Cloning and Disruption of *pgx4* Encoding an In Planta Expressed Exopolygalacturonase from *Fusarium oxysporum*

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Fusarium oxysporum f. sp. *lycopersici*, the causal agent of tomato vascular wilt, produces an array of pectinolytic enzymes, including at least two exo- α 1,4-polygalacturonases (exoPGs). A gene encoding an exoPG, *pgx4*, was isolated with degenerate polymerase chain reaction primers derived from amino acid sequences conserved in two fungal exoPGs. *pgx4* encodes a 454 amino acid polypeptide with nine potential N-glycosylation sites and a putative 21 amino acid N-terminal signal peptide. The deduced mature protein has a calculated molecular mass of 47.9 kDa, a pI of 8.0, and 51 and 49% identity with the exoPGs of *Cochliobolus carbonum* and *Aspergillus tubingensis*, respectively. The gene is present in a single copy in different formae speciales of *F. oxysporum*. Expression of *pgx4* was detected during in vitro growth on pectin, polygalacturonic acid, and tomato vascular tissue and in roots and stems of tomato plants infected by *F. oxysporum* f. sp. *lycopersici*. Two mutants of *F. oxysporum* f. sp. *lycopersici* with a copy of *pgx4* inactivated by gene replacement were as virulent on tomato plants as the wild-type strain.

Fungal plant pathogens secrete an array of enzymes capable of degrading the different components of the plant cell wall. Since most of these enzymes are also produced by saprophytic fungi, their importance in pathogenicity is uncertain and has been subject to numerous studies (Walton 1994). Particular attention has focused on enzymes that depolymerize pectin, a major component of the primary plant cell wall. Among these, endo- α 1,4-polygalacturonases (endoPGs) have received most of the attention, due to their massive induction by pectic substrates and their ability to macerate plant tissue (Collmer and Keen 1986). In most cases, however, inactivation of individual endoPG genes by targeted disruption had no apparent effect on virulence, probably due to the presence of additional PG genes (Gao et al. 1996; Scott-Craig et al. 1990). Nevertheless, two constitutively expressed endoPG genes were found to be required for full virulence in *Botrytis cinerea* and *Aspergillus flavus* (ten Have 1998; Shieh et al. 1997). In addition to

endoPGs, exo- α 1,4-polygalacturonases (exoPGs) have also been reported from plant-pathogenic fungi (Riou et al. 1992; Valsangiacomo and Gessler 1992), but their role in disease has only been studied in one fungal plant pathogen (Scott-Craig et al. 1998).

exoPGs may have an important function in pathogen-plant interactions, since they degrade elicitor-active oligogalacturonides released by endoPGs (Favaron et al. 1988) and are generally not inhibited by plant polygalacturonase-inhibiting proteins (PGIPs; Cervone et al. 1990, 1989). The tomato vascular wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, secretes a number of pectinolytic enzymes (Di Pietro and Roncero 1996a). Recently, *pgI* encoding the major endoPG secreted in pectin has been cloned (Di Pietro and Roncero 1998). In addition, *F. oxysporum* also produces at least two different exoPGs (Di Pietro and Roncero 1996b; García-Maceira et al. 1997). Activity corresponding to these exoPGs was observed in supernatants from liquid cultures of the fungus grown on pectic substrates but not in planta, in contrast to endoPG and endoPL, which were readily detected in infected tomato tissue (Di Pietro and Roncero 1996a). Failure to detect exoPG activity in planta does not rule out implication of exoPGs in pathogenicity, since the failure may be due to the limited sensitivity of the zymogram assay (Di Pietro and Roncero 1996a). The availability of the encoding gene(s) would provide a tool by which to study their expression during infection of the host plant and their importance in pathogenicity. Here we report the isolation of a gene encoding an exoPG (*pgx4*) from *F. oxysporum* f. sp. *lycopersici* and show that, although expressed during infection, the enzyme is not essential for pathogenicity on tomato plants.

RESULTS

Isolation and characterization of *pgx4*.

An alignment of the products of the two exoPG genes previously isolated from filamentous fungi, PGAX from *Aspergillus tubingensis* (Kester et al. 1996) and PGX1 from *Cochliobolus carbonum* (Scott-Craig et al. 1998), showed several amino acid regions that were identical in both enzymes. Two oligonucleotides (oligos 1 and 2), based on the amino acid sequences NTDYWQ and QCYGQKN, respectively, were used for polymerase chain reaction (PCR), using as a template cDNA derived from total RNA of mycelium of *F. oxysporum* f. sp. *lycopersici* strain 42-87 grown for 36 h on 0.5% poly-

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Nucleotide and/or amino acid sequence data for *pgx4* are available from the GenBank data base under accession number AF083075.

galacturonic acid sodium salt (PGA). These culture conditions were shown previously to induce optimum levels of exoPG3 of *F. oxysporum* f. sp. *lycopersici* (García-Maceira et al. 1997). The amplification product consisting of a number of bands was subjected to a second PCR with oligo1 and oligo3, the latter corresponding to the amino acid sequence EVDI-
VEN. A major amplification product of approximately 500 bp was purified, cloned, and sequenced, and its comparison with the databases showed homology to fungal exoPGs. The fragment was used as a probe to screen a genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 42-87. Three recombinant phage clones were isolated and characterized by restriction and Southern hybridization analyses, revealing that all of them encompassed the same genomic region. Two *Hind*III fragments of approximately the same size (2.1 kb) hybridizing to the probe were subcloned in Bluescript/KS⁺ and sequenced. The *pgx4* coding region consisted of an open reading frame of 1,362 bp encoding a 454 amino acid polypeptide with nine putative N-glycosylation sites. Five short introns (59, 47, 47, 51, and 50 bp) were identified based on alignment with fungal exoPGs and on conserved splice site consensus sequences of filamentous fungi. The 5' flanking region of *pgx4* contained putative TATA boxes at -64 and -69 and putative CAAT boxes at positions -103 and -109.

The N-terminal amino acid sequence of the predicted protein showed features of a signal peptide with a predicted cleavage site between residues 21 and 22 (von Heijne 1986). The deduced mature protein had a calculated molecular mass of 47.9 kDa and a pI of 8.0. The amino acid sequence of the *F. oxysporum* *pgx4* product had 51 and 49% identity with the exoPGs of *C. carbonum* and *A. tubingensis*, respectively (Fig. 1). Considerably lower identity was detected with bacterial or plant exoPGs. As in the exoPGs of *C. carbonum* and *A. tubingensis*, the Gly adjacent to the His residue at the active site of PGs (Caprari et al. 1996) was substituted by a Ser (residue 277 in Figure 1), a feature apparently characteristic of fungal exoPGs.

The copy number of *pgx4* was determined by Southern blot analysis of genomic DNA of *F. oxysporum* f. sp. *lycopersici* digested with nine different restriction enzymes. The hybridization pattern observed was consistent with the presence of a single copy of *pgx4* in the genome (results not shown). Furthermore, the occurrence of *pgx4* was studied by Southern blot analysis of genomic DNA from 11 *F. oxysporum* isolates belonging to six different formae speciales, digested with *Hind*III. The following isolates were used: *F. oxysporum* f. sp. *ciceris* 85-03; *F. oxysporum* f. sp. *conglutinans* 58110; *F. oxysporum* f. sp. *gladioli* 28-69; *F. oxysporum* f. sp. *lini* 21-59; *F. oxysporum* f. sp. *lycopersici* 4287, 218, 27-15, 281; and *F. oxysporum* f. sp. *melonis* 23M, 18M, 11-27. A hybridizing band of 2.1 kb was present in all isolates except isolate 23M (results not shown).

Expression of *pgx4* in culture and in infected tomato plants.

Expression of *pgx4* was determined by Northern hybridization analysis of total RNA obtained from mycelia of *F. oxysporum* f. sp. *lycopersici* grown for various periods of time in synthetic medium (SM) supplemented with different carbon sources. A single transcript with maximum expression at 12 h of growth was detected in mycelia from pectin, PGA, tomato vascular tissue (TVT) (Fig. 2), and D-galacturonic acid (not shown). No transcript was detected in mycelia grown on glucose as the sole carbon source, but when glucose was added together with pectin or PGA the *pgx4* transcript was still detectable, although at greatly reduced levels (results not shown).

To determine whether *pgx4* is expressed by *F. oxysporum* f. sp. *lycopersici* during infection of its host plant, tomato seedlings were inoculated with microconidia of the pathogen and planted in minipots containing sterile vermiculite. Reverse transcription (RT)-PCR with gene-specific primers was used to detect the presence of *pgx4* transcript in roots and lower stems at different times after infection, correlating with pro-

Fig. 1. Multiple alignment of the deduced amino acid sequences of *Fusarium oxysporum* f. sp. *lycopersici* PGX4 (AF083075), *Cochliobolus carbonum* PGX1 (L48982), and *Aspergillus tubingensis* PGAX (X99795). Identical amino acids are shaded. Amino acids corresponding to regions used to design degenerate oligonucleotides are indicated.

gressive stages of the disease (Huertas-González et al. 1999a). Total genomic DNA of *F. oxysporum* was used as a template for PCR to compare the sizes of the amplified fragments with or without the intron (319 and 272 bp, respectively). Southern analysis of the RT-PCR products with the *pgx4* probe showed expression of the gene in roots and stems of infected plants at all sampled time points, except for the first time point (3 days) in stems (Fig. 3). No amplified fragment hybridizing to *pgx4* was observed in the uninoculated control plants.

Inactivation of *pgx4* by gene replacement.

To produce mutants of *F. oxysporum* f. sp. *lycopersici* lacking PGX4, protoplasts of strain 42-87 were transformed

Fig. 2. Northern (RNA) hybridization analysis for *pgx4* transcript accumulation in *Fusarium oxysporum* f. sp. *lycopersici* mycelium grown for indicated time periods (hours) in synthetic medium containing 1% citrus pectin, 0.5% polygalacturonic acid sodium salt, 1% glucose, or 2.5% tomato vascular tissue (TVT) as the carbon source. **A**, Total RNA blotted onto nylon membrane and stained with 0.02% methylene blue. **B**, Filter shown in **A** was destained and hybridized with the *pgx4* probe marked in Figure 4A. Size of the *pgx4* transcript is indicated in kb.

Fig. 3. Southern hybridization analysis of reverse transcription-polymerase chain reaction (RT-PCR) products showing expression pattern of *pgx4* during infection of tomato plants by *Fusarium oxysporum* f. sp. *lycopersici*. First-strand cDNAs were generated from total RNA isolated at indicated time points from roots and stems of infected or uninfected plants and used as templates for PCR with primers specific for *pgx4* (see Materials and Methods). DNA control refers to the PCR performed with genomic DNA as the template. Aliquots of the PCR products were run on a 2% agarose gel, blotted onto nylon membrane, and hybridized with the *pgx4* probe marked in Figure 4A. Position and size of the *pgx4* fragment are indicated in bp.

with gene replacement vector pPgx4::hyg, in which 600 bp of the *pgx4* coding region had been removed and replaced by the hygromycin resistance cassette (Fig. 4A). For initial experiments, protoplasts were prepared from microconidia germinated in potato dextrose broth (PDB) as described previously (Di Pietro and Roncero 1998). Analysis of 128 hygromycin-resistant transformants by PCR and Southern hybridization indicated that all contained ectopically integrated copies of the vector (results not shown). In an attempt to increase the frequency of homologous integration events, protoplasts were prepared from conidia germinated in SM with 0.5% PGA under conditions that induce *pgx4* expression (see Figure 2). Southern hybridization analysis of eight transformants obtained from these protoplasts showed that two of them (CO16 and CO17) exhibited a banding pattern consistent with a homologous recombination event and replacement of the *pgx4* gene (Fig. 4B). Both transformants showed absence of the

Fig. 4. **A**, Physical map of the *pgx4* genomic region and gene replacement vector pPgx4::hyg. The *pgx4* coding region composed of five exons is indicated by striped boxes. The hygromycin resistance cassette in pPgx4::hyg is indicated by a white box. Arrows indicate position of the polymerase chain reaction primers used for construction. Fragment used as a probe is marked by a stippled bar. Restriction site abbreviations: A, *ApaI*; E, *EcoRV*; H, *HindIII*; K, *KpnI*; S, *SalI*. *ApaI* and *KpnI* sites in pPgx4::hyg are from cloning vector Bluescript/KS⁺. **B**, Southern analysis of *pgx4* replacement mutants of the *Fusarium oxysporum* f. sp. *lycopersici* strain 42-87. DNA of the wild-type strain (lane 4), of *pgx4* mutant strains CO16 and CO17 (lanes 2 and 3), of transformant CO15 carrying an ectopic insertion of the replacement vector (lane 1), and of plasmid pPgx4::hyg (lane 5) was digested with *HindIII* and hybridized with the *pgx4* probe marked in Figure 4A. Size of the hybridizing fragments is indicated in kb.

wild-type hybridizing band, which was replaced by a 6-kb fragment in CO17 and a 12-kb fragment in CO16. These banding patterns suggest the occurrence of a single-copy homologous recombination event in CO17 and a double tandem-copy recombination in CO16. Northern analysis of pectin-grown mycelia of the mutant strains CO16 and CO17, as well as of the wild-type strain and the ectopic transformant CO15 confirmed the absence in the mutants of the *pgx4* transcript that was readily detected in the other two strains (Fig. 5A). However, when supernatants from the wild-type strain and mutants grown in SM with 0.5% PGA were fractionated by preparative isoelectric focusing and fractions were analyzed for PG activity and protein bands, no differences were detected. Likewise, infection assays with tomato plants showed no significant loss of virulence in the mutants lacking PGX4, compared with the wild-type strain. Moreover, the pattern of colonization of the host plant, as determined by presence of the fungus in roots and stems of inoculated plants, was identical in the mutants and the wild-type strain (results not shown).

DISCUSSION

A gene encoding an exoPG of *F. oxysporum* f. sp. *lycopersici*, *pgx4*, has been isolated by PCR with degenerate oligonucleotide primers from conserved regions of fungal exoPGs. The PCR template was cDNA derived from RNA of the fungus grown in PGA, a substrate that was shown previously to induce exoPG production in *F. oxysporum* (García-Maceira et al. 1997). Two exoPGs, PG2 and PG3, have been purified and characterized previously from *F. oxysporum* f. sp. *lycopersici*. Since the N-terminal amino acid sequence of PG2 (Di Pietro

and Roncero 1996b) was not present in PGX4, we conclude that the product of the *pgx4* gene is not PG2. Sequencing of the purified PG3 protein has been unsuccessful so far, due to blockage of the N terminus or to heavy glycosylation of the enzyme, as reported for another fungal exoPG (Scott-Craig et al. 1998). Therefore, the question of whether the *pgx4* product is PG3 remains open.

pgx4 is the third exoPG gene cloned from a filamentous fungus and, as expected, shows homology to the other two fungal exoPGs. Identities of amino acid sequences were around 50%, indicating that the three enzymes belong to the same class of exoPGs. The calculated molecular mass of the mature PGX4 protein (47.9 kDa) is in the same range as those of the *A. tubingensis* and *C. carbonum* exoPGs and the calculated pI value (8.0) is close to that of the *C. carbonum* enzyme, whereas the *A. tubingensis* exoPG has an acidic pI. Similarly, the PG3 enzyme of *F. oxysporum* f. sp. *lycopersici* has a pI of 7.0, which is unusually high for an exoPG and lies in the range observed generally in endoPGs (García-Maceira et al. 1997). Southern analysis indicated that *pgx4* is widely distributed and structurally conserved among different formae speciales of *F. oxysporum*, except for one of the isolates studied, which apparently lacked the gene. Similar results have been reported for endoPG1, which was conserved in most *F. oxysporum* isolates, with a few exceptions (Di Pietro et al. 1998).

To determine the role of PGX4 in pathogenicity, we studied its expression during infection of the host plant and produced mutants of *F. oxysporum* lacking the enzyme, using transformation-mediated gene replacement. *F. oxysporum* has previously been reported to be recalcitrant to targeted gene disruption due to a high frequency of ectopic integration of the vector (Di Pietro and Roncero 1998). Similarly, no *pgx4* gene replacement event was detected in 128 transformants originating from protoplasts obtained from PDB-grown germlings. However, when protoplasts were produced from germlings grown in PGA, under conditions that induce *pgx4* expression, two among eight transformants showed replacement of the *pgx4* gene. The dramatic difference in the frequency of gene replacement may be due to remodeling of the chromatin structure at the *pgx4* locus when the gene is transcriptionally active, thus facilitating homologous recombination (Thomas and Rothstein 1989), a feature that may prove useful in future gene replacement experiments with *F. oxysporum*.

pgx4 was expressed by *F. oxysporum* f. sp. *lycopersici* during all stages of infection of tomato plants. Failure to detect expression in stems at 3 days after inoculation was probably due to absence of the fungal mycelium from this tissue. The pattern of *pgx4* expression in planta matched that previously observed with the *pg1* gene (Di Pietro and Roncero 1998), indicating that endoPG and exoPG are coordinately expressed during infection. Although both enzymes are present in infected plant tissue, the results of gene inactivation experiments suggest that their contribution is not essential for pathogenicity; it has been reported previously that PG1 is a major factor for competitive saprophytic growth on pectin but is not required for infection of melon plants (Di Pietro et al. 1998; Di Pietro and Roncero 1998). In the present work, mutants lacking PGX4 were fully pathogenic on tomato plants. Expression of endoPG and exoPG genes during infection but no change in pathogenicity of single and double mutants has been

Fig. 5. Northern (RNA) hybridization analysis for *pgx4* transcript accumulation in mycelia of the *Fusarium oxysporum* f. sp. *lycopersici* wild-type strain 42-87, *pgx4* mutant strains CO16 and CO17, and transformant CO15. Strains were grown for indicated time in synthetic medium containing 1% citrus pectin as carbon source. **A**, Total RNA blotted onto nylon membrane and stained with 0.02% methylene blue. **B**, Filters shown in **A** were destained and hybridized with the *pgx4* probe mentioned in Figure 2.

reported previously in the maize pathogen *C. carbonum* (Scott-Craig et al. 1998). The most likely explanation for the unaltered pathogenicity of the *F. oxysporum* mutants is that lack of PG1 and PGX4 activity is complemented by other pectinolytic enzymes present in the fungus. Thus, the fact that only one *pgx4* homologue was detected in the genome of *F. oxysporum*, while the fungus produces at least two exoPGs, suggests the presence of at least one additional, structurally unrelated exoPG gene. *F. oxysporum* also has, at least, one additional endoPG gene whose structure differs from that of *pg1* (F. I. García-Maceira, *unpublished results*) and one pectate lyase gene (Huertas-González et al. 1999b). It is currently unknown whether each of these different pectinolytic enzymes performs specific functions in the life cycle of the pathogen, or whether one activity can complement the lack of another without loss in overall fitness. In the case of exoPGs, it has been suggested that they may play a key role in releasing monomers from pectic substrates that are known to act as inducers of other pectinolytic enzymes. In fact, expression of both *pg1* (Di Pietro and Roncero 1998) and *pgx4* is induced by D-galacturonic acid. However, the majority of gene inactivation studies in fungal plant pathogens to date suggest that functional complementation is sufficient to compensate for the loss of one or even two pectinolytic isozymes, at least with respect to pathogenicity (Di Pietro and Roncero 1998; Gao et al. 1996; Scott-Craig et al. 1998, 1990). The only exceptions reported so far are two constitutively expressed endoPG genes from *A. flavus* and *Botrytis cinerea*, whose inactivation significantly decreased growth rates of the mutants in the infected host tissue, suggesting that these enzymes acted as virulence factors (ten Have 1998; Shieh et al. 1997). Thus, it cannot be ruled out that specific PG isozymes that are major determinants of virulence (Gao et al. 1996; Scott-Craig et al. 1998) have so far eluded detection in other fungal pathogens, including *F. oxysporum*.

Functional redundancy constitutes a major problem in determining the importance of fungal pectinolytic enzymes in pathogenicity by gene inactivation (Walton 1994). Ultimately, the only solution will be to construct mutants lacking all the genes encoding pectinase activity, as has been done in some bacterial plant pathogens (Barras et al. 1994; Collmer and Keen 1986). At present, such a task would appear extremely laborious considering both the expected number of genes and the technical limitations in fungal systems. An alternative approach could involve identification of regulatory pathways that control expression of pectinolytic enzymes, thus allowing manipulation of a group of coordinately expressed genes. Knowledge of such pathways will be extremely useful in elucidating the long-debated role of pectinolytic enzymes in fungal pathogenicity.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

The *F. oxysporum* f. sp. *lycopersici* strain 42-87 (race 2) was obtained from J. Tello, Universidad de Almería, Spain, and stored as a microconidial suspension (Di Pietro and Roncero 1998). The pathotype of the isolate was periodically confirmed by plant infection assays. Isolates 28-69 (*F. oxysporum* f. sp. *gladioli*), 218, 27-15 (*F. oxysporum* f. sp. *lycopersici* race 1), 281 (*F. oxysporum* f. sp. *lycopersici* race 2), 23M,

18M, and 11-27 (*F. oxysporum* f. sp. *melonis* races 0, 1, and 2, respectively) were also obtained from J. Tello; isolate 21-59 (*F. oxysporum* f. sp. *lini*) was from Colección Española de Cultivos Tipo, Valencia, Spain; 58110 (*F. oxysporum* f. sp. *conglutinans*) was from the American Type Culture Collection, Rockville, MD; 85-03 (*F. oxysporum* f. sp. *ciceris*) was provided by R. Jiménez-Díaz, Instituto de Agricultura Sostenible, Córdoba, Spain.

For extraction of DNA, mycelium was obtained from cultures grown for 4 days in PDB (Difco, Detroit, MI) in Erlenmeyer flasks on a rotary shaker at 150 rpm (Unitron; Infors, Bottmingen, Switzerland) and 28°C. For analysis of gene expression, microconidia obtained from 4-day-old PDB cultures were germinated for 14 h in fresh PDB medium. Germlings were washed twice in sterile water and transferred to SM (Di Pietro and Roncero 1996a) containing as the carbon source 1% (wt/vol) pectin from citrus fruits, 1% glucose, 0.5% PGA (all from Sigma) or 2.5% TVT, obtained as previously described (Di Pietro and Roncero 1996a).

Nucleic acid manipulations.

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelium grown as described (Di Pietro and Roncero 1998) by previously reported protocols (Chomczynski and Sacchi 1987; Raeder and Broda 1985). One microgram total RNA from mycelium grown 36 h in SM with 0.5% PGA was reverse-transcribed into cDNA with murine leukemia virus reverse transcriptase (Gibco BRL, Paisley, UK), with an oligo(dT) primer. The RT reaction was used for PCR amplification on a Perkin Elmer (Foster City, CA) GeneAmp System 2400 with the degenerate primers oligo1 (5'-AAYGAYACNGAYTAYTGGCAR-3') and oligo2 (5'-RTTYTTYTGNCCTARCAATG-3'), corresponding to the amino acid sequences NDTDYWQ and QCYGQKN, respectively, that are conserved in the exoPGs of *A. tubingensis* and *C. carbonum* (Kester et al. 1996; Scott-Craig et al. 1998). The following PCR conditions were used: 40 cycles with denaturation at 94°C for 35 s, annealing at 45°C for 60 s, and extension at 72°C for 60 s. An initial denaturation step of 3 min at 94°C and a final elongation step at 72°C for 6 min were performed. The PCR products were re-amplified with oligo1 and oligo3 (5'-RTTYTCNACDATRTCNACYTC-3', corresponding to the conserved amino acid sequence EVDIVN) under the above conditions. An amplified DNA fragment was cloned into pGEM-T (Promega, Madison, WI), sequenced, and used to screen a lambda EMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 42-87. Library screening, subcloning, and other routine procedures were performed as described in standard protocols (Sambrook et al. 1989). Sequencing of both DNA strands was performed at the Servicio de Secuenciación Automática de DNA, CIB, Madrid, with the Dyedeoxy Terminator Cycle Sequencing Kit (Perkin Elmer) on an ABI Prism 377 Genetic Analyzer apparatus (Applied Biosystems, Foster City, CA). Analyses of sequencing data were carried out with the Wisconsin Package, version 8.3 (Genetics Computer Group, Madison, WI), and the Lasergene Programs (DNASar, Madison WI). Southern and Northern analyses were carried out as described (Di Pietro and Roncero 1998) with the non-isotopic digoxigenin labeling kit (Boehringer Mannheim, Mannheim, Germany).

Pathogenicity assays and RT-PCR in infected tomato plants.

Seeds of tomato cultivar Vemar were kindly provided by Novartis Seeds, El Parador, Almería, Spain. Ten-day-old seedlings in the first true leaf stage were inoculated with *F. oxysporum* (Di Pietro and Roncero 1998). At different times after inoculation, severity of disease symptoms was recorded with an index from 1 (healthy plant) to 5 (dead plant) (Huertas-González et al. 1999a). For determination of plant colonization by *F. oxysporum*, roots and stems of infected tomato plants harvested at different times after inoculation were cut into two and four fragments, respectively, surface sterilized by soaking for 20 min in 0.7% sodium hypochlorite, and transferred to potato dextrose agar (Oxoid) plates containing, in the case of the transformants, 55 µg ml⁻¹ hygromycin B. Experiments were performed twice with similar results.

RT-PCR experiments were performed essentially as described (Di Pietro and Roncero 1998). Five tomato plants from each treatment were removed at 3, 7, 11, and 15 days; total RNA was isolated from roots and lower parts of the stems and subjected to RT-PCR with gene-specific primers flanking the third intron of *pgx4*. The antisense primer was 5'-GGAAGGCAGTCTCAACACCA-3', the sense primer was 5'-TGGTTGGGATACATAC-3', and PCR conditions were as described above except that annealing was at 50°C for 35 s. Aliquots of the PCR products were separated on 2% agarose gels, transferred to positively charged nylon membranes and subjected to Southern hybridization analysis with the labeled *pgx4* probe.

Transformation-mediated gene replacement and analysis of transformants.

The gene replacement vector pPgx4::Hyg was constructed as follows: a 2.1-kb *Hind*III fragment containing the 5'-flanking and part of the coding region of *pgx4* was cloned into Bluescript/KS⁺, followed by digestion with *Sall* and self ligation resulting in loss of the 300-bp fragment between the *Sall* and the *Hind*III site in the *pgx4* coding region. Into the *Sall* site of the resulting construct, we inserted an *Sall* fragment from the plasmid pHRC (Kistler and Benny 1988) containing the hygromycin resistance gene *hph* fused to the *A. nidulans* *trpC* promoter and terminator sequences (Cullen et al. 1987). Finally, a 1.3-kb fragment encompassing part of the *pgx4* coding region was amplified by PCR with the sense primer mentioned above and the antisense primer 5'-TTACGTTT TGTGCGACAG-3', cloned into pGEM-T (Promega), released by digestion with *Sall/Apa*I and cloned into the *Xho*I/*Apa*I sites of the previously mentioned construct. The final replacement vector contained 1.8 and 1.3 kb of colinear genomic DNA, with the hygromycin resistance cassette replacing approximately 600 bp of the *pgx4* coding region. The linearized fragment was released by digestion with *Hind*III/*Kpn*I, purified and used to transform protoplasts of the *F. oxysporum* strain 42-87 following a protocol described previously (Di Pietro and Roncero 1998). To obtain protoplasts induced in *pgx4* expression, microconidia were germinated for 14 h in SM with 0.5% PGA. Transformants appeared after 5 days and were transferred to hygromycin plates and subjected to two consecutive rounds of single-spore isolation before being stored as microconidial suspensions at -80°C.

For analysis of PG activity, transformants lacking PGX4 and the wild-type strain were grown in 400 ml of SM with 0.5% PGA as the sole carbon source for 48 h. Supernatants were harvested, fractionated by preparative isoelectric focusing, and assayed for PG activity as described. Fractions were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained as described previously to visualize protein bands (Di Pietro and Roncero 1996a).

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