Combined action of the major secreted exo- and endopolygalacturonases is required for full virulence of *Fusarium oxysporum*

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SUMMARY

The genome of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* encodes eight different polygalacturonases (PGs): four endoPGs and four exoPGs. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) revealed that endoPGs *pg1* and *pg5* and exoPGs *pgx4* and *pgx6* are expressed at significant levels during growth on citrus pectin, polygalacturonic acid or the monomer galacturonic acid, as well as during the infection of tomato plants. The remaining PG genes exhibit low expression levels under all the conditions tested. Secreted PG activity was decreased significantly during growth on pectin in the single deletion mutants lacking either *pg1* or *pgx6*, as well as in the double mutant. Although the single deletion mutants did not display a significant virulence reduction on tomato plants, the $\Delta pg1 \Delta pgx6$ double mutant was significantly attenuated in virulence. The combined action of exoPGs and endoPGs is thus essential for plant infection by the vascular wilt fungus *F. oxysporum*.

Keywords: endoPG, exoPG, *Fusarium*, tomato, virulence.

INTRODUCTION

Fungi secrete a battery of carbohydrate-active enzymes (CAZymes) responsible for the breakdown, biosynthesis or modification of glycoconjugates, oligo- and polysaccharides. CAZymes play a central role in the degradation of the plant cell wall, as well as during host–pathogen interactions (de Vries & Visser, 2001; Walton, 1994). Particular attention has focused on enzymes that efficiently depolymerize pectin, the structurally and functionally most important complex polysaccharide of the primary plant cell wall (Caffall and Mohnen, 2009). Pectin-degrading enzymes include pectin and pectate lyases (PLs), pectin methylesterases (PMEs), polygalacturonases (PGs), rhamnogalacturonases, rhamnogalacturonan lyases, as well as pectin methylesterases, pectin acetylsterases and rhamnogalacturonan acetylsterases

(reviewed by Glass *et al.*, 2013). PGs are present in all fungal genomes and are among the major secreted cell wall-degrading enzymes (CWDEs) in plant pathogens. PGs have been studied in detail in the saprophytic fungi *Aspergillus niger* and *Rhizopus oryzae* which are used in the food and feed industry, but also in certain phytopathogenic fungi (Kubicek *et al.*, 2014). Endo- α -1,4-polygalacturonase (endoPG), whose expression is often induced by pectic substrates, efficiently macerates plant tissue, whereas exo- α -1,4-polygalacturonase (exoPG) further degrades the oligogalacturonides (OGs) released from cell walls by the endoPGs (Cervone *et al.*, 1987).

Targeted knockout of CWDE encoding gene(s) has been carried out in a variety of plant-pathogenic fungi but, except in a few cases, deletion of a single CWDE in a plant-pathogenic fungus did not have a major impact on virulence (Di Pietro *et al.*, 2009). The first example of a fungal plant pathogen for which a functional role of pectinolytic enzymes during infection was demonstrated was *Fusarium solani* (Rogers *et al.*, 2000). This pea pathogen produces four endoPLs which may contribute to the penetration of cell wall carbohydrate barriers in the host during infection. Two genes are inducible: *pelA* by pectin (Gonzalez-Candelas and Kolattukudy, 1992) and *pelD* only by the host plant (Guo *et al.*, 1996). Targeted disruption of either *pelA* or *pelD* did not reduce virulence, whereas inactivation of both genes did. Virulence was restored by complementation of the double knockout with the intact *pelD* gene, or by supplementation of the infection droplets with either of the purified enzymes (Rogers *et al.*, 2000).

The role of PGs in plant pathogenicity has been studied extensively in the maize pathogen *Cochliobolus carbonum*. A double mutant lacking *PGN1*, encoding the major endoPG, and *PGX1*, encoding an exoPG, showed less than 0.8% of the wild-type PG activity, but was still fully pathogenic on maize, suggesting that PGs play only a minor role in the pathogenicity of *C. carbonum*. However, the double mutant was still able to utilize pectin for growth and retained PME and mycelium-associated PG activities, suggesting the presence of additional PG isozymes (Scott-Craig *et al.*, 1998). Likewise, the deletion of *enpg-1* encoding the major endoPG purified from culture filtrates of the chestnut blight fungus *Cryphonectria parasitica* did not cause a reduction in stem canker formation (Gao *et al.*, 1996). Analysis of PG activity extracted from diseased bark tissue revealed that ENPG-1 was

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only a minor activity component (less than 5%), whereas the predominant activity of the plant was associated with two acidic PG forms that were absent in the culture filtrate. In *Botrytis cinerea*, the causal agent of gray mould, deletion of *Bcpg1* encoding an endoPG resulted in mutants that were still pathogenic. However, although primary infections of the mutants were similar to the control strain, a significant decrease in secondary infection, i.e. growth of the lesion beyond the inoculation spot, was observed on three different host tissues, indicating that Bcp1 is required for full virulence (Ten Have *et al.*, 1998). Disruption of *pecA*, encoding an endoPG in the saprophytic fungus *Aspergillus flavus*, reduced lesion development in cotton balls, whereas its expression in a strain lacking PG increased lesion size (Shieh *et al.*, 1997). Interestingly, both *pecA* and *Bcpg1* showed constitutive expression. Importantly, simultaneous deletion of the two PG genes, *cpgg1* and *cpgg2*, in the ergot pathogen *Claviceps purpurea* rendered the fungus nearly non-pathogenic on rye without affecting its vegetative properties (Oeser *et al.*, 2002). endoPG also contributes to the colonization of tomato plants by the soil-borne bacterium *Ralstonia solanacearum*. A mutant deficient in the production of the 52-kDa endoPG PglA took twice as long as the wild-type to induce wilt in tomato plants (Schell *et al.*, 1988).

The vascular wilt pathogen *Fusarium oxysporum* secretes an array of CWDEs during root penetration and host plant colonization, including PGs, PLs, xylanases and proteases, which might contribute to infection (Roncero *et al.*, 2003). endoPG was the first secreted enzyme activity detected in *F. oxysporum* cultures on tomato cell walls (Jones *et al.*, 1972). PG1 is the major endoPG secreted during growth on pectin and during the infection of tomato plants (Di Pietro and Roncero, 1996). In addition, two exoPGs, PG2 and PG3, have been characterized (Di Pietro and Roncero, 1996; Garcia-Maceira *et al.*, 1997). All these PGs are secreted during growth on tomato vascular tissues as the sole carbon source. A second endoPG gene, *pg5*, is expressed during saprophytic growth on citrus pectin and *in planta* during early stages of infection, but targeted inactivation of *pg5* does not cause any measurable growth reduction on citrus pectin or alter virulence (Garcia-Maceira *et al.*, 2001). Mutants in the *pgx4* gene, encoding an exoPG, were not affected in growth on pectin or virulence, although *pgx4* is expressed during the infection of tomato plants (Garcia-Maceira *et al.*, 2000). PLs have also been suggested to be important in the development of vascular wilt diseases, and could be detected inside infected tomato root and stem tissue (Di Pietro and Roncero, 1996). Moreover, transcription of *pl1* encoding an endo-PL of *F. oxysporum* is observed during different stages of the disease cycle.

In this work, we conducted a genome-wide analysis of the PG encoding genes in *F. oxysporum* and generated targeted mutants in *pg1* and *pgx6* encoding the major secreted endoPG and exoPG, respectively. Although loss of either *pg1* or *pgx6* alone did not

affect virulence, double mutants displayed drastically reduced virulence, demonstrating that PGs are virulence factors in *F. oxysporum*.

RESULTS

Fusarium oxysporum f. sp. *lycopersici* contains eight different genes encoding predicted endo- or exoPGs

In order to characterize the PG system of *F. oxysporum* f. sp. *lycopersici* (isolate 4287), the genome database at http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html was searched using the deduced amino acid sequences of the previously described endo*pg1* (Di Pietro and Roncero, 1998) and exo*pgx4* (Garcia-Maceira *et al.*, 2000) genes. Using conservative criteria requiring *E* values equal to zero, we identified genes encoding four predicted endoPGs and four predicted exoPGs. These include the previously characterized genes *pg1*, *pg5* and *pgx4* (Garcia-Maceira *et al.*, 2001), as well as five additional genes: FOXG_15415 (named *pgx6*); FOXG_09893 (named *pgx7*); FOXG_10052 (named *pg8*); the tetra-plicated sequences FOXG_12535, FOXG_12585, FOXG_17088 and FOXG_14004 (collectively named *pg9*); and FOXG_13191 (named *pgx10*). The number of predicted PG genes in *F. oxysporum* was higher than in other *Fusarium* spp.; a total of five genes was detected in *F. graminearum* and *F. verticillioides*. Phylogenetic relationships among the *Fusarium* PG genes, as well as their orthologues in related fungal pathogens, were analysed. The sequences group into four major clades: two for the endoPGs (Fig. 1a) and two for the exoPGs, except *Pgx7* (Fig. 1b). Amino acid identities ranged from 40% to 54% within endoPGs and from 20% to 58% within exoPGs. The domains Asn-X-Asp (NXD; Asn, asparagine; Asp, aspartic acid), Asp-Asp (DD), Gly-His-Gly-X-Ser-Ile-Gly (GHGXSIG; Gly, glycine; His, histidine; Ile, isoleucine; Ser, serine), Arg-Ile-Lys (RIK; Arg, arginine; Lys, lysine) and Tyr (Y; Tyr, tyrosine), which are highly conserved in fungal PGs (Caprari *et al.*, 1993), were present in all predicted proteins with the exception of *Pgx7*. exoPGs show a serine/threonine (Ser/Thr) substitution of the first Gly within the conserved motif GHGXSIGS/T and two cysteine (Cys) residues next to the Tyr located in the catalytic site (Fig. 2) (Markovic and Janecek, 2001).

To identify conserved sequences with a putative role in transcription factor binding, upstream promoter regions of *pg* genes were analysed (Table 1). The TYATTGGTGGA sequence was reported to be important for *A. niger* *pgal1* induction (Visser *et al.*, 1994). Two copies of this consensus sequence are present in the promoter region of *pg1* and one copy in those of *pgx6* and *pg12*. A second sequence CCCTGA, previously described in *A. niger* as presumably implicated in pectin induction of hydrolytic genes (Benen *et al.*, 1996; de Vries *et al.*, 2002), is present in four *F. oxysporum* PG genes: *pg1*, *pgx4*, *pg8* and *pg9*. Most *pg* pro-

Fig. 1 Phylogram of predicted *Fusarium oxysporum* polygalacturonases (PGs). The PHYML 3.0 program (Guindon and Gascuel, 2003) was used to perform a 1000 non-parametric bootstrap phylogenetic analysis of the alignment of the indicated amino acid characters with the maximum likelihood method after optimization of the settings by the Modelgenerator program, version 0.85 (Keane *et al.*, 2006). Results are depicted in a phylogenetic tree constructed using MEGA version 4 (Tamura *et al.*, 2007). (a) The analysis includes the alignment of 158 amino acid characters from endoPGs of *F. oxysporum* (FOXG), as well as orthologues from *F. graminearum* (FGSG), *F. verticillioides* (FVEG), *Nectria haematococca* (NECHA), *Magnaporthe oryzae* (MGG), *Colletotrichum graminicola* (GLRG), *Cochliobolus carbonum* (COCCA), *Botryotinia fuckeliana* (BC1G) and *Sclerotinia sclerotiorum* (SS1G). PGs group within two clades; clade I contains Pg1 and clade II contain the rest. (b) The analysis includes the alignment of 112 amino acid characters from the exoPGs of *F. oxysporum* (FOXG), as well as orthologues from *F. graminearum* (FGSG), *F. verticillioides* (FVEG), *Colletotrichum graminicola* (GLRG), *Nectria haematococca* (NECHA), *Magnaporthe oryzae* (MGG), *Botrytis cinerea* (BC1G), *Sclerotinia sclerotiorum* (SS1G) and *Cochliobolus carbonum* (COCCA). exoPGs group within two clades; clade I contains Pgx6 and Pgx10, and clade II contains Pgx4. Pgx7 appears not to belong to any clade.

motors harbour CREA motifs (G/CYGGGG) related to carbon catabolite repression (Kulmburg *et al.*, 1993), and four promoters contain binding sequences of transcription factors related to development, such as Ste12 (TGAAACA) (Madhani and Fink, 1998) and AbaA (CATTCT) (Andrianopoulos and Timberlake, 1994).

Expression of PG encoding genes in *F. oxysporum*

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with gene-specific primers was used to measure the abundance of transcripts in the *F. oxysporum* wild-type strain grown on the inducing carbon sources galacturonic acid (GA), polygalacturonic acid (PGA) and pectin (Fig. 3a). The

highest expression levels (close to 40-fold relative to the actin gene) were detected for the *pg1* gene in GA after 10–14 h of induction, followed by the *pgx4* gene (1.7-fold). Expression profiles in pectin were similar, but at lower levels, whereas PGA was a less effective inducer. The remaining PG genes showed very low transcript levels (<0.02-fold of actin, results not shown). In the presence of pectin and glucose, all PG genes showed very low transcript levels, suggesting the presence of carbon catabolite repression (data not shown).

To investigate the expression of the different PG genes during infection, the abundance of transcripts was quantified in the roots and stems of tomato plants at different times after inoculation, using the fungal actin gene for transcript normalization. Transcript

Fig. 2 Conserved amino acid domains of eight predicted polygalacturonases (PGs) from *Fusarium oxysporum*. Sequence alignment was performed with the Bioedit 7.0.0 program (Hall, 1999) using the CLUSTALW method. Boxes indicate conserved domains, and the essential residues for PG function are shaded. Conserved residues specific for exoPGs, which are absent in endoPGs, are shaded in black.

Table 1 Putative recognition motifs for binding of transcription factors in polygalacturonase (PG) gene promoter regions. Position relative to the ATG start codon is indicated. Putative pectin response box (TTGGGCAA) (Sancho R, Mammalian Genetics Laboratory, Cancer Research, London Research Institute, UK. *et al.*, unpublished data); *Aspergillus niger* pectin response region (CCCTGA) (Benen *et al.*, 1996); binding motifs for transcriptional regulators CREA (G/CYGGGG) (Kulmburg *et al.*, 1993), Aba/Tec1 (CATTCT) (Andrianopoulos and Timberlake, 1994) and Ste12 (TGAACA) (Madhani and Fink, 1998).

Gene	Pectin box	<i>A. niger</i> region	CREA	Aba/Tec1	Ste12
<i>pg1</i>	−731; −431	−421	−876	N.I.	N.I.
<i>pgx4</i>	−454	−865	−527; −200	−353	N.I.
<i>pg5</i>	N.I.	N.I.	−623	−825; −74; −27; −11	−423
<i>pgx6</i>	−894	N.I.	−209; −134	N.I.	N.I.
<i>pgx7</i>	N.I.	N.I.	−668; −295	−902	−589
<i>pg8</i>	N.I.	−736	−403	−168	−454
<i>pg9</i>	N.I.	−669	−894	N.I.	−527
<i>pgx10</i>	N.I.	N.I.	N.I.	N.I.	N.I.

N.I., not identified.

levels of the different PG genes varied over a 60-fold range, with *pg1* showing the overall highest levels between 2 and 6 days after inoculation in roots. A second group of genes, including *pgx4*, *pg5* and *pgx6*, showed medium expression levels (Fig. 3b), whereas *pgx7*, *pg8*, *pg9* and *pgx10* exhibited very low expression levels (Fig. 3c). No fungal *pg* gene transcripts were detected in the non-inoculated control plants.

Targeted deletion of the major endo- and exoPG genes

The gene expression profiles observed during growth in both *in planta* and *in vitro* conditions indicated that *pg1*, *pgx4*, *pg5* and *pgx6* contribute to most of the total PG activity. As deletion mutants in *pgx4* or *pg5* were already available (Garcia-Maceira

Fig. 3 Transcript levels of predicted polygalacturonase (PG) encoding genes in *Fusarium oxysporum* wild-type strain relative to actin measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (a) Mycelia were transferred for different time periods (h) to the indicated carbon source. GA, galacturonic acid; PGA, polygalacturonic acid. (b, c) Roots of tomato plants were inoculated with *F. oxysporum* and RNA was obtained after the indicated time periods (days). Graphs show transcript levels relative to actin of PG genes expressed at high (b) or low (c) levels. Bars represent standard errors calculated from three biological replicates, each including three technical replicates.

et al., 2000, 2001), we performed targeted deletion of *pg1* and *pgx6* in *F. oxysporum*. Analysis by PCR and Southern blot identified one and two transformants carrying targeted deletions at the *pg1* (#12) or *pgx6* (#5 and #21) locus, respectively (Fig. S1a, b, see Supporting Information). To generate a $\Delta pg1 \Delta pgx6$ double mutant, targeted deletion of *pgx6* was carried out in the $\Delta pg1$ background using nourseothricin resistance (Nat^R) as selective marker (Hoff *et al.*, 2010). In one transformant (#20), the 12.2-kb band containing the *pgx6* gene was replaced by a 7.5-kb fragment hybridizing with the Nat^R cassette (Fig. S1c). Complementation of the $\Delta pg1 \Delta pgx6$ double mutant was carried out by co-transformation with a 3.9-kb frag-

ment containing the intact *pg1* gene, using the Phl^R (phleomycin resistance) marker. Two complemented transformants (#2 and #21) were identified and confirmed by Southern blot analysis (Fig. S1d).

To determine the effect of gene deletion on the expression of the remaining PG genes, transcript levels in the different PG-deficient mutants were compared with those of the wild-type strain, either during tomato plant infection (Fig. 4a) or during growth on pectin. Collectively, the results of these experiments suggest the existence of compensatory effects among PG genes in the different mutant backgrounds during the infection of tomato plants. For example, the $\Delta pg1$ mutant showed a five-fold

Fig. 4 Relative expression of predicted polygalacturonase (PG) encoding genes in *Fusarium oxysporum* $\Delta pg1$, $\Delta pgx6$ and $\Delta pg1\Delta pgx6$ mutants in comparison with the wild-type strain determined by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA samples were obtained from roots (R) or stems (S) of tomato plants at the indicated days (D) after inoculation (a), or from mycelia transferred for 6 h to 0.5% pectin (b). Bars represent standard errors calculated from three biological replicates, each including three technical replicates.

increase in transcript levels of *pgx4*, *pg5* and *pgx6* in infected tomato stems compared with the wild-type (Fig. 4b).

One exo- and one endoPG gene are responsible for >90% of the total secreted PG activity

Inspection of the amino acid sequences of the predicted *F. oxysporum* PGs suggested that they fall into two groups, one containing proteins with acidic to neutral and the other containing proteins with alkaline isoelectric point (Fig. 5a). To experimentally validate the predicted extracellular enzyme profile, concentrated and dialysed culture filtrates of *F. oxysporum* grown on pectin were subjected to preparative isoelectric focusing (IEF) over the pH range 3–10. As shown in Fig. 5b, the fractions with acidic pI (3–5) contained around 10% of the total PG activity, whereas the fractions with neutral and alkaline pI values contained nearly 90% of the total activity (Fig. 5b).

We next determined the relative contributions of the major PG genes to the secreted PG activity. Pectin-induced culture supernatants of the wild-type strain were compared with those of the different deletion strains by measuring their total PG or specific endoPG activity (Fig. 5c,d). The $\Delta pg1$ mutant showed a strong decrease in total PG and endoPG activity, whereas $\Delta pgx6$ showed a 16% reduction in total relative to the wild-type, which was largely a result of the decrease in exoPG activity. The double mutant showed a further reduction in total activity (up to 90%), which was largely reversed by reintroduction of the intact *pg1* allele (Fig. 5c). Analysis of the concentrated culture supernatants by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5e) revealed the absence of specific protein bands in the single and double deletion mutants (indicated by asterisks in lanes 3, 4 and 5), as well as the presence of the Pg1 protein band in the complemented strain (lane 6).

No apparent differences in dry weight accumulation were observed between the mutants and wild-type strain during growth in 0.5% pectin, except for a certain delay in the double mutant at early time periods (Fig. 6a). The generation and consumption of reducing sugars in media containing complex (PGA or pectin) and simple (GA) carbon sources were determined in the wild-type (Fig. 6b).

Next, the presence of degradation products was monitored in the culture supernatants of the wild-type strain and the mutants grown on pectin or PGA using thin layer chromatography (TLC). The supernatants from the $\Delta pg1\Delta pgx6$ mutant contained much less intermediate (oligoGA, di-GA) and end-product (GA) than those of the single mutants and the wild-type strain (Fig. 6c,d). This indicates a strong cooperative effect between endoPG and exoPG activities, where the oligomeric GA forms are generated by the major endoPG Pg1 and further degraded by the major exoPG Pgx6 (Fig. 6c,d).

Loss of the major endo- and exoPG causes a significant decrease in virulence

To determine the role of the two major PGs in the virulence of *F. oxysporum*, roots of 2-week-old tomato plants were inoculated with the different fungal strains. Most plants inoculated with the wild-type strain, the single mutants or the ectopic transformants (not shown) were dead 30 days after inoculation (Fig. 7). The mortality of plants inoculated with the $\Delta pg1\Delta pgx6$ double mutant was significantly reduced according to the log-rank (Mantel–Cox) test ($P = 0.0004$), with 60% of the plants still alive after 30 days. Importantly, complementation of the $\Delta pg1\Delta pgx6$ mutant with the *pg1* wild-type allele fully restored the virulence phenotype. Real-time qPCR of infected tomato plants did not detect significant differences in the amount of fungal biomass between the different strains, suggesting that the decrease in virulence of the $\Delta pg1\Delta pgx6$ mutant is not associated with a reduction in plant colonization (Fig. S2, see Supporting Information).

We next monitored the transcript levels of the defence-related genes *lin6* (extracellular invertase), *gluB* (basic glucanase), *chi3* and *chi9* (acidic and basic chitinase, respectively) and *pgip1* (polygalacturonase-inhibiting protein) (Stotz *et al.*, 1994) in roots of tomato plants inoculated with the different fungal strains using real-time qRT-PCR (Pareja-Jaime *et al.*, 2010). At 2 or 4 days after inoculation, the transcript levels of the six defence genes did not differ significantly between the plants inoculated with the different fungal strains and the non-inoculated control plants (Fig. S3, see Supporting Information).

DISCUSSION

Fungal pathogens secrete an array of virulence factors into the surrounding environment to promote infection and to facilitate the assimilation of nutrients from the host (Espino *et al.*, 2010; Zhang *et al.*, 2014). The root-infecting pathogen *F. oxysporum* produces a number of endo- and exoPGs during growth on pectin, some of which are also detected during tomato infection (Di Pietro and Roncero, 1996). We found that the genome of *F. oxysporum* contains a higher number of predicted PG genes than those of the related species *F. graminearum* and *F. verticillioides*, as reported previously for other gene families in the *F. oxysporum* species complex (Bravo Ruiz *et al.*, 2013; Ma *et al.*, 2010). The difference in the number of PG genes between the three *Fusarium* species probably reflects the divergences in the adaptation to the infection process in the roots and aerial parts of the plant.

The expression of PG encoding genes is generally induced by pectin or by its monomer GA, and is repressed in the presence of carbon catabolites, such as glucose (Li *et al.*, 2004). We found that, in *F. oxysporum*, the monomer GA was the most efficient inducer of *pg* gene expression. Substitution of GA with pectin or PGA affected *pg* gene transcript levels, which were 10- to 100-fold

Fig. 5 Biochemical analysis of secreted polygalacturonases (PGs) from *Fusarium oxysporum*. (a) Features of predicted PG gene products identified in the *F. oxysporum* genome database at http://www.broadinstitute.org/annotation/genome/fusarium_group/. Prediction of signal peptides was performed using SignalP v4.0 <http://www.cbs.dtu.dk/services/SignalP>. Prediction of molecular mass (kDa) and isoelectric point was calculated using the webpage http://scansite.mit.edu/calc_mw_pi.html *Genes manually curated after alignment with related orthologues and paralogues. [#]Identical tetra-plicated gene sequences. (b) Extracellular PG activity profile from pectin-induced wild-type cultures after isoelectric focusing with a Rotofor apparatus. (c) Total extracellular PG activity from culture supernatants in the wild-type and indicated mutant strains after 6 h of growth in pectin, using the Nelson–Somogyi method. (d) Specific endoPG activity from culture supernatants from the wild-type and indicated mutant strains after 6 h of growth in pectin, using the ruthenium red dye assay (Torres *et al.*, 2011). (e) Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracellular proteins in cultures of the wild-type and indicated mutant strains after 6 h of growth in pectin. Black asterisks show the absence of Pg1 protein, white asterisks show the absence of Pg6 protein, and the white circle shows the restored Pg1 protein in the complement strain. wt, wild-type.

Fig. 6 Pectinolytic activity of *Fusarium oxysporum* polygalacturonase (PG) mutants. (a) Mycelial dry weight of the indicated strains grown for different time periods (days) on pectin. Bars indicate standard errors calculated from three biological replicates each including three technical replicates. Asterisk indicates significant difference between the double mutant and the rest of the strains (Student's *t*-test, $P \leq 0.01$). (b) Presence of reducing sugars over time in culture supernatants of the wild-type strain grown on the indicated carbon sources was followed using the Nelson–Somogyi method. (c, d) Thin layer chromatography (TLC) from 50-fold-concentrated supernatants of the different PG mutants and the wild-type strain grown for the indicated time periods (h) on pectin (c) or PGA (d). DGA, digalacturonic acid; GA, galacturonic acid; P, pectin; PGA, polygalacturonic acid.

reduced, respectively, in comparison with GA. The presence of multiple secreted PGs in *F. oxysporum* suggests that they may serve distinct functions during nutrient acquisition and infection of tomato plants. Previous studies in plant-pathogenic fungi have suggested that the pattern of CWDE gene expression *in planta* may differ substantially from that observed *in vitro*, as certain inducing factors present in the plant tissue may be lacking in the artificial substrates (Annis and Goodwin, 1997). Analysis of the different *pg* genes during infection of tomato plants revealed

three different expression patterns. One group comprising the *pg1* and *pgx6* genes is highly induced during plant infection. The second group includes genes with a medium level of expression (*pgx4* and *pg5*), whereas the third group comprises the remaining genes which are expressed at very low levels. The maximum transcript levels were observed at 2–6 days after inoculation, suggesting that PGs function at early stages of infection. However, it cannot be excluded that PGs may also contribute to later stages of disease. The finding that only four of the eight *pg* genes are

Fig. 7 Virulence of *Fusarium oxysporum* wild-type and mutant strains on tomato plants. Groups of 2-week-old tomato plants (cv. Monika) were inoculated by dipping roots into a suspension of 5×10^6 freshly obtained microconidia/mL of the indicated fungal strains; the tomato plants were planted in vermiculite and maintained in a growth chamber (Di Pietro and Roncero, 1998). Percentage survival was plotted for 30 days. Data shown are from one representative experiment. Experiments were performed three times with similar results.

significantly expressed *in planta* suggests differences in the regulation of individual genes. So far, no general transcription factor for pectin- or GA-mediated induction of *pg* genes has been identified in filamentous fungi.

Pg1 has been identified previously as the major endoPG secreted by *F. oxysporum* during growth in pectin medium and during infection of tomato roots (Di Pietro and Roncero, 1996, 1998). A natural melon-infecting *F. oxysporum* isolate carrying a *pg1* allele inactivated by a transposon insertion showed a dramatic decrease in PG activity on pectin, but was still able to infect muskmelon plants (Di Pietro and Roncero, 1998). Expression of a wild-type *pg1* allele in the *pg1*-deficient isolate resulted in a strong increase in secreted PG activity, but had no significant effect on plant infection, suggesting that Pg1 by itself is not essential for virulence in *F. oxysporum* (Di Pietro and Roncero, 1998). Likewise, individual deletion of a second endoPG gene, *pg5*, or of the exoPG gene *pgx4*, which are both expressed during the early stages of infection, did not affect the virulence of *F. oxysporum* on tomato plants (Garcia-Maceira *et al.*, 2000, 2001). Likewise, we found here that targeted deletion of either *pg1* or *pgx6* did not affect the infectious capacity of *F. oxysporum*. However, simultaneous disruption of both genes caused a severe reduction in virulence, suggesting that different PGs, although partially redundant with respect to total PG activity, carry out different and specific functions during pathogenicity. Interestingly,

the double mutant, but not the two single mutants, generated less final (GA) or intermediate hydrolysis products than the wild-type strain. This supports the idea that endo- and exoPG act synergistically during substrate degradation. Collectively, these results demonstrate that the coordinated action of endo- and exoPGs plays an important role during the development of vascular wilt disease caused by *F. oxysporum* on tomato plants.

The exact role of the interplay of PGs during infection remains to be elucidated. Increased elicitation of plant defence responses has been observed on alterations in the pattern of secreted CWDEs (Di Pietro *et al.*, 2009). Recently, transgenic *Arabidopsis* plants expressing a particular polygalacturonase inhibiting protein (PGIP)-PG chimera, referred to as the 'OG machine' (OGM), were found to accumulate OGs and to exhibit enhanced resistance to a variety of pathogens, providing direct evidence for the function of OGs as *in vivo* elicitors (Benedetti *et al.*, 2015). The upregulation of plant defence genes by avirulent *F. oxysporum* mutants has been reported previously (Benhamou and Garand, 2001; Duijff *et al.*, 1998; Fuchs *et al.*, 1997; Olivain *et al.*, 2003; Panina *et al.*, 2007; Pareja-Jaime *et al.*, 2010). We therefore tested whether the induction of tomato defence responses by the $\Delta pg1 \Delta pgx6$ mutant could account for the observed decrease in virulence. However, the mutant did not trigger an increased expression level of tomato defence genes, indicating that suppression of host immunity is not a major role of these two PGs during infection. Other yet unknown mechanisms must therefore account for the reduced virulence of the $\Delta pg1 \Delta pgx6$ mutant. The finding that host colonization is not significantly affected in the mutant suggests that PGs may play a crucial role in the induction of disease symptoms on infected plants.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum f.sp. *lycopersici* wild-type strain 4287 (race 2) was obtained from J. Tello (Universidad de Almeria, Spain). Microconidial suspensions from wild-type and mutant strains were stored in 30% glycerol at -80°C . The pathotype of the isolates was periodically confirmed by plant infection assays. For extraction of DNA and microconidia production, cultures were grown in potato dextrose broth (PDB) (Difco, Franklin Lakes, New Jersey, USA) at 28°C , as described previously (Di Pietro and Roncero, 1998).

For induction conditions 5×10^8 *Fusarium* microconidia were grown in PDB medium for 14 h. The germlings obtained were collected and transferred to flasks containing synthetic medium (SM) (Di Pietro and Roncero, 1998) supplemented with one of the following substrates: 0.5% (w/v) pectin from citrus fruit, 0.5% (w/v) PGA, 0.5% (w/v) GA, 0.5% (w/v) glucose or a combination as specified, and cultures were maintained in a rotary shaker at 170 rpm and 28°C for the indicated time periods.

Dry weight was determined from freeze-dried mycelia obtained from 1×10^8 spores grown under induced conditions, SM supplemented with 0.5% pectin, for the indicated time periods.

Reducing sugars from wild-type culture supernatants induced on SM containing different carbon sources, pectin (0.5%), GA (0.5%) or PGA (0.5%), were determined by the Nelson–Somogyi method, as described previously (Di Pietro and Roncero, 1996), for the indicated time periods.

Nucleic acid manipulation

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelia according to previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). Plant RNA was isolated using the RNeasy plant minikit (Qiagen Iberia, Madrid, Spain). Southern analyses and probe labelling were carried out as described previously (Di Pietro and Roncero, 1998) using the non-isotopic digoxigenin labelling kit (Roche, Barcelona, Spain). Sequencing of DNA clones used in fungal transformation experiments was performed at STAB-VIDA (Caparica, Portugal). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (Bethesda, MD, USA), at the Broad Institute *Fusarium* comparative website www.broadinstitute.org. PCRs were routinely performed with the High Fidelity Template PCR system (Roche).

Gene transformation-mediated replacement and complementation

Targeted gene replacement was performed using the split marker technique (Catlett *et al.*, 2003). Two overlapping gene deletion constructs were used for replacement, one containing the promoter of the target gene obtained by amplification with specific primers (Table 2) fused to the 3'-end (approximately 75%) of the Hyg^R (hygromycin resistance) cassette (Carroll *et al.*, 1994), and the other fragment containing the terminator region of the target gene obtained by amplification with specific primers fused to the 5'-end (approximately 75%) of the Hyg^R cassette (Table 2). For construction of single disruption mutants, each set of overlapping fragments was used to transform protoplasts of the wild-type strain 4287, as reported previously (Di Pietro and Roncero, 1998). The resulting Hyg^R transformants were identified by PCR, and homologous recombination events were further confirmed by Southern analysis of selected clones using promoter or internal gene fragments as probes (Fig. S1a, b).

The double deletion mutants were obtained by co-transformation of protoplasts from the Δ FOXG_14695 (Δ pg1) mutant using the appropriate construct for targeted deletion of FOXG_15415 (*pgx6*), by the split marker technique, together with the Nat^R cassette as selective marker (Hoff *et al.*, 2010), using 75 µg/mL of the antibiotic (Werner BioAgents, Jena, Germany). The resulting Nat^R transformants were identified by PCR and Southern analyses (Fig. S1c).

Complementation of *pg1* was achieved by reintroduction of the *pg1* allele into a Δ pg1 Δ pg6 mutant genetic background using the Phl^R cassette (Punt and van den Hondel, 1992) as selective marker; the complemented strains harbouring *pg1* wild-type version were confirmed by PCR and subsequently by Southern analyses (Fig. S1d).

Purification and biochemical characterization of secreted PGs

All purification steps were carried out at 4 °C unless stated otherwise. For total PG activity, germlings from the wild-type and mutant strains were

collected from PDB cultures 14 h after inoculation, and transferred for induction to 250-mL Erlenmeyer flasks containing 50 mL of SM (Di Pietro and Roncero, 1998) supplemented with the indicated carbon source. Cultures were induced for 6 h, filtered through Monodur (pore size, 10 µm) and centrifuged to discard microconidia; the supernatants were transferred to dialysis tubing (12-kDa cut-off), dialysed overnight against distilled water and then concentrated 50-fold by placing the tubing on solid polyethylene glycol ($M_r = 35\,000$) (Fluka Chemika-Biochemika, St Louis, Missouri, USA). Ten-microlitre aliquots from diluted (1:10) supernatants were used as source for activity assays.

Total PG activity was determined by measurement of the release of reducing groups from PGA using the Nelson–Somogyi method as described previously (Di Pietro and Roncero, 1996). Appropriate controls without either enzyme or substrate were performed simultaneously. The quantity of reducing sugar released was calculated from standards of GA. Enzyme activity is expressed in micromoles of GA released min⁻¹ and µL⁻¹ of supernatant used. Each assay was carried out at least three times in 96-well microtitre plates. For the pl profile, germlings from the wild-type were transferred for induction to 500-mL Erlenmeyer flasks containing 100 mL of SM supplemented with 0.5% pectin for 6 h; the supernatants were obtained and concentrated two-fold as described above. The concentrates were then subjected to preparative IEF on a Rotofor apparatus (BioRad, Munich, Germany) carried out in a total volume of 55 mL containing 2% (w/v) carrier ampholytes (Bio-Lyte 3/10; BioRad) at 4 °C for 3–6 h at a constant power of 12 W. Twenty fractions were collected and analysed for PG activity. The profile of extracellular proteins induced by pectin was compared between the different mutants and the wild-type strain by SDS-PAGE performed in 12% discontinuous (w/v) acrylamide gels (Bio-Rad). Similar protein quantities were used as determined by Bradford using the BioRad protein assay, with bovine serum albumin as standard.

Specific endoPG activity was determined by measuring the remaining PGA after PG action using the ruthenium red (RR) colorimetric assay, as described by Torres *et al.* (2011) with minor modifications. Aliquots containing 10 µL of enzyme preparation were mixed with 90 µL of 1% PGA and incubated for 30 min at 37 °C. After incubation, 900 µL of distilled water and 15 µL of RR stock solution (5 mg/mL) were added and homogenized. The mixture was then diluted to 2 mL, and centrifuged at 5000 *g* for 5 min to discard the precipitated RR–PGA complex. The resulting supernatants were transferred to 96-well microtitre plates and A_{535} was determined. Appropriate controls without either enzyme or substrate were performed simultaneously. Enzyme activity is expressed in nanograms of PGA converted to smaller fragments unable to precipitate with the dye per minute and per microlitre of enzyme preparation used under the standard assay conditions.

PG activities were measured both as the amount of reducing ends generated during the enzyme reaction, determined by the Somogyi–Nelson method, and as the remaining substrate after the enzyme hydrolysis by the novel RR dye method. These endo- and exoPG activities were further confirmed by the detection of GA by TLC.

TLC

The different mutants and the wild-type strain were grown with pectin (0.5%) or PGA (0.5%) as unique carbon source for 2, 4 or 6 h, and 50 mL of cell-free supernatant were freeze-dried and resuspended in 1 mL of

Table 2 Oligonucleotides used in this study. Lowercase is the overlapping region with the 3' end of the Hyg^R/Phl^R/Nat^R cassettes to generate DNA fragment fusions by polymerase chain reaction (PCR) amplification. Positions relative to the start codon, either downstream (+) or upstream (–) of ATG, are indicated. (s) sense; (as) antisense.

Name	Sequence 5'–3'	Position to ATG	Experimental use
<i>Fusarium pg</i>			
pg1-1	GTCACCTCGGGTACAAACATC	+790 (s)	qRT-PCR/gene identification
pg1-2	CCTTGATGAACCTTGATGCCGC	+1169 (as)	qRT-PCR/gene identification
pg1-3	GCAGCGCTACTGACTACTCC	+120 (s)	Southern
pg1-4	GTTAGAACCTTGCCATCCCA	+428 (as)	Southern
pg1-5	AGCTCCGAGTAAGAGAACAAAG	–1630 (s)	Split marker
pg1-6	GGAATAATGTAATCGCATCACT	–1596 (s)	Split marker/complementation
pg1-7	ttaccagaatgcacagggtacactgtttCGAGTGAGACAAGACAAGAG	–30 (as)	Split marker/Southern
pg1-8	ATTTAGGGTGGCTTTGTGGAT	+2722 (as)	Split marker
pg1-9	GCTTTGGTTGGCTTTTGTAAAG	+2659 (as)	Split marker
pg1-10	tggtcgtttaggggctgtattaggtctcgCTAAGCGGGACGATCATCAC	+1320 (s)	Split marker
pg1-11	TTATTACCCGTAACCCGCA	–1076 (s)	Southern
pg1-12	ACGGTGCTGTGATCTATGAAG	+2298 (as)	Complementation
pgx4-1	GTACAGCATTGCCTGCCAC	+275 (s)	qRT-PCR
pgx4-2	CGGGTTTCTCATTCGAGGTT	+662 (as)	qRT-PCR
pg5-1	GCCTGGTCGCTCTCGTACT	+28 (s)	qRT-PCR
pg5-2	TCTTCTTGGCCCGTTGCTGCCCTTGCCGT	+418 (as)	qRT-PCR
pgx6-1	GAAGTCATCGCAAGGTCTATAC	+136 (s)	qRT-PCR/Southern/gene identification
pgx6-2	AGAACAAGATAGGTGCGAGGTA	+635 (as)	qRT-PCR/Southern/gene identification
pgx6-3	AGTAATGAGCTTGTGGTCCGG	–1093 (s)	Southern
pgx6-4	gctgttcctgtgtgaaattgttatccgctGACGGGGTTTGTGGTAATTAG	+1594 (s)	Split marker
pgx6-5	TTCATATCCTTAGCGGTGATT	–1588 (s)	Split marker
pgx6-6	TTCTCTGGCTCAACGGTCT	–1542 (s)	Split marker
pgx6-7	ttaccagaatgcacagggtacactgtttCAGAAGATGAGTTGGCTTGAG	–40 (as)	Split marker/Southern
pgx6-8	CTTCGGGAGCATCAGTGGC	+3190 (as)	Split marker
pgx6-9	GTCGTGGTTTCATCTTGAGG	+3107 (as)	Split marker
pgx6-10	tggtcgtttaggggctgtattaggtctcgGACGGGGTTTGTGGTAATTAG	+1594 (s)	Split marker
pgx6-11	tacaacgtcgtgactgggaaacccctggcgCAGAAGATGAGTTGGCTTGAG	–40 (as)	Split marker
pgx7-1	TCCAAGTACACGACCCTTTCAA	+417 (s)	qRT-PCR
pgx7-2	GCTGTTCACAATAAGACTCGTC	+750 (as)	qRT-PCR
pg8-1	CTCACATCAACCAATTCACCTC	+516 (s)	qRT-PCR
pg8-2	CACTGCCGATGGAGAGACC	+759 (as)	qRT-PCR
pg9-1	GCCGTTTCCGAGATAGTAAT	+379 (as)	qRT-PCR
pg9-2	AGTTCAGACTCCCATCCAGCC	+52 (s)	qRT-PCR
pgx10-1	GGGGAGGAAAGGACATTAGAA	+460 (s)	qRT-PCR
pgx10-2	GTTGTTGACAGTCCGGTTG	+806 (as)	qRT-PCR
<i>Fusarium</i> qRT-PCR primers			
act-q7	ATGTCACCACCTTCAACTCCA	+1278 (s)	qRT-PCR
act-q8	CTCTCGTCGTAATCTCTGCTT	+1578 (as)	qRT-PCR
chsV-3	ACAGCTCCAACGAATCTCTT	+2910 (s)	qRT-PCR
chsV-26	GGAGGTACTTGGTCATGTCGT	+3417 (as)	qRT-PCR
Hygromycin resistance (Hyg ^R) cassette			
hyg-G	CGTTGCAAGACCTGCCTGAA	+289 (s)	Split marker
hyg-Y	GGATGCCTCCGCTCGAAGTA	+735 (as)	Split marker
TtrpC-8B	AAACAAGTGATCTGTGCATTG	+1727 (as)	Split marker
PgpdA-15B	CGAGACCTAATACAGCCCT	–1193 (s)	Split marker
Phleomycin resistance (Phl ^R) cassette			
TtrpC-8B	AAACAAGTGATCTGTGCATTG	+1133 (as)	Complementation
PgpdA-15B	CGAGACCTAATACAGCCCT	–1193 (s)	Complementation
phl-1	CGGAGCGGTGAGTTCTGG	+50 (s)	Probe Southern
phl-2	CGAACTCCCGCCCCACG	+301 (as)	Probe Southern
Nourseothricin resistance (Nat ^R) cassette			
m13-F	CGCCAGGGTTTCCAGTCACGAC	+715 (as)	Split marker
m13-R	AGCGGATAACAATTCACACAGGA	–503 (s)	Split marker
nat-1	TCCTTCAACACCGACACCG	+81 (s)	Split marker
nat-2	ACCCATCCGCGGTACG	+463 (as)	Split marker
Tomato plant qRT-PCR primers			
Efa1-1	TACTGGTGGTTTTGAAGCTGG	+2809 (s)	qRT-PCR
Efa1-2	AACTTCCTTACGATTTATCA	+3055 (as)	qRT-PCR
pgip-1	TGCTTTGCTTCTCTTCACTAT	+36 (s)	qRT-PCR
pgip-2	GGAAGACGGTGAGAGCATTTA	+223 (as)	qRT-PCR
lin6-1	GAAACACTAAGAAAGAAAAGGT	+1137 (s)	qRT-PCR
lin6-2	AGACCACCTTGAACCGTTGA	+1352 (as)	qRT-PCR
gluB-1	ATTCTGTTATGCTGCGATGG	+866 (s)	qRT-PCR
gluB-2	CTTCTCGGACTACCTTCTTT	+1009 (as)	qRT-PCR
chi3-1	TCTGTCTCTTTTCTTGTCC	+61 (s)	qRT-PCR
chi3-2	GCAGTATCATCACAGCAGT	+236 (as)	qRT-PCR
chi9-1	GCCTTCTGTGACGATGTA	+767 (s)	qRT-PCR
chi9-2	CTCCAAGAATTCCGCAATACC	+935 (as)	qRT-PCR

qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

distilled water. Aliquots of 10 µL were applied and submitted to TLC separation using pre-coated TLC-plates (silica gel) (Macherey-Nagel, Düren, Germany) run vertically in 2:1:1 (v/v/v) *n*-butanol : acetic acid : water, and the final products were visualized by spraying the plates with 1.8% aniline, 1.8% diphenylamine in acid acetone, as described previously (Garcia-Maceira *et al.*, 1997). GA (3%, w/v) and diGA (3%, w/v) were used as standards.

Phylogenetic analysis of predicted PG encoding genes

Amino acid sequences were aligned using the CLUSTALW algorithm (Thompson *et al.*, 1994) with the Bioedit 7.0.0 program (Hall, 1999) and cleaned by GBLOCKS v0.91b (Castresana, 2000). The PHYML 3.0 program (Guindon and Gascuel, 2003) was used to perform a 1000 non-parametric bootstrap phylogenetic analysis of the resulting alignment with the maximum likelihood method after optimization of the settings by the Modelgenerator program, version 0.85 (Keane *et al.*, 2006). Phylogenetic relationships among sequences were depicted in phylogenetic trees constructed using MEGA version 4 (Tamura *et al.*, 2007).

Expression analyses of *Fusarium* PG encoding genes

The expression analysis of the predicted PG encoding genes [*pg1*, FOXG_14695 (Di Pietro and Roncero, 1998); *pgx4*, FOXG_08862 (Garcia Maceira *et al.*, 2000); *pg5*, FOXG_13051 (Garcia Maceira *et al.*, 2001)] and the five newly identified genes (FOXG_15415, *pgx6*; FOXG_09893, *pgx7*; FOXG_10052, *pg8*; the tetra-plicated sequence FOXG_12535, FOXG_12585, FOXG_17088, FOXG_14004, all corresponding to *pg9*; FOXG_13191, *pgx10*) was performed *in vitro* under inducing conditions. RNA from *F. oxysporum* wild-type germlings grown for different time periods (2–10 h on 0.5% pectin or 0.5% PGA and 2–24 h on 0.5% GA) was analysed by qRT-PCR. *In vivo* expression of the predicted PG genes during plant colonization was also analysed. Roots were maintained immersed in water containing 5×10^6 spores/mL with rotary shaking at 28 °C, and five tomato plants from each treatment were removed at the indicated time periods (1–10 days after inoculation). The isolated RNA obtained from germlings, or from the roots and stems of non-inoculated and inoculated plants, was treated with DNase I (Life Technologies, Alcobendas, Madrid, Spain) and then used to synthesize cDNA using the Transcriptor Universal cDNA Master mix (Roche). Total cDNA obtained was subjected to qRT-PCR using the appropriate gene-specific primers (Table 2) and FastStart Essential DNA Green Master (Roche). Relative levels of the RT-PCR products were determined using the $\Delta\Delta Ct$ or ΔCt method (Livak and Schmittgen, 2001). Expression values are presented as relative to those of the wild-type strain or non-inoculated plants, and normalized to the *Ct* value of the *Fusarium* actin gene or the tomato elongation factor alpha 1 (*EFα1*), as indicated. Samples without cDNA were used as negative control reactions. The assay was repeated three times with independent biological samples.

Plant infection assays

Tomato root inoculation assays were performed as described previously (Di Pietro and Roncero, 1998) using 2-week-old tomato seedlings (cultivar

Monika, seeds kindly provided by Syngenta, Almería, Spain) and *F. oxysporum* strains by immersing the roots in a suspension of 5×10^6 spores/mL for 30 min; the tomato plants were planted in vermiculite and maintained in a growth chamber. Ten plants were used for each treatment. The severity of disease symptoms and plant survival were recorded daily over 30 days as described previously (López Berges *et al.*, 2012). The Mantel–Cox method was used to assess the statistical significance of differences in survival among groups. Data were plotted using Graph Pad Prism software version 4 for Windows. Differences showing $P < 0.05$ were considered to be significant. Experiments were performed three times with similar results. The data presented are from one representative experiment.

The quantification of *in planta* fungal biomass was performed as described previously (Pareja-Jaime *et al.*, 2010) using total DNA from tomato roots or stems infected with the *F. oxysporum* PG mutants or the wild-type strain. Real-time PCR assays were performed using the primer pairs CHSV-3 and CHSV-26 (Table 1) (Pareja-Jaime *et al.*, 2010) for the specific detection of *F. oxysporum*, and EFα1 and EFα2 (Table 1) for tomato detection, defining amplicons of 511 and 246 bp, respectively. Five plants were used for each treatment. After the indicated time periods (5 or 10 days post-inoculation), the roots and stems were collected separately and DNA extracted. The DNA concentration of each sample was extrapolated from standard curves, which were developed by plotting the logarithm of known concentrations (10-fold dilution series from 100 ng to 1 pg) of *F. oxysporum* mixed with a fixed concentration (100 ng) of non-inoculated tomato plant DNAs against the *Ct* values. Samples without *Fusarium* DNA were used as negative control reactions. The assay was repeated three times with independent biological samples.

qPCR of defence-related genes

In vivo expression of the predicted tomato plant defence-related genes during plant colonization of the different PG mutant or wild-type strains was analysed as described above. Roots were maintained immersed in water containing 5×10^6 spores/mL with rotary shaking at 28 °C for 2 and 4 days. Total cDNA obtained from roots was subjected to qRT-PCR using the appropriate gene-specific primers (Table 2) and FastStart Essential DNA Green Master (Roche). Relative levels of the RT-PCR products were determined using the $\Delta\Delta Ct$ or ΔCt method (Livak and Schmittgen, 2001). Expression values are presented relative to non-inoculated plants and normalized to the *Ct* value of tomato plant *EFα1*. Samples without cDNA were used as negative control reactions. The assay was repeated three times with independent biological samples.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Strategy for deletion of polygalacturonase (PG) genes by replacement of the coding region with the split marker method (Catlett *et al.*, 2003) using the Hyg^R (hygromycin resistance), Phl^R (phleomycin resistance) or Nat^R (nourseothricin resistance) cassette as selective marker. Identification of mutants by Southern and polymerase chain reaction (PCR) analysis of *Fusarium oxysporum* transformants. Genomic DNAs were treated with the indicated restriction enzymes, separated in a gel and hybridized with the indicated probe 'a' or 'b'. The positions of the restriction sites *Pst*I (a), *Xho*I (b, c) and *Hind*III (d) are shown. The scale bar indicates 1 kb. Black (forward) and red (reverse) arrowheads indicate the positions of the primers used for amplification of each overlapping fragment. (a) Deletion of *pg1*. (b) Deletion of *pgx6*. (c) Construction of $\Delta pg1 \Delta pgx6$ double mutant. (d) Complementation of the $\Delta pg1 \Delta pgx6$ mutant by co-transformation with the *pg1* wild-type allele and the Phl^R cassette.

Fig. S2 Quantification of genomic DNA (gDNA) from *Fusarium oxysporum* wild-type strain and polygalacturonase (PG) mutants was performed with total DNA obtained from inoculated tomato roots (a) or stems (b) at 5 and 10 days post-inoculation (dpi) using real-time polymerase chain reaction (PCR) analysis. Fungal DNA is expressed as the ratio to plant DNA extracted from infected roots or stems. Each column represents the mean of three independent experiments which included three replications for each DNA sample. Standard error bars represent three independent experiments with three technical replicates each.

Fig. S3 Relative expression of tomato defence encoding genes from plants infected with *Fusarium oxysporum* $\Delta pg1$, $\Delta pgx6$ and $\Delta pg1 \Delta pgx6$ mutants or the wild-type, in comparison with uninfected plants (H₂O), determined by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA samples were obtained from roots of tomato plants at 2 days (a) or 4 days (b) post-inoculation. Bars represent standard errors calculated from three biological replicates, each including three technical replicates.