Molecular Characterization of a Subtilase from the Vascular Wilt Fungus *Fusarium oxysporum*

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The gene *prt1* was isolated from the tomato vascular wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*, whose predicted amino acid sequence shows significant homology with subtilisin-like fungal proteinases. *Prt1* is a single-copy gene, and its structure is highly conserved among different formae speciales of *F. oxysporum*. *Prt1* is expressed constitutively at low levels during growth on different carbon and nitrogen sources and strongly induced in medium containing collagen and glucose. As shown by reverse transcription-polymerase chain reaction and fluorescence microscopy of *F. oxysporum* strains carrying a *prt1*-promoter–green fluorescent protein fusion, *prt1* is expressed at low levels during the entire cycle of infection on tomato plants. *F. oxysporum* strains transformed with an expression vector containing the *prt1* coding region fused to the inducible endopolygalacturonase *pg1* gene promoter and grown under promoter-inducing conditions secreted high levels of extracellular subtilase activity that resolved into a single peak of pI 4.0 upon isoelectric focusing. The active fraction produced two clearing bands of 29 and 32 kDa in sodium dodecyl sulfate gels containing gelatin. Targeted inactivation of *prt1* in *F. oxysporum* f. sp. *lycopersici* had no detectable effect on mycelial growth, sporulation, and pathogenicity on tomato plants.

The vascular wilt fungus *Fusarium oxysporum* is a soil-borne facultative parasite that causes economically important losses in a wide variety of crops. The fungus enters the host roots directly through penetration hyphae and colonizes the cortex by intracellular and intercellular growth. Once it reaches the vascular tissue, the pathogen spreads rapidly upward through the xylem vessels, provoking the characteristic wilt symptoms (Beckman 1987). During root penetration and host plant colonization, *F. oxysporum* secretes an array of

enzymes such as polygalacturonases, pectate lyase, and xylanases that may contribute to the degradation of the structural barriers constituted by plant cell walls (Di Pietro and Roncero 1996a; Di Pietro and Roncero 1996b; Di Pietro and Roncero 1998; García-Maceira et al. 2000; Huertas-González et al. 1999; Ruíz-Roldán 1999).

Although made up mainly of polysaccharides, plant cell walls contain different classes of structural proteins and enzymes important for defense (Bowles 1990; Showalter 1993). Degradation of these proteins may be critical for pathogens to successfully colonize plant hosts. Extracellular proteases have been identified in a number of plant pathogenic fungi (Ball et al. 1991; Carlile et al. 2000; Choi et al. 1993; Dobinson et al. 1997; Murphy and Walton 1996; Robertsen 1984; Sreedhar et al. 1999), and attempts to address their role in pathogenesis have yielded contradictory results. No pathogenicity loss was detected in trypsin-deficient mutants of the cereal pathogens *Cochliobolus carbonum* and *Stagonospora nodorum* (Bind-schedler et al. 2000; Murphy and Walton 1996). Conversely, extracellular cysteine protease and subtilase activity correlated with virulence and symptom expression in *Pyrenopeziza brassicae* and *Magnaporthe poae*, respectively (Ball et al. 1991; Sreedhar et al. 1999).

Within the proteases, the subtilase family (EC 3.4.21.14.) is distributed widely in bacteria, fungi, yeast, plants, and animals. Subtilases are distinguished by the characteristic arrangement of the histidine, asparagine, and serine residues that conform the catalytic triad (Siezen and Leunissen 1997). Subtilases have been associated with pathogenicity of fungi to humans (Kolattukudy et al. 1993), insects (Goettel et al. 1989) and nematodes (Tunlid et al. 1991), and mycoparasitism (Germia et al. 1993) as well as colonization of grasses by endophytes (Reddy et al. 1996) and pathogens (Sreedhar et al. 1999).

Here, we present the molecular characterization of a subtilase, *Prt1*, from the vascular wilt fungus *F. oxysporum*. We show that targeted inactivation of *prt1* does not affect pathogenicity of *F. oxysporum* on tomato plants, although the gene is expressed at low levels during the entire infection process.

RESULTS

Isolation and characterization of *prt1*.

A recombinant phage clone from the genomic library of *F. oxysporum* f. sp. *lycopersici* previously was isolated based on

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homology with a pectate lyase gene from *Fusarium solani* (Huertas-González et al. 1999). Sequencing of the 3' adjacent region on the same phage clone revealed an open reading frame (ORF) with homology to subtilases from filamentous fungi. A 3.5-kb *Pst*I fragment containing the complete gene (*prt1*) was subcloned in pBluescript, characterized by restriction and Southern analysis, and sequenced. The *prt1* coding region consisted of an ORF of 1,194 bp encoding a 397 amino acid polypeptide. Two short introns (59 and 52 bp) were identified based on the alignment with sequences of other fungal subtilase genes and on conserved splice site consensus sequences of filamentous fungi. The exact position of the introns was confirmed by sequencing cloned reverse transcription-polymerase chain reaction (RT-PCR) fragments. The 5' flanking region of *prt1* contained the WGATAR consensus binding sequence of major nitrogen regulatory proteins (Kudla et al. 1990) at position -149. The sequence AATAAA, typically proceeding the polyadenylation signal, was found in the 3' flanking region 129 bp downstream of the stop codon.

The deduced amino acid sequence revealed a precursor protein of 397 amino acids with a calculated molecular mass of 41 kDa. The first 15 residues show features of a signal peptide (Von Heijne 1986). Moreover, a putative propeptide of 93 amino acids was identified from sequence alignments with fungal subtilases (Fig. 1). The putative mature protein has a molecular mass of 29 kDa and a pI of 4.6. Two potential N-glycosylation sites were found at amino acid positions 133 and 283 of the mature protein.

Sequence comparison of the *prt1* product gave significant homology with subtilisin-like serine proteinases from filamentous fungi (Fig. 1). The highest identities (50 to 60%) were

detected with proteinase R from *Tritirachium album* (Samal et al. 1990), Alp from *Fusarium* spp. (Morita et al. 1994), Pr1 from the entomopathogen *Metarhizium anisopliae* (St. Leger et al. 1992), and At1 from the endophyte *Acremonium typhinum* (Reddy et al. 1996). The positions of the Asp41, His72, and Ser227 residues at the catalytic sites were conserved completely in Prt1. Three conserved Cys residues at positions 36, 181, and 251 in Prt1 indicate the possible conservation of a disulfide bridge (Jany et al. 1986). Conversely, a free Cys residue near the active site His, which is highly conserved in other serine proteases and has been postulated to play a role in catalysis (Betzel et al. 1986), is a Val in Prt1. Interestingly, the corresponding residue also is a Val in the proteinases from the fungal endophyte *A. typhinum* (Reddy et al. 1996), the mycoparasite *Trichoderma harzianum* (Geremia et al. 1993), and the plant parasite *Aspergillus oryzae* (Tatsumi et al. 1989).

To determine the copy number of *prt1*, Southern analysis of *F. oxysporum* f. sp. *lycopersici* genomic DNA digested with different restriction enzymes was performed with two different probes: *prt1p1*, a PCR fragment corresponding to the region between nucleotides 195 and 429 of the *prt1* coding region, and *prt1p2*, a 0.3-kb PCR fragment corresponding to the region immediately upstream of the *prt1* start codon. The hybridizing banding patterns indicate that probe *prt1p1* hybridizes to two different genes, whereas *prt1p2* hybridizes only to a single gene, *prt1* (results not shown). We conclude that *prt1* is a single-copy gene and that the *F. oxysporum* genome contains at least one additional structurally related proteinase gene. Furthermore, the occurrence of *prt1* in 14 *F. oxysporum* isolates belonging to formae speciales *ciceris*, *conglutinans*, *gladioli*, *lini*, *lycopersici*, *melonis*, *niveum*, *radicis-*

FoPRT1	1	MRSATLLALLP	FALAAPS	- - - RRA	- - - - -	-EPAPILRPRGV	KLV	DGKYIVK	MKN	GFQA
AcCAH	1	MRAATLLALLP	LALAAPS	AV - KRD	- - - - -	-APAPVLAPRDA	KLV	PGKYIVK	FKK	KDSVS
TaPRTR	1	MRLSILLLGLP	LAPRPPAV	- - - DAVE	QRSE	EPAPLIEAQ	GEM -	IAD	KYIVK	KLKESAL
MaPR1	1	MHLSALLTLLP	AVLAAPATIG	RRRA	- - - - -	-EPAPLFTPQA	ES -	IAD	KYIVK	FKKDDIAR
FoALP1	1	MRLSI - IAVLP	LALAAPVL	- - - - -	- - - - -	-EPAPLLEARG	SQPIAG	KYIVK	LK	DKTAKI
FoPRT1	50	SSIESWVDK	MIESIEAD	ADYTYSK	GF	GGFAASL	KDDE	LNKLKH	DPN	VEYIEQ
AcCAH	52	TAVSS - - -	AIQSI	AAADYT	YAKH	FNGFAAS	LTDAE	IKKL	RDDP	NVEYIEQ
TaPRTR	54	ASLDA - - -	AMEKLS	GKADH	VYKNI	FKGFAAS	LDEK	MVEV	LRAH	DPNVEYIEQ
MaPR1	53	IATDD - - -	TVSALT	SADH	VYKNI	FKGFAAS	LDEK	MVEV	LRAH	DPNVEYIEQ
FoALP1	47	GIMEA - - -	TAK - -	VANPER	VYQNV	IKGFSAS	LCKEE	VERL	RHDP	DVESIEQ
FoPRT1	109	ATTQQS	NAPWG	IARVSS	QSPGG	STYT	YD	NSAGE	GTC	AYVIDTG
AcCAH	107	AT - -	QENAP	WGLAR	ISSQ	EPGG	STYT	YD	NSAGE	GTC
TaPRTR	109	A - -	EQRN	APWGL	AR	ISSQ	EPGG	STYT	YD	NSAGE
MaPR1	108	GITE	QS	GAPWG	LGR	IS	HR	KGST	TY	RYDD
FoALP1	100	AITQ	QSG	ATWGL	TR	SHR	QK	GST	TY	RYDD
FoPRT1	168	NFAGGS	DS	DGQGH	GTHV	AGTIGS	T	YGV	AKK	TS
AcCAH	164	NFADEN	SD	VQGH	GTHV	AGTIGS	T	YGV	AKK	TS
TaPRTR	166	TYA -	SSRD	GNH	GTHC	AGTIGS	R	YGV	AKK	TS
MaPR1	167	SFIS	QNTD	GNH	GTHC	AGTIGS	R	YGV	AKK	TS
FoALP1	159	TFA	S -	TARD	GNH	GTHC	S	GTIGS	K	YGV
FoPRT1	227	GHA	KDEN	CPK	GVV	NMS	LG	GETS	DA	VNQ
AcCAH	223	DNA	GED	CPN	GVV	NMS	LG	GETS	DA	VNQ
TaPRTR	224	NDY	RNR	CPN	GVV	NMS	LG	GETS	DA	VNQ
MaPR1	226	QDS	KTR	CPN	GVV	NMS	LG	GETS	DA	VNQ
FoALP1	217	SDY	RSR	NC	PR	GVV	NMS	LG	GETS	DA
FoPRT1	286	ES	ACT	VGA	T	TRD	T	LA	EYS	N
AcCAH	282	PS	ACT	VGA	T	TRD	T	LA	EYS	N
TaPRTR	283	SS	ICT	VGA	T	DRS	S	FS	NY	G
MaPR1	285	PS	ACT	VGA	T	DRS	S	FS	NY	G
FoALP1	276	P	S	V	CT	VGA	T	D	S	DRS
FoPRT1	345	AA	YFL	GL	GQ	KA	-	-	EGL	CDY
AcCAH	341	AA	YLM	GT	GA	SV	-	-	SGL	CDY
TaPRTR	342	AA	YLM	GT	GA	SV	-	-	SGL	CDY
MaPR1	344	AA	YLS	AL	Q	GK	T	TPA	NA	CK
FoALP1	335	G	A	Y	L	L	A	L	E	G

Fig. 1. Comparison of the deduced amino acid sequences of *Fusarium oxysporum* f. sp. *lycopersici* PRT1 (AF074391), *Acremonium chrysogenum* cephalosporin C acetylhydrolase (AJ238108), *Tritirachium album* protease R (X56116), *Metarhizium anisopliae* PR1 (M73795), and *Fusarium* spp. ALP1 (S71812). Identical amino acids are shaded. Active-site Asp, His, and Ser residues are indicated by asterisks. The putative prosequence cleavage site is indicated by #.

lycopersici, and *tuberosi* was studied by Southern blot analysis of genomic DNA digested with *Hind*III and probed with *prt1p1*. Two hybridizing bands, a 1.2-kb band corresponding to *prt1* and an additional band of 5 kb (results not shown), were detected in all isolates. We conclude that *prt1* and the second structurally related protease gene are widely distributed and highly conserved within the species *F. oxysporum*.

Expression of *prt1* in culture and during infection of tomato plants.

Expression of *prt1* in culture was determined by Northern hybridization analysis of total RNA from mycelia of *F. oxysporum* f. sp. *lycopersici* strain 4287 grown for 24 h in synthetic medium supplemented with different substrates, including collagen, skim milk, glucose, muskmelon roots, and tomato vascular tissue. High levels of a single 1.4-kb transcript were detected in mycelia grown on collagen plus glucose, whereas under all remaining growth conditions, low levels of expression were observed (Fig. 2). These results were confirmed by fluorescence microscopy of transformants of strain 4287 carrying a *prt1* promoter–green fluorescent protein (GFP) fusion construct. Mycelia and microconidia from medium with collagen plus glucose showed strong green fluorescence, whereas those from the remaining media exhibited low levels of fluorescence (results not shown).

To determine whether *prt1* is expressed by *F. oxysporum* f. sp. *lycopersici* during infection of its host plant, RT-PCR with gene-specific primers was used to detect transcripts in total RNA extracted from roots and lower stem parts of infected tomato plants at different times after inoculation. Electrophoretic analysis of the RT-PCR products showed a fragment of the expected size (192 bp) in roots and stems of infected plants at all sampled time points (Fig. 3). The fragment was absent in the uninoculated control plants, indicating that it was of fungal origin. Cloning and sequencing of the amplified band confirmed that the sequence completely matched that of the *prt1* genomic clone, except for the absence of the intron.

Fig. 2. Northern hybridization analysis for *prt1* transcript accumulation in *Fusarium oxysporum* f. sp. *lycopersici* mycelium grown for 24 h in synthetic medium supplemented with collagen (C), glucose (G), collagen + glucose (C+G), skim milk (SM), muskmelon roots (MR), and tomato vascular tissue (TVT). **A**, Total RNA blotted onto nylon membrane and stained with 0.02% methylene blue. **B**, Filter shown in **A** destained and hybridized with a 1.2-kb internal *Hind*III fragment of the *prt1* gene. Size of the observed transcript is indicated in kb.

The presence of two additional fragments observed in stems at 14 days after inoculation may be a result of a reduced expression level of *prt1* and most likely corresponds to genomic DNA (upper fragment) and a nonspecific amplification product (lower fragment).

To further investigate *prt1* expression in planta in a quantitative manner, a transformant of strain 4287 carrying the *prt1* promoter–GFP fusion was inoculated on tomato plants. Roots and stems of infected plants were observed under a fluorescence microscope at different times after inoculation to determine expression of the GFP gene. Low levels of fluorescence were detected in fungal structures at all stages of infection, from root attachment and penetration and colonization of the cortex and vascular tissue to the final stages of disease (results not shown). In contrast, a strain carrying the GFP gene fused to the strong constitutive *Aspergillus nidulans* *gpdA* promoter had strong fluorescent hyphae during all stages of plant infection. We conclude that *prt1* is expressed at low constitutive levels in most conditions of fungal growth, including infection of tomato plants.

Expression of *prt1* under an inducible promoter and partial purification of the gene product.

The low constitutive expression pattern of the native *prt1* gene makes it difficult to characterize its product secreted by *F. oxysporum*. Therefore, we employed a strategy to express *prt1* under the control of a strongly inducible promoter. To this purpose, hygromycin resistance vector Ppg1::prt1 (Fig. 4A) containing the *prt1* coding region fused to the promoter of the endopolygalacturonase *pgl* gene (Di Pietro and Roncero 1998) was used to transform *F. oxysporum* f. sp. *melonis* strain 18M. This strain previously was shown to contain an inactive allele of *pgl* (Di Pietro et al. 1998), thus avoiding interference of the native *pgl* gene product in the transformants. Microconidia from three independent hygromycin-resistant transformants were germinated in potato dextrose broth (PDB; Difco, Detroit MI, U.S.A.) and transferred to synthetic medium containing citrus pectin as the sole carbon source where the *pgl* promoter is strongly activated (Di Pietro and Roncero 1998). Supernatants from the Ppg1::prt1 transformants contained

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) products showing *prt1* expression pattern during infection of tomato plants by *Fusarium oxysporum* f. sp. *lycopersici*. First-strand cDNAs generated from total RNA isolated at the indicated time points (days after inoculation) from roots and stems of infected or uninfected plants were used as templates for PCR with primers specific for *prt1* (see text). Aliquots of the PCR products were run on a 2% agarose gel with a 100-bp ladder marker. Position of the 200- and 400-bp marker bands is indicated.

high levels of protease activity, as determined with the use of azocasein as a substrate (Fig. 4B). In contrast, supernatants from wild-type 18M or hygromycin-resistant transformant CO4 carrying an unrelated construct (an internal PCR fragment of the *pgl* coding region) (Di Pietro and Roncero 1998) contained very low levels of protease activity. To test whether the protease activity produced by the transformants corresponded to a subtilisin-like enzyme, activity against two subtilisin A substrates, Boc-Gly-Gly-Leu-pNA and Z-Ala-Ala-Leu-pNA, was determined. The same three transformants produced high subtilisin activity, whereas the remaining strains had activities close to zero (Fig. 4B). When the *pgl* promoter-*prt1* transformants were grown with glucose instead

of pectin as the carbon source, very low protease activity was detected in all of the supernatants (results not shown), which is consistent with repression of the *pgl* promoter (Di Pietro and Roncero 1998).

To further characterize the secreted product of *prt1*, dialyzed and concentrated supernatant from transformant Ppg1::prt1-3 was subjected to preparative isoelectric focusing in a pH range from 3 to 10 and fractions were analyzed for protease activity against azocasein. Fractions 2 and 3 contained a single peak of activity corresponding to a pI of 4.0 (Fig. 4C). A very similar activity profile was obtained with the specific subtilisin assay (data not shown). As a control, supernatant obtained in the same way from the untransformed strain 18M also was subjected to isoelectric focusing and analyzed. No protease activity peak was detected with azocasein or the subtilisin assay (results not shown).

Fractions 2 and 3 from the Ppg1::prt1-3 supernatant were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing gelatin, followed by renaturing and staining to visualize clear zones produced by protease activity. Two closely spaced activity bands of 29 and 32 kDa were detected in the zymograms (Fig. 4D). No activity bands were detected in zymograms from fractions 2 and 3 of the 18M supernatants.

We conclude that the transformants expressing the *prt1* gene under control of the *pgl* promoter secrete high levels of subtilisin activity under promoter-inducing conditions. This activity resolves in a single peak of pI 4.0 upon isoelectric focusing and corresponds to two protein bands with molecular weights of 29 and 32 kDa.

Targeted replacement of *prt1*.

Mutants carrying a disrupted copy of the *prt1* gene were generated with a one-step gene replacement. Gene replacement vector pDprt1 was constructed (Fig. 5A) by replacing a 0.65-kb *Bam*HI fragment within the *prt1* coding region with the hygromycin B resistance cassette (*hph*). A linear PCR fragment containing the disrupted *prt1* gene was used to transform *F. oxysporum* f. sp. *lycopersici* strain 4287, which is highly virulent on tomato plants. Hygromycin-resistant transformants were selected and their genomic DNA isolated and digested with *Sal*I and subjected to Southern analysis with

Fig. 4. **A**, Physical map of vector Ppg1::prt1 containing the *prt1* coding region and terminator (black box) fused to the *pgl* promoter (stippled box) in plasmid pAN7Blue3 with the hygromycin resistance cassette (white boxes). **B**, Protease activities in supernatants from pectin-grown cultures of control strains 18M and CO4 and three Ppg1::prt1 transformants (Ppg1::prt1-1, 1-2, and 1-3) measured against azocasein (striped bars) and subtilisin A substrate I (filled bars). Protease activities are expressed as a percentage of the highest activity measured. **C**, Preparative isoelectric focusing (IEF) of culture supernatant of transformant Ppg1::prt1-3 on a Rotofor apparatus. Filled circles indicate protease activity against azocasein expressed as a percentage of the highest activity measured. Empty circles indicate fraction pH. **D**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of culture supernatants from transformant Ppg1::prt1-3 and control strain 18M in a 10% polyacrylamide gel containing 0.5% gelatin. After fractionation, the gel was incubated in phosphate buffer, pH 8, and stained with Coomassie brilliant blue to visualize clear protease activity bands. Lanes 1 and 2, IEF fractions 2 and 3, respectively, from the Ppg1::prt1-3 supernatant (Fig. 4C); lane 3, molecular mass standards; lanes 4 and 5, IEF fractions 2 and 3, respectively, from the 18M supernatant. Molecular mass standard sizes, in kDa, are shown.

probe prt1p2. Strain 4287 and transformants pDprt1-2, pDprt1-3, and pDprt1-5 contained an 0.8-kb *Sal*I fragment corresponding to the wild type *prt1* allele, whereas in pDprt1-1 and pDprt1-4, this fragment was replaced by a larger 5-kb fragment (Fig. 5B). These results indicate that transformants pDprt1-1 and pDprt1-4 contain a single copy of the replacement vector that has been integrated by double homologous recombination, thus generating a disrupted copy of *prt1*. Consistent with gene replacement, the same single fragment hybridized with a *hph* probe (Fig. 5C). Transformants pDprt1-2, pDprt1-3, and pDprt1-5 contained additional hybridizing fragments of different sizes that were indicative of ectopic insertion of the replacement vector.

Inactivation of *prt1* did not cause any measurable growth defect in PDB. Hyphal morphology and the extent of microconidia production were indistinguishable from the wild type (data not shown). Extracellular protease activity of wild-type strain 4287, the *prt1* replacement mutants, and the ectopic insertion transformants was determined by measuring production of a clear halo on plates containing skim milk. Experiments were performed at alkaline (7.8) or acidic pH (4.5), with or without an additional carbon (glucose) and/or nitrogen source (ammonium sulfate). Production of a large halo was observed at alkaline and acidic pH on plates without an additional carbon and nitrogen source (Table 1). Halo production was only slightly reduced by glucose but was completely repressed in the presence of ammonium sulfate. In all conditions tested, colony growth and halo production of *prt1* mutants was very similar to that of the control strains. We conclude that *prt1* does not contribute measurably to total extracellular protease activity in *F. oxysporum* under the conditions tested.

Infection assays on tomato plants were performed to determine the effect of *prt1* inactivation on pathogenicity. Two-week-old tomato plants were inoculated by immersing the roots in a microconidial suspension of the *prt1* mutants and the control strains. After planting them in vermiculite, plants were scored for disease symptoms at different time intervals. The *prt1* mutants were as virulent as the wild type and the ectopic insertion strains (results not shown). To test whether *prt1* contributes to invasive growth of *F. oxysporum* on living host plant tissue, tomato fruits were inoculated by puncturing the epidermis with a sterile pipette tip and injecting a microconidial suspension into the fruit tissue. After 6 days incubation at 100% humidity, the *prt1* mutants colonized and rotted the fruit tissue surrounding the site of inoculation to the same extent as the wild-type strain and ectopic insertion transformants, forming a dense mycelial mat on the surface of the fruit (results not shown). We conclude that the subtilase *prt1* is not required for pathogenicity of *F. oxysporum* on tomato plants and for invasive growth on living plant tissue.

DISCUSSION

This report describes the isolation of *prt1* encoding an extracellular subtilisin-like serine protease from *F. oxysporum*. The predicted primary structure of Prt1 indicates that it is synthesized as a pre-proenzyme consisting of a 15 amino acid signal peptide, a 93 amino acid propeptide, and a mature polypeptide of 289 amino acids. The presence of a prosequence is typical of zymogens, and its cleavage has been shown to be mandatory for the generation of active protease from the inactive zymogen (Zhou et al. 1995). Within the sub-

tilase superfamily, Prt1 falls into the proteinase K family, which is composed of a number of endopeptidases, mainly of fungal origin, that share a high degree of similarity (Siezen and Leunissen 1997).

The expression of *prt1* was shown to be constitutive at low levels during growth on a variety of substrates, including glucose, tomato vascular tissue, and collagen, a hydroxyproline-rich glycoprotein that bears similarity to plant extensins. Such low levels of constitutive protease activity have been reported previously in the plant pathogens *C. carbonum* and *Verticil-*

Fig. 5. Targeted replacement of the *Fusarium oxysporum* *prt1* gene. **A**, Physical map of the *prt1* genomic region and gene replacement vector pDprt1. The *prt1* coding region is shown as a black box and the putative introns are white boxes. Arrow shows the orientation of the *prt1* open reading frame. Arrowheads show the position of the primers for polymerase chain reaction amplification of the linear fragment used for transformation. The location of probe prt1p2 is marked by an empty box. **B**, Analysis of pDprt1 transformants by Southern blot. Genomic DNA from wild-type strain 4287 (lane 1) and transformants pDprt1-1, pDprt1-2, pDprt1-3, pDprt1-4 and pDprt1-5 (lanes 2 to 6) were digested with *Sal*I, separated in a 0.7% agarose gel, blotted onto a nylon membrane, and probed with prt1p2 (upper panel), then stripped and reprobed with the hygromycin resistance cassette (lower panel).

lium dahliae (Dobinson et al. 1997; Murphy and Walton 1996). In contrast, we observed high levels of *prt1* transcript in the presence of collagen and glucose. This suggests that, similar to *C. carbonum* (Murphy and Walton 1996), *prt1* induction in *F. oxysporum* is conditioned by an appropriate supply of carbohydrates rather than by nutrient depletion, as reported for the *prt1* orthologs from *M. anisopliae* and *A. typhinum* (Reddy et al. 1996; St. Leger et al. 1992).

The low expression level of *prt1* under most of the growth conditions tested made it difficult to identify its product. Therefore, the *prt1* coding region was fused to the promoter of the *pgl* gene, which previously has been shown to be induced strongly by citrus pectin (Di Pietro and Roncero 1998), and the construct was introduced into a *F. oxysporum* strain lacking a functional copy of the native *pgl* gene (Di Pietro et al. 1998). Transformants grown in the presence of pectin produced significant extracellular protease activity, and several lines of evidence suggest that this activity corresponds to the product of the *prt1* gene. First, control strains lacked such activity, indicating that growth on pectin does not generally induce extracellular protease production in *F. oxysporum*. Second, the extracellular protease activity was caused by a subtilase because it was active against two different subtilase A substrates. Third, the general protease and the subtilase activity resolved into a single peak upon preparative isoelectric focusing, strongly suggesting that they correspond to a single enzyme. The pI of the active fraction (4.0) was close to the calculated pI of the mature Prt1 polypeptide (4.6). SDS-PAGE of this fraction showed that it contained two closely spaced protease activity bands, with molecular weights of 29 and 32 kDa. These may correspond to differentially glycosylated isoforms of the Prt1 enzyme, in accordance with the presence of two potential sites for N-glycosylation in the predicted Prt1 polypeptide. Multiple isoforms differing in their glycosyl moiety previously have been reported for *F. oxysporum* endopolygalacturonase PG1 and endoxylanase XYL1 (Di Pietro and Roncero 1996a; Ruiz et al. 1997).

Extracellular subtilases have been associated with fungus–host interactions. The entomopathogen *M. anisopliae* secretes high amounts of the subtilase Pr1 at the site of penetration, which is coincident with hydrolysis of cuticle proteins (Goettel et al. 1987). An extracellular subtilase from *Arthrobotrys oligospora* has been implicated in the penetration of nematode hosts (Tunlid and Jansson 1991). The presence of a subtilase also has been reported during the interaction of the mycoparasite *T. harzianum* with fungal hosts (Geremia et al. 1993). Finally, an elastolytic serine protease is a virulence factor in *Aspergillus fumigatus*, the causal agent of invasive aspergillosis.

In the latter case, the contribution of a single protease to virulence has been demonstrated by targeted gene inactivation (Kolattukudy et al. 1993), whereas in the remaining interactions, evidence remains circumstantial.

A role of proteases also has been proposed in pathogenicity to plants. Possible targets include hydroxyproline-rich glycoproteins such as extensins, which play a key role in plant cell wall self-assembly and extension (Showalter 1993). In addition, proteases also may contribute to the inactivation of proteinaceous components of the plant defense response such as chitinases and β -1,3-glucanases (Alexander et al. 1994). In fact, many plants contain protease inhibitors that may serve as a defense against microbial proteolysis (Ryan 1990). Recently, a metalloprotease from the rice blast fungus *Magnaporthe grisea* has been shown to function as a gene-for-gene-specific avirulence factor by directly binding the plant resistance gene product and triggering a signaling cascade leading to resistance (Jia et al. 2000). Although production of fungal proteases during plant infection has been reported in a number of systems (Ball et al. 1991; Carlile et al. 2000; Choi et al. 1993; Dobinson et al. 1997; Lindstrom et al. 1994; Murphy and Walton 1996; Reddy et al. 1996; Robertsen 1984; Sreedhar et al. 1999), an essential role of a single fungal protease in pathogenicity has not been demonstrated. Targeted inactivation of trypsin-encoding genes had no effect on virulence in the cereal pathogens *C. carbonum* and *S. nodorum* (Bindschedler et al. 2000; Murphy and Walton 1996). Here, we provide what we believe to be the first report on mutational analysis of a subtilisin in a plant-pathogenic fungus. As in the above cases, we observed no change in virulence in *F. oxysporum* mutants lacking Prt1, suggesting that the enzyme is dispensable for pathogenesis. In contrast to *C. carbonum* and *S. nodorum*, where extracellular protease activity of the mutants was significantly reduced, we found no measurable reduction in clear halo production of the *prt1*-mutants either on alkaline or acidic media and under nitrogen-repressing or derepressing conditions. This result strongly suggests that Prt1 does not contribute significantly to total extracellular protease activity in *F. oxysporum*, probably as a result of the generally low level of gene expression. In fact, as shown by RT-PCR and promoter–GFP fusions, expression of *prt1* also remains at basal levels during plant infection, favoring the interpretation that this protease might be involved in basic metabolic functions rather than participate in pathogenesis. Thus, other protease genes are likely to account for most of the extracellular activity of *F. oxysporum*. Southern analysis with a probe from the *prt1* coding region suggested the presence of at least one structurally related gene in *F. oxysporum*. A second alkaline

Table 1. Production of a clear halo around colonies of *Fusarium oxysporum* f. sp. *lycopersici* strains grown on plates containing skim milk^a

Strain	pH 7.8				pH 4.5			
	–	C	N	CN	–	C	N	CN
4287	4.3 ± 0.6	3.0 ± 0.0	0	0	4.7 ± 0.6	3.3 ± 0.6	0	0
pDprt1-1	4.7 ± 0.6	3.0 ± 0.0	0	0	4.7 ± 0.6	3.7 ± 0.6	0	0
pDprt1-2	4.0 ± 0.0	3.3 ± 0.6	0	0	5.0 ± 0.0	3.3 ± 0.6	0	0
pDprt1-4	4.7 ± 0.6	2.7 ± 0.6	0	0	4.7 ± 0.6	3.3 ± 0.6	0	0
pDprt1-5	4.3 ± 0.6	3.0 ± 0.0	0	0	5.0 ± 0.0	3.3 ± 0.6	0	0

^a Media had pH 7.8 or 4.5 and, when appropriate, were supplemented with glucose as a carbon source (C) and/or ammonium sulfate as an additional nitrogen (N) source. Clear halo size is expressed as the difference between halo and colony radius in millimeters. Values represent the mean of three replicates ± standard errors.

protease gene from *Fusarium* spp. that is different from *prtI* has been reported previously, although its contribution to extracellular protease activity has not been studied (Morita et al. 1994). In addition, acidic proteases such as trypsin also are present in this fungus (Rypniewski et al. 1993; Urbanek and Yirdaw 1978). As is the case in carbohydrate-degrading enzymes such as polygalacturonases (Di Pietro and Roncero 1998; García-Maceira et al. 2000) and xylanases (Ruíz-Roldan et al. 1999), functional redundancy of proteases in *F. oxysporum* poses a major challenge to understanding the role of these enzymes in pathogenesis.

MATERIALS AND METHODS

Fungal strains and culture conditions.

F. oxysporum f. sp. *lycopersici* strain 4287 (race 2) and *F. oxysporum* f. sp. *melonis* strain 18M (race 1), obtained from J. Tello (Universidad de Almería, Spain), were stored as microconidial suspension in 30% glycerol at -80°C . The pathotype of the isolates was confirmed periodically by plant-infection assays in the growth chamber. A detailed origin of the other *F. oxysporum* isolates used for Southern analysis is described elsewhere (Di Pietro et al. 1998).

For extraction of genomic DNA, mycelium was obtained from cultures grown in PDB in Erlenmeyer flasks on a rotary shaker at 150 rpm and 28°C . For analysis of gene expression, microconidia were germinated in PDB, washed in sterile water, and transferred to synthetic medium (SM), as described (Di Pietro and Roncero 1998). Prior to autoclaving, SM was supplemented with one of the following substrates (wt/vol): 1% collagen (Sigma, St. Louis, MO, U.S.A.), 1% glucose, 1% skim milk (Difco), 2.5% roots from 20-day-old muskmelon plants (cv. Pancha), and 2.5% tomato vascular tissue (cv. Vemar), obtained as described previously (Di Pietro and Roncero 1996b). Plant seeds were provided by Sluis & Groot, Almería, Spain.

For visual determination of extracellular protease activity, a 5- μl droplet of a microconidial suspension (10^8 per ml) of wild-type strain 4287, the *prtI* replacement mutants, and the ectopic insertion transformants was deposited on plates containing 1% (wt/vol) skim milk, 0.05% (vol/vol) Triton X-100 (Sigma), and 1.5% (wt/vol) agar (Mattern et al. 1992). The pH of the media was adjusted either to 7.8 with 50 mM M Tris-HCl or to 4.5 with 50 mM sodium acetate and, when appropriate, media were supplemented with 0.5% (wt/vol) of glucose and/or ammonium sulfate. Production of a clear halo around colonies was determined after 4 days growth at 28°C and expressed as the difference between the halo and colony radius in millimeters. Data represented the mean from three replicates, and the experiment was performed twice with similar results.

Isolation and characterization of *prtI*.

A recombinant phage clone from a lambda EMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 4287 was isolated previously, following standard screening protocols (Sambrook et al. 1989), and shown to contain a gene encoding a pectate lyase (Huertas-González et al. 1999). Subcloning of restriction fragments and other routine procedures followed standard procedures. Sequencing of both strands of cloned DNA was performed at the Servicio de Secuenciación

Automática de DNA (CIB, Madrid, Spain) with the Dye-deoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, U.S.A.) on an ABI Prism 377 Genetic Analyzer apparatus (PE Applied Biosystems). Analyses of sequencing data were carried out with Lasergene programs (DNASTar, Madison, WI, U.S.A.). DNA and protein sequence databases were searched with the BLAST algorithm (Altschul et al. 1990) at the National Center for Biotechnology Information (Bethesda, MD, U.S.A.).

Nucleic acid isolation and analysis.

Genomic DNA was extracted from *F. oxysporum* mycelium, as described previously (Raeder and Broda 1985). Two micrograms of DNA were digested with appropriate restriction enzymes and subjected to Southern hybridization analysis following standard protocols (Sambrook et al. 1989) with probes labeled with the nonisotopic dig-dUTP labeling kit (Roche Biochemicals, Mannheim, Germany), in accordance with the manufacturer's instructions. Total RNA was prepared from mycelia, according to the protocol of Chomczynski and Sacchi (1987). Five micrograms of RNA were fractionated on a formaldehyde 1% agarose gel and transferred to positively charged nylon membranes (Roche Biochemicals) by capillary transfer. For quantification, transferred RNA was stained on the membrane for 5 min in 0.02% methylene blue in 0.3 M sodium acetate, pH 5.2. After destaining in 20% ethanol, filters were subjected to Northern hybridization analysis. A non-isotopically labeled, single-stranded antisense DNA probe was generated by asymmetric PCR as described (Di Pietro and Roncero 1998), with the 0.8-kb *SalI* fragment containing part of the *prtI* coding region cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.) as template.

Pathogenicity assays on tomato plants and fruits.

Infection of tomato plants was performed as reported (Di Pietro and Roncero 1998). Briefly, tomato seedlings of Vemar were inoculated with *F. oxysporum* f. sp. *lycopersici* strains by dipping the roots in a microconidial suspension, planting seedlings in minipots with vermiculite, and maintaining them in a growth chamber at 25°C with 14 h of light and 10 h of dark. Plants immersed in sterile water were used as controls. For pathogenicity assays, the severity of disease symptoms was recorded at different times after inoculation with a scale of 1 (healthy plant) to 5 (dead plant) (Di Pietro and Roncero 1998). Twenty plants were used for each treatment.

To assay invasive growth of *F. oxysporum* strains, tomato fruits (cv. Daniela) were washed under running tap water and surface sterilized by immersing for 5 min in ethanol. After air drying, the epidermis was punctured with a sterile pipette tip, and 10 μl of a microconidial suspension (5×10^8 per ml) was injected into the fruit tissue. Fruits were incubated at 28°C under conditions of 100% humidity. Colonization of the fruit tissue and formation of a mycelial mat on the fruit surface was determined at different times after inoculation. All pathogenicity assays were performed at least twice with similar results.

RT-PCR.

Five plants from each treatment were inoculated as described above and sampled after different time periods. Total RNA was isolated from the roots and lower parts of the stems, and RT-PCR was performed, as reported previously (Di Pietro

and Roncero 1998). Briefly, total RNA was treated with RNase-free DNase (Roche Biochemicals), reverse transcribed into cDNA with murine leukemia virus reverse transcriptase (GIBCO-BRL, Paisley, U.K.) with the specific antisense primer 5'-GACACGAGCAATACCCCAAGG-3', and used for PCR amplification with the sense primer 5'-TGATGCCGATTACACCTATTC-3' and the antisense primer described above, both flanking the first intron of *prt1*. PCR conditions were denaturation at 94°C for 35 s, annealing at 56°C for 35 s, and extension at 72°C for 90 s. An initial denaturation step of 2 min at 94°C and a final elongation step at 72°C for 7 min were performed. Total genomic DNA of *F. oxysporum* was used as a template for PCR reaction to compare the size of the amplified fragments, with or without the intron. Aliquots of the PCR products were analyzed on 2% agarose gels. The amplified 192-bp cDNA fragment was cloned into pGEM-T and sequenced to confirm its identity.

Construction of plasmid vectors and fungal transformation.

A vector containing the *prt1* 5' flanking region fused to the sGFP gene was constructed as follows. A PCR fragment containing the *prt1* promoter was amplified from *F. oxysporum* genomic DNA with primers 5'-CCTACTGCTGTTCTCTG TCA-3' (sense) and 5'-ATCCCGGGTTGAAGAAAGATC GTTGTAAGT-3', thus introducing a *SmaI* site at the 3' end. This fragment was cloned into pGEM-T, released with *SmaI*-*SacI*, and inserted into the *SmaI*-*SacI* sites of the hygromycin resistance vector pAN7Blue3 (Di Pietro and Roncero 1998), thus fusing it to the sGFP gene that had been subcloned previously into pAN7Blue3 from plasmid pHSP70-SG (Spellig et al. 1996) with *ApaI*-*SmaI* digestion. Fidelity of the promoter-GFP fusion was checked by sequencing, and the resulting vector was used to transform protoplasts of strain 4287 to hygromycin resistance following a protocol described previously (Di Pietro and Roncero, 1998). A transformant of strain 4287 carrying a construct with the sGFP gene fused to the strong constitutive *gpdA* promoter from *A. nidulans* (Fernández-Ábalos et al. 1998) was used as a positive control. Transformants were selected on plates of minimal medium containing 3 µg of hygromycin B (Sigma) per ml, purified by monoconidial isolation, and stored as microconidial suspensions at -80°C.

Expression vector Ppg1::prt1 containing the *prt1* coding region fused to the promoter of the endopolygalacturonase *pgl* gene (Di Pietro and Roncero 1998) was constructed as follows. A PCR fragment containing the *prt1* coding region and terminator was amplified from *F. oxysporum* genomic DNA with primers 5'-CCCGGGATGCGATCTGCTACTCTTCTC-3' (sense) and 5'-GCAGCACTCAAGTCCCAGT-3' (antisense), thus introducing a *SmaI* site immediately upstream of the ATG start codon. The fragment was cloned into pGEM-T, released with *ApaI*-*SmaI*, and inserted into the *ApaI*-*SmaI* sites of pAN7Blue3, thus fusing it to the *pgl* promoter that had been amplified previously by PCR and subcloned into pAN7Blue3 from pGEM-T with *SmaI*-*SacI* digestion. Fidelity of the *pgl* promoter-*prt1* translational fusion was checked by sequencing, and the resulting vector was used to transform protoplasts of strain 18M to hygromycin resistance.

Gene-replacement vector pDprt1 was constructed as follows. A 1.8-kb fragment containing the hygromycin B resis-

tance gene (*hph*) under control of a *Cochliobolus heterostrophus* promoter was amplified from plasmid pH1B (Turgeon et al. 1987) with the use of PCR with primers hph1B (5'-TAGGATCCGCTCCACCGGCTGCACATGTC-3') and hph-2B (5'-TAGGATCCTATTCCTTTGCCCTCGGACGAGT-3'), thus introducing a *BamHI* site at both ends. The fragment was cloned into pGEM-T, released by digestion with *BamHI*, and used to replace a 0.65-kb *BamHI* fragment within the *prt1* coding region. To this purpose, a 1.64-kb PCR fragment encompassing the *prt1* gene was amplified previously from *F. oxysporum* genomic DNA with sense primer 5'-TCGTCATC-TCAGGCTCTGTTG-3' and antisense primer 5'-GCAGCAC-TCAAGTCCCAGT-3', cloned into pGEM-T, and cut with *BamHI*. The resulting replacement construct was used as a template for PCR with sense primer 5'-TGATGCCGATTAC-ACCTATTC-3' and antisense primer 5'-CGGCTTCACCAC-AACATCC-3' to amplify a linearized 2.2-kb fragment containing the hygromycin resistance cassette flanked by 0.1 and 0.9 kb of the *prt1* gene. This fragment was used to transform protoplasts of *F. oxysporum* f. sp. *lycopersici* strain 4287 to hygromycin resistance.

Fluorescence microscopy.

Roots of tomato plants were inoculated with conidia of transformants of *F. oxysporum* strain 4287 carrying the GFP gene, as described above. After different time periods, plants were removed from the vermiculite and the roots and stems were observed directly under a DMRB epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a filter set for fluorescein isothiocyanate (BP450 to 490 excitation and 510 to 520 emission).

Characterization of transformants carrying the *pgl* promoter-*prt1* fusion construct.

Microconidia (4×10^6 per ml) of hygromycin-resistant transformants carrying the *pgl* promoter-*prt1* fusion construct were germinated in PDB, washed in sterile water, transferred to SM supplemented with 1% (wt/vol) citrus pectin (Sigma) with pH adjusted to 5.6 after autoclaving, and incubated in a shaker at 28°C and 180 rpm for 20 h. The parental strain 18M and hygromycin-resistant transformant CO4 carrying an internal fragment of the *pgl* gene (Di Pietro and Roncero 1998) were used as negative controls. The cultures were filtered through 3MM paper (Whatman, Kent, U.K.), centrifuged at 8,000 g for 10 min, and the supernatant was passed through a Millipore filter (0.2 µm pore size; Bedford, MA, U.S.A.). For enzyme assays, proteins in the supernatant were precipitated with 2.5 vol of acetone at -80°C and dissolved in an appropriate volume of 50 mM sodium phosphate buffer, pH 8.

For preparative isoelectric focusing, culture-filtered supernatants, obtained as described above, were concentrated in dialysis tubing against polyethylene glycol (Di Pietro and Roncero 1996a), dialyzed against water, and subjected to preparative isoelectric focusing in pH from 3 to 10 on a Rotor apparatus (BioRad, Hercules, CA, U.S.A.), under the conditions reported earlier (Di Pietro and Roncero 1996a). Protein determination by the Bradford method, fractionation of protein samples by SDS-PAGE in 12% (wt/vol) acrylamide resolving gels, and silver staining of proteins were performed as previously described (Di Pietro and Roncero 1996a). For visualization of protease activity, 10% SDS gels containing

0.5% gelatin (Difco) were transferred for 90 min to phosphate buffer, pH 8, supplemented with 0.1% (vol/vol) Triton X-100, after completing fractionation, then incubated for 60 min at 37°C in phosphate buffer, pH 8, and stained overnight with Coomassie brilliant blue.

Nonspecific protease activity was assayed with azocasein (Sigma) in 50 mM phosphate buffer, pH 8, as described (Murphy et al. 1996), with 50 µl of enzyme solution in the same buffer in a total volume of 1 ml. Samples were incubated at 37°C for 30 min and the optical density (OD) at 410 nm was measured in a Spectronic 1001 spectrophotometer (Bausch & Lomb, Rochester, NY, U.S.A.). Subtilisin activity was assayed with chromogenic subtilisin A substrates I and II (Boc-Gly-Gly-Leu-*p*-nitroanilide and Z-Ala-Ala-Leu-*p*-nitroanilide, respectively; Calbiochem-Novabiochem, Bad Soden, Germany). Standard assays contained 1 mM substrate in 50 mM sodium phosphate buffer, pH 8, and 50 µl of enzyme solution in the same buffer in a total volume of 100 µl in microtiter plate wells. Reactions were incubated at 37°C for 2 h, and the OD₄₀₅ was measured in a Model 2100 enzyme-linked immunosorbent assay plate reader (Rosys Anthos, Wals, Austria).

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