereby suppressing I2CD. Yet, its capacity to suppress I2CD is insufficient to suppress I-2 function in tomato, because I-2 plants are resistant to *SIX6*-expressing *F. oxysporum* f. sp. *lycopersici* strains. However, I-2-mediated resistance to *F. oxysporum* f. sp. *lycopersici* is not absolute and growth of the fungus in the xylem vessels of roots and stems of resistant plants is frequently observed (Elgersma et al. 1972; our unpublished results). Possibly, some of this fungal growth is due to partial, Six6-mediated suppression of I-2, implying that fungal growth of a *SIX6* knockout on a resistant plant will be reduced. Unfortunately, the reduction of fungal biomass already observed upon infection of susceptible plants by the *SIX6* knockouts (Fig. 4) prevented us from testing this hypothesis. This virulence phenotype on susceptible plants also suggests that Six6 has effects on host susceptibility other than (partial) suppression of I-2-mediated host responses.

Future work should include experiments aimed at identification of the Six6 host targets, to uncover how this effector contributes to virulence of *F. oxysporum* f. sp. *lycopersici* toward tomato and suppression of I2CD in *N. benthamiana*. It will also be interesting to see whether the Six6 homologs found in other plant pathogens are also required for full pathogenicity.

MATERIALS AND METHODS

Alignment.

The Six6 protein alignment was performed with ClustalW and Seaview.

Plant material and fungal and bacterial strains.

Tomato 'C32' was used for the *F. oxysporum* f. sp. *lycopersici* inoculation assays and is susceptible to all *F. oxysporum* f. sp. *lycopersici* races (Kroon and Elgersma 1993). *A. thaliana* ecotype Col-0 was used for transformation and the disease assays with *V. dahliae* JR2 (provided by B. Thomma, WUR, Wageningen, The Netherlands), *P. syringae* pv. *tomato* DC3000 (Whalen et al. 1991), and Fo5176 (originally isolated by Queensland Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia). All *F. oxysporum* f. sp. *lycopersici* gene-deletion lines were generated in a Fol007 background (provided by L. Davidse, France), a race 2 isolate nonpathogenic on tomato plants carrying I-2 or I-3.

Agroinfiltrations were performed using the disarmed *Agrobacterium tumefaciens* strain GV3101::pMP90 (Koncz and Schell 1986). *A. tumefaciens*-mediated transformation of *F. oxysporum* f. sp. *lycopersici* was done with the strain EHA105 (Hood et al. 1993) and, for *Arabidopsis thaliana* transformation, the strain Agl-0 was used (Lazo et al. 1991).

RNA isolation and RT-PCR.

Ten-day-old tomato seedlings were inoculated with Fol007, the *SIX6* knockout strain, or the knockout strain complemented with *SIX6* with the root-dip method (Wellmann 1939) and then potted in vermiculite (Agra-vermiculite; Eveleens, Aalsmeer, The Netherlands). At 10 dpi, the roots were cut below the hypocotyls, rinsed twice with water, and dried with tissues. The root samples were ground in liquid nitrogen. Total RNA from the samples was extracted with TRIzol LS reagent (Invitrogen, Life Technologies, Grand Island, NY, U.S.A.) and subsequently purified with RNeasy Mini kit (Qiagen, Düsseldorf, Germany). DNA was removed by on-column treatment with RNase-free

DNase (Qiagen). cDNA was synthesized using the M-MuLV reverse-transcriptase RNase H minus kit (Fermentas, Thermo Scientific, Pittsburgh PA, U.S.A.). Either primer pair FP1995/FP1996 or FP2155/FP2156 (list of all primer sequences in Supplementary Table S3) were used to amplify *SIX6* from cDNA. *FEM1* was amplified using primer pair FP157/FP158.

To assess *SIX6* expression of *F. oxysporum* f. sp. *lycopersici* grown in artificial medium, 0.5 ml of Fol4287 (race 2) at 10^7 spores/ml was added to 4.5 ml of a 1-week-old tomato MSK8 cell culture grown in BY medium (Felix et al. 1991) or to NO_3 medium (100 mM KNO_3 , 3% sucrose, and 0.17% yeast nitrogen base without amino acids or ammonia). After 24 h of incubation at 22°C, the material was harvested, washed two times with sterile water, and freeze dried. RT-PCR was performed as described above but DNase (Fermentas) treatment was executed before cDNA synthesis.

Construction of the *SIX6* knockout and complementation constructs.

To generate a *SIX6* gene deletion, a 1,328-bp upstream sequence (starting 36 bp 5' of the *SIX6* start codon) was amplified by PCR using primers FP1509 and FP1510, carrying either an *Xba*I or *Hind*III site. The PCR product was inserted into the binary vector pRW2h (Houterman et al. 2008) upstream of the hygromycin resistance cassette. An 836-bp fragment starting 124 bp after the *SIX6* start codon containing an internal *Bss*HII site was amplified using the primers FP1511 and FP1512 and *Bss*HII digested. The resulting 676-bp fragment was inserted into a *Stu*I/*Bss*HII-digested vector downstream of the Hygromycin cassette.

The complementation construct was generated using primers FP1727 carrying a *Bcu*I (*Spe*I) linker and FP1728. PCR amplification resulted in a 1.9-kb fragment starting 1,023 bp upstream of the *SIX6* start codon and ending 261 bp downstream of the stop codon. The fragment was integrated into an *Xba*I/*Eco*1471 (*Stu*I)-digested pRW1p vector.

Construction of binary vectors for agroinfiltration.

SIX6 was PCR amplified from genomic DNA of Fol007 (race 2). Forward primer FP2423 generated a truncated *SIX6* gene lacking the sequence encoding its signal peptide. To allow C-terminal tagging of Six6, a PCR amplification was also done with the FP2204 reverse primer that removes the stop codon. To generate a similar fragment for *SIX8*, the gene was amplified using primers FP2838 and FP2206 from cDNA isolated from Fol007-infected tomato roots. *AVR1* lacking the sequence encoding its signal peptide and prodomain was obtained by PCR amplification using primers FP2292 and FP2202 on cDNA of Fol001 (race 1)-infected tomato roots. To all PCR products, Gateway *attB* flanks were added in a subsequent PCR amplification with primers FP872 and FP873. The obtained *attB* PCR products were recombined via entry clone pDONR207 (Invitrogen) into binary vectors CTAPi (Rohila et al. 2004) and pGWB451 following the Gateway protocol (Invitrogen). All primers were purchased from MWG (Ebersberg, Germany). *AVR2*- and *I-2*-carrying constructs have been described previously (Houterman et al. 2009; van Ooijen et al. 2008).

Agroinfiltration of *N. benthamiana*.

Agroinfiltrations were performed according (Ma et al. 2012) as described. In brief, *A. tumefaciens* strain GV3101 carrying the construct of interest was grown until an optical density at 600 nm (OD_{600}) of 0.8 in LB_{um} (tryptone at 10 g liter⁻¹, yeast extract at 5 g liter⁻¹, and NaCl at 2.5 g liter⁻¹) medium supplemented with 20 μM acetosyringone and 10 mM morpholinethanesulfonic acid (MES), pH 5.6. The cells were resuspended in infiltration medium (Murashige-Skoog [MS] salts at 5 g/liter,

sucrose at 20 g/liter, 200 μM acetosyringone, and 10 mM MES, pH 5.6) to a final OD_{600} of 1.0 when used for in planta protein expression or localization studies. For suppression assays, *Agrobacterium tumefaciens* suspensions were diluted to an OD_{600} of 0.5 or 0.3.

Protein extraction and Western blotting.

To determine accumulation of GFP- or TAPi-tagged recombinant proteins, agroinfiltrated *N. benthamiana* leaves were harvested 24 to 48 hpi and frozen in liquid nitrogen. Protein extraction was done as described previously (Ma et al. 2012; van Ooijen et al. 2008) and 0.5 g of frozen plant tissue was ground in liquid nitrogen and allowed to thaw slowly in 1 ml of extraction buffer (25 mM Tris [pH 7.5], 1 mM EDTA, 150 mM NaCl, 5 mM dithiothreitol, 1× Roche Complete protease inhibitor cocktail, 2% polyvinyl-polypyrrolidone, and 0.1% NP-40). The extract was centrifuged at 13,000 rpm at 4°C for 30 min and the supernatant was filtered through Miracloth (Calbiochem, San Diego, CA, U.S.A.). The protein concentration was determined by measuring the OD_{280} on a spectrophotometer (Hitachi U-2900) and equal amounts of protein were loaded on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. For protein detection, proteins were wet blotted on polyvinylidene difluoride membranes and equal loading and transfer were confirmed by staining the blot with 2% Ponceau S in 5% acetic acid. Subsequently, membranes were blocked with 5% milk in Tris-buffered saline and GFP or TAPi tag fusion proteins were detected with an anti-GFP (VXA6455; Invitrogen) (1:3,000 diluted) or anti-PAP antibody (Sigma P1291) (1:5,000 diluted), which is linked to horseradish peroxidase. In case of GFP detection, a goat-antirabbit antibody (Pierce P31430) (1:8,000 diluted) was used as secondary antibody. In either case, the signal was detected using enhanced chemiluminescence (ECL Plus Western Blotting Detection System; GE Healthcare, Piscataway, NJ, U.S.A.).

Confocal microscopy.

In planta accumulation of Six6-GFP after agroinfiltration was visualized using confocal microscopy (Zeiss LSM 510). An Ar-laser (intensity set at 22%) was used for excitation at 488 nm. Fluorescence was detected with a 520- to 550-nm-band path filter for GFP and autofluorescence of the plant tissue was recorded using a 650-nm-long path filter. Scans were taken with the C-Apochromat 40X1.2 Wcorr objective.

Visualization and quantification of the HR.

For quantification of cell death, three leaf discs with a diameter of 7 mm were collected per agroinfiltrated sector 27 h after agroinfiltration. The leaf discs were washed in 50 ml of water for 30 min while shaking at 100 rpm. Afterward, the discs were placed with their abaxial sides facing downward in a well containing 3 ml of 0.01% Silwet-L77. The leaf discs were vacuum infiltrated and the 12-well culture plates (Greiner) were incubated at room temperature under fluorescent lamps (25 lux) while shaking at 100 rpm. The solution (25 μl) was sampled every 1 to 2 h for 12 h and conductivity was determined with a TwinCond conductivity meter (Horiba, Kyoto, Japan).

To visualize cell death, leaves were stained with trypan blue (Ma et al. 2012). Then, 50 ml of staining solution (100 ml of lactic acid [DL Sigma L-1250], 100 g of phenol, 100 ml of glycerol, 100 ml of H_2O , and 100 mg trypan blue) was mixed with 50 ml of 96% EtOH and heated in a water bath until boiling. Up to three leaves were added together to the boiling solution and allowed to stain for 5 min. Leaves were destained with chloral hydrate solution at 2.5 mg/ml overnight and scanned using an Perfection 1200 Scanner (Epson, Long Beach, CA, U.S.A.).

A. tumefaciens growth curves.

A. tumefaciens growth curves were measured as described (Hann and Rathjen 2007). *A. tumefaciens* GV3101 carrying the appropriate constructs was agroinfiltrated at an OD₆₀₀ of 0.05. Two leaf discs were harvested from each agroinfiltrated area 0, 2, or 4 dpi. Bacteria were extracted in 500 µl of 10 mM MgSO₄ after homogenizing the leaf material by adding two metal beads (2.5 mm in diameter) and placing the tube for 2.5 min in a paint shaker (Fast and Fluid management; IDEX Corporation, Lake Forest, IL, U.S.A.). Serial dilutions of the homogenate were made and 20 µl of each dilution (typically 10⁻³, 10⁻⁴, and 10⁻⁵) was plated on LB_{tum} supplemented with rifampicin (20 µg/ml). After 2 days of incubation at room temperature, the CFU were counted and the CFU/cm² value was calculated. Each sampling was performed in three technical and five biological replicates. Significance was tested with a paired student *t* test (*P* < 0.05).

Plant inoculations.

Ten-day-old tomato seedlings were uprooted from the soil. The seedlings were placed for 5 min in a 5-day-old *F. oxysporum* f. sp. *lycopersici* spore suspension (10⁷ spores/ml) and potted. Disease progression was evaluated after 3 weeks. Plant weight and disease index (Rep et al. 2004) were scored for 20 plants/treatment. Significant differences between treatments were tested using ANOVA.

Inoculation of *Arabidopsis thaliana* plants with *V. dahliae* JR2 and scoring of the disease index was done as described (Yadeta et al. 2011). *A. thaliana* was grown under short-day conditions in a climate chamber (21°C, 70% relative humidity, and 11-h photoperiod). After 21 days, the plants were carefully removed from the soil and the roots were placed in a *V. dahliae* spore suspension (10⁶ spores/ml) for 5 min. Thereafter, the plants were transferred to fresh soil. After 2 to 3 weeks, the first disease symptoms developed. Disease symptoms were scored visually by measuring the size of the plants and evaluating development of chlorosis on the leaves (Yadeta et al. 2011).

The spore suspension (5 ml at 10⁶ spores/ml) of an *F. oxysporum* Fo5176 culture was added directly to the soil of 13-day-old *A. thaliana* seedlings growing on a soil/sand mix (ratio 1:1). The disease index was scored (on a scale of 0 = no disease symptoms; 1 = one or two leaves with yellow vessels; 2 = lowest full developed leaves show chlorosis; 3 = all full developed leaves show chlorosis; 4 = all leaves show chlorosis, including the rosette of new developed leaves; and 5 = plant is dead) at 9 and 15 dpi.

P. syringae was cultured overnight in King's B medium (King et al. 1954) and resuspended in 10 mM MgSO₄ to a final OD₆₀₀ of 0.0007. The bacteria were syringe infiltrated into the leaves of 22-day-old *A. thaliana* plants. At 3 dpi, leaf discs (0.5 cm) were collected and the bacteria were extracted in 10 mM MgSO₄. Different dilutions (typically 10⁻³, 10⁻⁴, and 10⁻⁵) of the bacteria suspension were plated on King's B.

Transformation of *F. oxysporum* f. sp. *lycopersici*, targeted deletion, and complementation of *SIX6*.

Transformation of *F. oxysporum* f. sp. *lycopersici* was mediated by *A. tumefaciens* transformation (Takken et al. 2004). Spores of a 5-day-old *F. oxysporum* f. sp. *lycopersici* culture were isolated and resuspended in IM (10 mM glucose, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 2.5 mM NaCl, 4 mM [NH₄]₂SO₄, 0.7 mM CaCl₂, 2 mM MgSO₄, 9 µM FeSO₄, 0.5% [wt/vol] glycerol, and 40 mM MES, pH 5.3) supplemented with 200 µM acetosyringone to a final concentration of 2 × 10⁶ spores/ml. *A. tumefaciens* containing the binary vector of interest was grown for 24 h in 2× yeast extract and tryptone me-

dium supplemented with the appropriate antibiotics. Then, the cells were resuspended in 10 ml of IM to an OD₆₀₀ of 0.45 and incubated for 6 h at 28°C. Then, 2 ml of each of the *F. oxysporum* f. sp. *lycopersici* and *A. tumefaciens* resuspensions were mixed and aliquots of 25 µl were transferred to ME25 filters placed on co-cultivation plates (the same as IM but containing 5 mM glucose) solidified with 1.5% Bacto agar. After 2 days of incubation at 25°C, the filters were transferred to Czapek Dox agar containing cefotaxime and hygromycin or zeocin (both 100 µg/ml). Plates containing with zeocin were buffered with 0.1 M Tris, pH 8.0. Putative transformants appeared after 2 to 4 days and monospores were made for further analysis. Absence of *SIX6* in hygromycin-resistant transformants was tested using primer pair FP1490/FP1491. In locus insertion of the knockout cassette was confirmed by PCR using primer pairs FP745/FP2264 (right border) and FP2266/FP659 (left border). Presence of *SIX6* in the *SIX6* knockout complementation strains was also confirmed by PCR using three primer pairs, of which one (FP745/FP1961) amplified the region upstream of *SIX6* and a part of the *SIX6* coding region and the other two primer sets (FP1995/FP2218 and FP1959/FP746) amplified the region downstream of *SIX6* and a part of the *SIX6* coding region.

Transformation of *A. thaliana* using the floral dip method.

A. thaliana plants were grown in a 12-h photoperiod at 70% relative humidity and 21°C. The first inflorescence was removed to boost flower production. *Agrobacterium tumefaciens* carrying the dSPSIX6-TAPi was grown in 250 ml of LB_{tum} supplemented with spectinomycin (100 µg/ml) overnight. The bacteria were resuspended in 5% sucrose to a final OD₆₀₀ of 0.8 and 0.02% Silwet was added. The flowers were dipped in the bacterial suspension, wrapped in plastic foil to increase humidity, and protected against direct light. The dipping was repeated after 1 week. After seed harvest and sterilization, the seed were plated on MA plates (MS including Gamborg B5 vitamins, at 2.2 g/liter; FeNaEDTA at 36.7 mg/liter; MES [pH 5.8] at 1g/liter; and 0.7% Daishin agar) containing nystatin at 100 µg/ml and phosphinothricin at 10 µg/ml. Resistant plants were propagated and homozygous plant lines carrying single *SIX6* T-DNA insertions were selected after segregation analysis. Six6 protein expression was analyzed by extracting the proteins from leaf material (100 mg) in urea buffer (9.5 M urea, 100 mM Tris [pH 6.8], 2% SDS, and 2% β-mercaptoethanol) following detection of their attached TAPi tag by Western blot analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ClustalW database: www.ebi.ac.uk/Tools/msa/clustalw2
 Seaview, a multiplatform, graphical user interface for multiple sequence alignment and molecular phylogeny:
pbil.univ-lyon1.fr/software/seaview.html