Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* Defines a New Class of Saponinases

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Plants produce a variety of secondary metabolites, many of which have antifungal activity. Saponins are plant glycosides that may provide a preformed chemical barrier against phytopathogenic fungi. *Fusarium oxysporum* f. sp. *lycopersici* and other tomato pathogens produce extracellular enzymes known as tomatinases, which deglycosylate α -tomatine to yield less toxic derivatives. We have cloned and characterized the cDNA and genomic DNA encoding tomatinase from the vascular pathogen of tomato *F. oxysporum* f. sp. *lycopersici*. This gene encodes a protein (FoTom1) with no amino acid sequence homology to any previously described saponinase, including tomatinase from *Septoria lycopersici*. Although FoTom1 is related to family 10 glycosyl hydrolases, which include mainly xylanases, it has no detectable xylanase activity. We have over-expressed and purified the protein with a bacterial heterologous system. The purified enzyme is active and cleaves α -tomatine into the less toxic compounds tomatidine and lycotetraose. Tomatinase from *F. oxysporum* f. sp. *lycopersici* is encoded by a single gene whose expression is induced by α -tomatine. This expression is fully repressed in the presence of glucose, which is consistent with the presence of two putative CRE binding sites in the promoter region of the tomatinase gene. The tomatinase gene is expressed in planta in both roots and stems throughout the entire disease cycle of *F. oxysporum* f. sp. *lycopersici*.

Tomato plants synthesize α -tomatine, a steroidal glycoalkaloid (Roddick 1974) belonging to a class of compounds commonly known as saponins (Hostettmann and Marston 1995). Saponins are glycosylated molecules that consist of an aglycone, either steroidal or triterpenoid, linked to one or more sugars (Price et al. 1987). Antifungal saponins, which are produced by many plants (including many major food crops; Price et al. 1987), have been implicated as preformed determinants of resistance to fungal attack (Arneson and Durbin 1968; Schönbeck and Schlösser 1976; Fenwick et al.

1992; Bowyer et al. 1995; Osbourn et al. 1995; Osbourn 1996; Suleman et al. 1996; Quidde et al. 1998). α -Tomatine is produced in all parts of tomato plants, including roots, calyxes, leaves, stems, flowers, and green fruits, at levels of 14 mg (roots) and 131 mg (green fruits) per 100 g fresh weight (Friedman and Levin 1995). α -Tomatine is considered a phytoanticipin, a term proposed to describe saponins and other antimicrobial compounds that are produced as part of normal plant development and that might function to protect plants from disease (VanEtten et al. 1994).

The fungitoxic effects of α -tomatine and other saponins are attributed to their interactions with 3β -hydroxyl sterols, which result in an increase in fungal membrane permeability, pore formation, and leakage of cell contents (Safe et al. 1977; Dow and Callow 1978; Roddick 1979; Roddick and Drysdale 1984; Steel and Drysdale 1988; Keukens et al. 1995). Previous studies have shown that tomato pathogens are generally less sensitive to α -tomatine than fungi that do not infect tomato (Arneson and Durbin 1968; Suleman et al. 1996; Sandrock and VanEtten 1998). Several fungal pathogens of tomato have previously been shown to be tolerant to this glycoalkaloid, and to possess enzymes involved in its detoxification (Sandrock and VanEtten 1998). α -Tomatine consists of an aglycone moiety (tomatidine), and a tetrasaccharide moiety (β -lycotetraose) that contains two molecules of D-glucose and one each of D-galactose and D-xylose (Fig. 1). Many fungal pathogens of tomato produce specific glycosyl hydrolases that detoxify α -tomatine by removal of sugars from the tetrasaccharide chain of the saponin. These enzymes, collectively known as tomatinases, differ in their mechanism of action. Tomatinases from *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium solani* cleave the β 1-linked galactose and release the tetrasaccharide β -lycotetraose and tomatidine (Ford et al. 1977; Lairini et al. 1996; Lairini and Ruiz-Rubio 1998). *Alternaria solani* also degrades α -tomatine to tomatidine but does so by a release of monosaccharides rather than the tetrasaccharide (Schönbeck and Schlösser 1976). Tomatinases from *Septoria lycopersici* and from *Verticillium albo-atrum* remove the terminal β 1,2-linked glucose from α -tomatine (Durbin and Uchytel 1969; Pegg and Woodward 1986; Osbourn et al. 1995; Sandrock et al. 1995). Finally, *Botrytis cinerea* eliminates the terminal β 1-3-linked D-xylose (Quidde et al. 1998).

Here we report the cloning and characterization of the cDNA and genomic DNA encoding tomatinase from the vascular pathogen of tomato *F. oxysporum* f. sp. *lycopersici*. This gene encodes a protein with no amino acid sequence homol-

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ogy to any previously described saponinase, including tomatinase from *S. lycopersici* (Osbourn et al. 1995). The enzyme has been overexpressed in a bacterial system and maintains its activity. Tomatinase from *F. oxysporum* f. sp. *lycopersici* (FoTom1) is encoded by a single gene whose expression is induced by α -tomatine and fully repressed in the presence of glucose. Tomatinase is expressed in planta, in both roots and stems, throughout the entire disease cycle of *F. oxysporum* f. sp. *lycopersici*, suggesting that it may play a role in the pathogenicity of *F. oxysporum*.

RESULTS

Cloning of the *F. oxysporum* tomatinase gene.

A cDNA expression library was prepared from mycelium of *F. oxysporum* f. sp. *lycopersici* isolate 42-87 grown in the presence of 40 μ g of α -tomatine per ml, in the vector Lambda ZAP Express. This cDNA library was screened with rabbit polyclonal antisera raised against the purified tomatinase enzyme (Lairini and Ruiz-Rubio 1997). Three positive clones were isolated and the phagemid excised. Only one of the positive clones gave tomatinase activity in transformed bacteria, and this was called pBKTomB. Cell lysates from DH5 α *Escherichia coli* transformed with pBKTomB showed a single band that cross-reacted with anti-tomatinase antiserum when analyzed by Western blot (immunoblot; data not shown).

Southern hybridization analysis of genomic DNA digested with *Hind*III from two different isolates of *F. oxysporum* f. sp. *lycopersici*, strains 42-87 and 281, with *FoTom1* cDNA as a probe, showed a single 2.1-kb hybridizing band (data not shown). This result suggests that the genome of *F. oxysporum* contains a single copy of the tomatinase gene, which was named *FoTom1*.

A lambda-EMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 42-87 was screened with *FoTom1* cDNA and two positive clones were isolated. One of them, which gave a consistent hybridization signal, was further characterized by restriction and Southern hybridization analyses. A 2.5-kb *Xba*I fragment that hybridized to the cDNA was subcloned

in pBluescript KS⁻, and sequenced on both strands. The tomatinase coding region contains a 1,008-bp open reading frame with no introns that is identical to the cDNA (Fig. 2). The tomatinase gene encoded a predicted protein of 335 amino acid residues with a calculated molecular mass of 37.1 kDa. The deduced protein sequence contained five potential N-glycosylation sites and a 16-amino-acid N-terminal signal peptide (von Heijne 1986) followed by 7 residues (VKIPCTG), which matched the N-terminal amino acid sequence of the mature tomatinase enzyme (amino acids 17 to 23 in Figure 2; Lairini et al. 1996). The 5' noncoding region shows characteristic features of a lower eukaryotic promoter including a perfect TATA box (Gannon et al. 1979) at position -83 relative to the start codon and several putative CAAT boxes (positions -19, -73, -514, -520, and -565; Fig. 2). Two CREA protein-binding site consensus sequences (Felenbok et al. 1992) that may mediate carbon catabolite repression were also found at positions -296 and -508.

A data base search with the deduced amino acid sequence of the *FoTom1* gene revealed a high degree of similarity to microbial endo-1,4- β -xylanases belonging to the family 10 glycosyl hydrolases (Henrissat 1991; Henrissat and Bairoch 1993; Davies and Henrissat 1995; Henrissat and Bairoch 1996), with amino acid identities ranging from 38% (*Aspergillus nidulans*) to 43% (*Dictyoglomus thermophilum*) (Fig. 3).

Overexpression and purification of tomatinase.

A polyhistidine (His₁₀) affinity tag was attached to the amino terminus of the mature tomatinase protein by subcloning the cDNA, without the signal peptide coding sequence, into the pET16b expression vector to obtain the recombinant plasmid pFoTom1. Tomatinase was expressed at a high level by growing the *E. coli* strain BL21 (DE3) transformed with the plasmid pFoTom1 at 37°C in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG), but most of the protein was insoluble and found in inclusion bodies. However, when cells were grown at 30°C in the absence of IPTG, leaky expression was still observed and soluble tomatinase activity was pro-

Fig. 1. Structure of α -tomatine and cleavage sites for tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and *Septoria lycopersici* (SI).

duced. The polyhistidine-tagged tomatinase protein was purified to apparent homogeneity by Ni^{2+} -NTA affinity chromatography. Elution with 500 mM imidazole revealed a major protein band, as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which migrated with an apparent molecular mass of 38.1 kDa (Fig. 4A). The purified protein was detected as a single band by Western blot analysis with anti-tomatinae antiserum (Fig. 4B), and had a specific tomatinase activity of 1,300 U mg^{-1} of protein as determined spectrophotometrically with the di-

nitrosalicylic acid reagent for determination of reducing sugars. *E. coli* BL21 (DE3) transformed with the vector (pET16b) alone was used as a negative control and showed no detectable tomatinase activity. The analysis of the enzymatic activity by thin-layer chromatography (TLC) showed that the purified protein catalyzed the hydrolysis of α -tomatine to tomatidine (Fig. 5), in agreement with the mode of action of the native enzyme (Lairini et al. 1996). When shorter assays were carried out in which α -tomatine was not fully degraded, the only product detected was tomatidine, suggesting that no

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gtggctctcagatcccgagacgcgtaattctcagctgcaagaacggggctcatgattgattaatggagacatagggaca -564
attaccgacttgaagtcaccagatccgggtacactaacaccgggcaattacaatgagtggggtaattcaggggtatgacgaa -484
gccccgcgggtgaccccatgggagaagactggctactgagtctggaagaggggtgcatagccacgggtgatgtccccgga -404
taacgacggttagaggaatatcggttaattgtctgaggacgctaagtgtctatttgcggagggcgccaaatccatcacia -324
actacacactagactagccgtcggtgactggggaattgtattacaagactgggcttcaacgagacgctacagtacttcgc -244
tcgaattcgttctgagaagagtatattgacagagtatcctcctcgtccttagtccccacaccttttagtctcgttctcgtat -164
aacgctctccccggcaaaaacaaacgtcattcgttaggaagcaaggttgacgttatgacgaagaggccacaccacaacc -84
tataaaa ccccaatgatgtccgggcaagaagtgtattgcaacatcacagccaacgacacggagagcaatcctagaaacaga -4
aacATGAAGGCCACTATCATCATCGGAGCGCTCGTCGCCGTTGCTCAAGCAGTCAAATCCCCTGCACCGGAACACCTC 77
M K A T I I I G A L V A V A Q A V K I P C T G T T S 26
ACTGAGAGAGGAGGCGGCCAAGAAGAACCTTCTCTTTGGGTCCGGGGCGATCAATCCGTCCTACCTCGATGATGCACAGT 157
L R E E A A K K N L L F G S G A I N P S Y L D D A Q 52
TCAGAGCCGTCCTTCCGAGCAGTTCAACAGTCTTTCTCTCTGAGAACGAGTTGAAGTGGAACTTCTTCCATCAATCCCCA 237
F R A V L S E Q F N S L S P E N E L K W N F F H Q S P 79
GATCACTACGACTGGCATAAGCTCGACCGCCTCGTCAAATTCGCAGAGGCCAATAACATGGCAGTCAAGGGACATGGGCT 317
D H Y D W H K L D R L V K F A E A N N M A V K G H G L 106
TATCTCAGGCTGCTGTAACCCAGACTATGTGCTTAACATCACCAGCCAGCAGCTCTTCGCGCTGCCATAACTACCCACT 397
I S G C C N P D Y V L N I T S P A A L R A A I T T H 132
TCGAGGCTGTCTATGCACCGGTACCGCGGAAGATGCATGGGATGTTGTCTCTGAGGCCCTTAAAACGAATGGCAGT 477
F E A V M H R Y R G K M D R W D V V S E A L K T N G S 159
GGCCTGGCGTCCAACATTTTCTACGATACGCTCGGTCCCGGCTACGTTGAAGAGGCATTTTCGCATCGCCCCAGCGGCAGA 557
G L A S N I F Y D T L G P G Y V E E A F R I A R A A D 186
CCCAGATGCCAAGCTGTTCTCAACGAGAACCTTGTCGAGGTACTGCCGAAAAGCGCCAAGAACTCTACGAGATGGTAG 637
P D A K L F L N E N L V E V L P K K R Q E L Y E M V 212
CACAACCTCGTCGCGAAGGGCGTCCCATCGATGGGATTGCCTTGACAGATGCACATCACCTCCAGCCCCTGGTGCCTGGT 717
A Q L V A K G V P I D G I A L Q M H I T L Q P L V P G 239
GTTATTAGAGAAATGGTTAACTCTTATAAGGCGCTCGGGCTCGAGGTCAACATCGCTGAAATGGATGTCCATACGTACAA 797
V I R E M V N S Y K A L G L E V T I A E M D V H T Y N 266
CGCTACCCCAACAGACCGAGATCTACGGCGATGTTATCAAGGAAGCTCTTGACTCAGGCATTACCGATATCAGCTTCTGGG 877
A T Q Q T E I Y G D V I K E A L D S G I T D I S F W 292
GCTTCACAGATAAACACGCCTACACCTGGCTCCCGGGAGCGAAGCCCTTGATGTTCAACGAGACCTACTATCCCAAGGGC 957
G F T D K H A Y T W L P G A K P L M F N E T Y Y P K G 319
GCATTCTATGCAACTCATAGTGCCTTGCCAACCTTGTTCGGGCGTCTTGAActccagttaatcttgcoctagtactaccc 1037
A F Y A T H S A L A N F V R A S * 335
agagattttgacccttaggggtcatgatactttcttttcaggatatttgatcctcgttagttgtggatattatatagcaat 1117
taaactttttattactgtatgacttgactttcattccagtgccaagcttcaogtaataa 1197

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Fig. 2. Nucleotide and deduced amino acid sequence of the cloned tomatinase gene (EMBL accession no. AJ012668). Nucleotide numbering begins at the A of the predicted start codon. Amino acids 1 to 16 constitute the signal peptide (light gray box). Amino acids 17 to 23 represent the experimentally determined N-terminal sequence (VKIPCTG). Dots indicate five possible sites of N-glycosylation. TATA and CREA sequences are marked with a black box and dark gray boxes, respectively. Six possible CAAT boxes are underlined. Stop codon is indicated by one asterisk. Noncoding sequences are shown in lowercase letters. A putative polyadenylation consensus sequence is marked as an open box; polyadenylation site is indicated by an upward arrow.

intermediate metabolites are formed during the reaction (data not shown). This observation agrees with those obtained for native tomatinase in different formae speciales of *F. oxysporum* including *lycopersici* (Lairini et al. 1997). The tomatinase amino acid sequence shows high homology to xylanases, including a glutamic acid residue (Glu-258) that in *Cellulomonas fimi* exoglucanase has been shown to be directly involved in glycosidic bond cleavage (Tull et al. 1991). However, purified tomatinase showed no degrading activity on xylan from different sources (data not shown).

The presence of two CREA consensus sequences in the *FoTom1* gene promoter suggests that a carbon-catabolite-repression regulatory system similar to CREA may regulate its transcription. The effect of glucose as a carbon source upon tomatinase expression was investigated by Northern blot analysis of total RNA isolated from mycelia grown in the presence of 1% glucose. The induction of a transcript (1 kb) was observed in mycelia grown in the absence of glucose both 1 and 3 h after addition of α -tomatine (Fig. 7). However, no signal was detected when RNA was extracted from glucose-grown mycelia (Fig. 7). This is in agreement with the presence of CREA sequences in the *FoTom1* gene promoter.

To determine whether tomatinase is expressed by *F. oxysporum* f. sp. *lycopersici* in infected tomato plants, reverse transcription–polymerase chain reaction (RT-PCR) with gene-specific primers was carried out to detect the presence of *Fo-Tom1* transcripts in roots and lower stems at different infection stages. To distinguish false positives arising from amplification of possible contaminant traces of the intron-less genomic

Tomatinase gene expression.

Northern (RNA) blot analysis showed that the *FoTomI* gene encodes an mRNA of approximately 1 kb that is inducibly expressed in the presence of α -tomatine (Fig. 6A). The tomatinase transcript was not detected in the absence of the glycoalkaloid, but transcript increased in its presence, reaching a maximum between 2 and 4 h after tomatine addition and decreasing markedly afterward (Fig. 6B). The level of tomatinase activity in the extracellular filtrate also showed an α -tomatine-dependent induction, with maximum activity

Figure 1. Multiple sequence alignment of the *hsp70* gene from *A. nidulans*, *A. awamori*, *T. alba*, *C. fimi*, *S. halstedii*, *Thermotoga* sp., *D. thermophilum*, and *F. oxysporum*. The alignment shows the conserved regions of the gene across the different species. The sequences are presented in blocks, with the species names and their corresponding sequence positions listed on the left. The alignment is color-coded to highlight conserved regions (green) and variable regions (red). The sequences are as follows:

A. nidulans (1-36)
A. awamori (37-48)
T. alba (49-50)
C. fimi (51-52)
S. halstedii (53-54)
Thermotoga sp. (55-56)
D. thermophilum (57-58)
F. oxysporum (59-60)

A. nidulans (92-93)
A. awamori (94-95)
T. alba (96-97)
C. fimi (98-99)
S. halstedii (100-101)
Thermotoga sp. (102-103)
D. thermophilum (104-105)
F. oxysporum (106-107)

A. nidulans (149-150)
A. awamori (151-152)
T. alba (153-154)
C. fimi (155-156)
S. halstedii (157-158)
Thermotoga sp. (159-160)
D. thermophilum (161-162)
F. oxysporum (163-164)

A. nidulans (205-206)
A. awamori (207-208)
T. alba (209-210)
C. fimi (211-212)
S. halstedii (213-214)
Thermotoga sp. (215-216)
D. thermophilum (217-218)
F. oxysporum (219-220)

A. nidulans (263-264)
A. awamori (265-266)
T. alba (267-268)
C. fimi (269-270)
S. halstedii (271-272)
Thermotoga sp. (273-274)
D. thermophilum (275-276)
F. oxysporum (277-278)

A. nidulans (301-302)
A. awamori (303-304)
T. alba (305-306)
C. fimi (307-308)
S. halstedii (309-310)
Thermotoga sp. (311-312)
D. thermophilum (313-314)
F. oxysporum (315-316)

Fig. 3. Comparison of amino acid sequence of *Fusarium oxysporum* f. sp. *lycopersici* tomatinase (EMBL accession no. AJ012668) with xylanases belonging to family 10 glycosyl hydrolases. Amino acids that are identical in at least five sequences are boxed. Xylanase GenBank accession nos.: *Aspergillus nidulans*, Q00177; *Aspergillus awamori*, P33559; *Thermomonospora alba*, P74912; *Cellulomonas fimi*, Q59277; *Streptomyces halstedii*, Q59922; *Thermotoga* sp., Q60044; *Dactyloglomus thermophilum*, Q12603.

DNA sequence, parallel RT-PCRs were performed, substituting the upper primer with an oligonucleotide annealing to a promoter-specific sequence that is absent in the mRNA transcript. Gel electrophoresis and Southern hybridization analysis of the RT-PCR products from roots and stems of infected plants showed a fragment of the expected size (325 bp) that hybridized specifically to a *FoTom1* cDNA probe and was absent in the uninoculated controls (Fig. 8A). This band was absent in reactions performed with the set of genomic-specific primers (Fig. 8B), and therefore represents amplification of the mRNA transcript. Expression of transcript was detected in both roots and stems at all four stages of infection.

DISCUSSION

Saponin-detoxifying enzymes are produced by a number of phytopathogenic fungi and seem to be important factors implicated in determining fungal pathogenicity to plants (Bowyer et al. 1995). Here we report the isolation of a saponinase-encoding gene from *F. oxysporum* f. sp. *lycopersici* implicated in the degradation of α -tomatine. This gene encodes a protein with no sequence homology to any previously described saponinase, but which is highly similar to xylanases (family 10 glycosyl hydrolases; Gilkes et al. 1991; Henrissat 1991; Henrissat and Bairoch 1993; Davies and Henrissat 1995; Henrissat and Bairoch 1996). Enzymes that detoxify the tomato steroidal glycoalkaloid α -tomatine are collectively named tomatinases, but their mechanisms of action differ and this is probably reflected in their amino acid sequences. A gene encoding a tomatinase-detoxifying enzyme has recently been cloned from the fungal pathogen of tomato *Septoria lycopersici* (Osbourne et al. 1995). The corresponding protein has shown to be closely related to another saponin-detoxifying

enzyme, avenacinase, that is produced by *Gaeumannomyces graminis*, a pathogen of wheat, barley, and oats (Bowyer et al. 1995). Both the tomatinase from *S. lycopersici* and avenacinase from *G. graminis* remove single terminal glucose molecules from the sugar moiety of their corresponding substrates. These two saponinases are closely related to family 3 glycosyl hydrolases, which include mainly β -glucosidases, and may represent a subgroup of this family (Osbourne et al. 1995). In contrast, tomatinase from *F. oxysporum* f. sp. *lycopersici* does not remove terminal glucose molecules from α -tomatine but instead releases the intact lycotetraose group to give the aglycone, tomatidine. *F. oxysporum* f. sp. *lycopersici* tomatinase belongs to a different family of glycosyl hydrolases, family 10, which includes mainly xylanases (Henrissat 1991; Henrissat and Bairoch 1993; Davies and Henrissat 1995; Henrissat and Bairoch 1996). Although *F. oxysporum* tomatinase does not have any detectable xylanase activity under the assay conditions employed here, we do not know yet if any of the xylanases listed in Figure 3 have activity toward α -tomatine. In any case, it is conceivable that even closely related enzymes may have very different substrate specificities. This is illustrated by *G. graminis* avenacinase and *S. lycopersici* tomatinase, which share 53% amino acid identity but show negligible activity against each other's substrates.

The demonstration that *F. oxysporum* tomatinase has no sequence homology to tomatinase from *S. lycopersici* suggests that different tomato pathogens have developed the ability to cleave α -tomatine by convergent evolution. This may also explain the different molecular masses of tomatinases characterized so far: 110 kDa for *S. lycopersici*

Fig. 4. Purification of *Fusarium oxysporum* f. sp. *lycopersici* recombinant tomatinase. **A**, Tomatinase was overexpressed in *Escherichia coli*, and proteins were visualized on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel by Coomassie staining. Lane 1, a lysate of BL21(DE3) cells containing the pET16b vector; lane 2, vector carrying tomatinase cDNA; lane 3, purified tomatinase eluted from nickel-chelate column in the presence of 0.5 M imidazole. Positions of protein size markers in kDa (Bio-Rad, Spain) indicated on left. **B**, Western blot (immunoblot) of SDS-polyacrylamide gel electrophoresis of purified tomatinase with rabbit polyclonal antisera raised against tomatinase from *F. oxysporum* f. sp. *lycopersici* (Lairini and Ruiz-Rubio 1997).

Fig. 5. Thin-layer chromatography of tomatinase reaction products. Purified tomatinase (8 mg) was incubated at 45°C for 30 min in the presence of 2 mM α -tomatine. After incubation, α -tomatine and tomatidine were precipitated by increasing the pH to 10 with sodium hydroxide. The pellet was resuspended in 20 μ l of methanol and loaded in lane 1. Tomatidine (2 mM) and α -tomatine (2 mM) were loaded in lanes 2 and 3, respectively, and used as markers. Assay was repeated on two independent occasions; figure shows result from a representative experiment.

(Osbourn et al. 1995; Sandrock et al. 1995), 70 kDa for *Botrytis cinerea* (Quidde et al. 1998), around 50 kDa for *F. oxysporum* (Lairini et al. 1996), and about 32 kDa for *F. solani* (Lairini and Ruiz-Rubio 1998).

The results presented here indicate that tomatinase from *F. oxysporum* is encoded by a single gene whose expression is induced by α -tomatine and repressed when mycelium is grown in the presence of glucose. This observation is in contrast to that reported for tomatinase from *B. cinerea*, which is not subjected to carbon catabolite repression (Quidde et al. 1998). DNA sequence analysis of the promoter of the *FoTom1* gene revealed motifs resembling those involved in carbon catabolite repression in yeast and filamentous fungi (Felenbok et al. 1992). The presence of two copies of the CREA-binding site in the promoter of the gene, together with the absence of tomatinase expression in glucose medium, suggests that regulation may be modulated by a CREA protein. Therefore, from the results presented here it can be concluded that, as a general mechanism, tomatinase production is subject to induction by the substrate and to catabolite repression. Signals derived from the interaction with the plant must regulate the expression of this activity. α -Tomatine is present in healthy plants in its biologically active form. The amount of α -tomatine found in roots and in stems (14 and 54 mg per

100 g fresh weight, respectively; Friedman and Levin 1995) may approach concentrations in excess of 100 and 500 μ M, respectively, in these organs. According to Sandrock and Van-etten (1998), these concentrations could be sufficient to inhibit the growth of *F. oxysporum* to some extent, since they found a 50% of radial growth inhibition in a petri dish with 912 μ M of α -tomatine. On the other hand, the concentration of α -tomatine present in roots and stems is clearly able to induce the tomatinase gene from *F. oxysporum*, since extracts from roots or stems in minimal medium (estimated α -tomatine concentrations 7 and 16 μ M, respectively) induce tomatinase activity (Lairini and Ruiz-Rubio 1997). α -Tomatine resistance may be, therefore, a prerequisite for successful fungal infection. However, the high sequence homology between tomatinase and glycosyl hydrolases, which are enzymes required presumably for nutritional purposes, and the fact that tomatinase gene expression is fully repressed in the presence of glucose, do not exclude the possibility that tomatinase might have also a nutritional function. RT-PCR experiments in planta indicated that the *FoTom1* gene is indeed expressed throughout the entire disease cycle of *F. oxysporum* f. sp. *lycopersici* in both roots and stems, suggesting that this enzyme may be important for pathogenicity of *F. oxysporum*.

Fig. 6. Induction of tomatinase in the presence of α -tomatine. **A**, Northern (RNA) hybridization analysis of tomatinase transcript accumulation in *Fusarium oxysporum* f. sp. *lycopersici* mycelium in medium containing α -tomatine. Total RNA was purified from mycelium grown in the absence or presence of α -tomatine (20 μ g/ml): lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8 h; lane 7, 12 h; and lane 8, 24 h, after addition of the glycoalkaloid. The RNA was blotted onto a nylon membrane and stained with 0.02% methylene blue (left panel). The filter was destained and hybridized with a digoxigenin-dUTP-labeled, single-stranded, antisense DNA probe of 1,008 bp corresponding to complete *tomatinase* cDNA (right panel). **B**, Tomatinase mRNA level time course. Images of stained nylon membrane and the autoradiograph films obtained in **A** were digitalized and intensity of each band quantified by densitometry with the NIH Image program. For each time point the level of mRNA obtained in the absence (open symbols) or presence (closed symbols) of α -tomatine was referred to the total amount of RNA loaded as estimated from the stained nylon membrane. **C**, Tomatinase activity levels. At each time point the tomatinase activity from filtrates obtained from mycelium grown in the absence (open symbols) or presence (closed symbols) of α -tomatine was determined spectrophotometrically at 520 nm with the dinitrosalicylic acid reagent (DNS) for determination of reducing sugars. Inserts show the tomatinase mRNA level or tomatinase activity at short times after addition of α -tomatine. The experiment was repeated on at least two independent occasions; figure shows result from a representative experiment.

MATERIALS AND METHODS

Culture methods for *F. oxysporum*.

For cDNA library construction and gene expression analysis, cultures inoculated with microconidia from *F. oxysporum* f. sp. *lycopersici* isolate 42-87 (Lairini et al. 1997) were grown with shaking for 36 h at 28°C in CA medium (10 g of casamino acids per liter, 10 mM ammonium sulfate, and 0.5 g of yeast nitrogen base per liter). The culture was filtered through nylon cloth (Monodur, Albus, Spain; mesh size 10

µm) to separate mycelia from spores, and 0.3 g of mycelia was reinoculated in 100 ml of fresh CA media (for carbon catabolite repression experiments the fresh CA media was supplemented with 1% glucose). After 2 h of incubation at 28°C, α-tomatine (Sigma, St. Louis, MI) dissolved in 50 mM potassium citrate buffer (pH 4.0) was added aseptically to the fungal growth medium to a final concentration of 20 to 40 µg/ml, and the incubation continued for 10 min to 72 h. The mycelia were then collected by filtration through nylon cloth, frozen in liquid nitrogen, and stored at -80°C, and the filtrate was used to determine tomatinase activity. At each time point flasks inoculated with α-tomatine-free buffer were grown in parallel and used as a control. For DNA extraction, mycelium from *F. oxysporum* f. sp. *lycopersici* (strains 42-87 and 218; Lairini et al. 1997) was obtained from cultures grown during 4 days at 28°C in potato dextrose broth (PDB; Difco, Detroit, MI).

Tomatinase and xylanase activity assays.

Tomatinase and xylanase activities were measured spectrophotometrically at 520 nm with the dinitrosalicylic acid reagent (DNS) for determination of reducing sugars (Miller 1959; Baker et al. 1968). For tomatinase activity a reaction mixture (200 µl) containing 20 mM sodium acetate buffer (pH 5.5), 2 mM α-tomatine, and an adequate amount of protein extracts or enzyme preparation was incubated at 30°C for 30 min. After incubation, 200 µl of 3, 5-dinitrosalicylic acid solution was added, the mix boiled for 5 min, and the final volume brought to 1 ml with water. One unit of enzyme activity is defined as the amount of enzyme that forms 1 µmol of β-lycotetraose per min under optimal assay conditions. Specific activity is expressed in U/mg of protein. Xylanase activity was measured either with larchwood (USB Corp., Cleveland, OH) or oat

Fig. 7. Carbon catabolite repression of tomatinase transcription. *Fusarium oxysporum* f. sp. *lycopersici* mycelium was grown in the (lanes 1 and 2) absence or (lanes 3 and 4) presence of glucose. After 3 h of incubation, α-tomatine (20 µg/ml) was added (lane 2 and 4). Total RNA was isolated (left panel) 1 or (right panel) 3 h after addition of α-tomatine. RNA was blotted onto nylon membrane and stained with 0.02% methylene blue (upper panels). Filter was destained and hybridized with a digoxigenin-dUTP-labeled, single-stranded, antisense DNA probe of 1,008 bp corresponding to complete *FoTom1* cDNA (lower panels).

Fig. 8. Reverse transcription–polymerase chain reaction (RT-PCR) products showing expression of the *FoTom1* gene during infection of tomato plants by *Fusarium oxysporum* f. sp. *lycopersici*. First-strand cDNA was generated from total RNA isolated from roots or stems at stage 0 (no symptoms), stage 1 (yellowing or wilting of lower leaves), stage 2 (yellowing or wilting of all leaves), stage 3 (entire plant showing wilting). Reactions without RNA (C), containing total RNA from uninfected plants (–) or containing genomic DNA (genomic DNA) were carried out in parallel and used as control. Aliquots of the PCR products were analyzed on 2% agarose gels and subjected to Southern blot analysis with the 1,008-bp *FoTom1* cDNA as a probe. In **A**, primers TRT-R1 and TRT-F1, which anneal inside the open reading frame, were used. In **B**, RT-PCRs were performed, substituting TRT-F1 with an oligonucleotide (TRT-F2) annealing to a promoter-specific sequence that is absent in the mRNA in order to distinguish false positives arising from amplification of possible contaminant traces of the intron-less genomic DNA sequence. The whole procedure was repeated at least three times; figure shows result from a representative experiment.

spelt xylan (Sigma). A reaction mixture (100 μ l) containing 0.5% xylan in 50 mM sodium acetate buffer (pH 4.5), and enzyme preparation (1, 2, 4, 8, 16, and 32 μ g of protein) was incubated at 37°C for 30 min. After incubation, 100 μ l of 3, 5-dinitrosalicylic acid solution was added, the mix boiled for 5 min, and the final volume brought to 1 ml with water. The amount of β -lycotetraose formed from α -tomatine or reducing sugars formed from xylan was then measured spectrophotometrically, determining the absorbance at 520 nm. Negative controls were prepared with protein extracts incubated in buffer alone or following inactivation by heating.

TLC analysis.

A reaction mixture (200 μ l) containing 20 mM sodium acetate buffer pH 5.5, α -tomatine (2 mM) and 8 μ g of enzyme preparation was incubated at 45°C for 30 min. After incubation, α -tomatine and tomatidine were precipitated by increasing the pH to 10 with sodium hydroxide. The pellet (α -tomatine and/or tomatidine) was resuspended into 20 μ l of methanol and separated on Silica Gel 25 TLC plates. The plates were developed in a solvent system consisting of acetic acid, ethyl acetate, methanol, and water (10:30:20:1). Spots were visualized after TLC plates were sprayed with 50% sulfuric acid and heated at 110°C. The metabolites were identified by comparison with standards (α -tomatine and tomatidine, both 2 mM), which were treated in the same way as the incubation mixtures. α -Tomatine appeared as a black spot near to the origin, while tomatidine was localized as a dark green spot near the solvent front.

cDNA library construction and screening.

Total RNA was prepared from liquid cultures mycelium (200 ml) of *F. oxysporum* f. sp. *lycopersici* grown for 6 h in the presence of 40 μ g of α -tomatine per ml as described (Farrell 1993). Polyadenylated RNA was isolated from 0.5 mg of total RNA with the Stratagene Poly (A) Quik mRNA Isolation Kit (Stratagene, La Jolla, CA). cDNA was synthesized with the Stratagene ZAP Express cDNA Synthesis Kit, with oligo(dT) primers containing an *Xho*I restriction site. After second-strand synthesis, an *Eco*RI adapter was added and the cDNA was ligated into the vector lambda ZAP Express (predigested with *Eco*RI and *Xho*I restriction enzymes) following the manufacturer's instruction (Stratagene). The ligated DNA was packaged with the Gigapack III Gold Cloning Kit, yielding approximately 10^6 primary PFU. An amplified aliquot (5×10^3 PFU) was plated on *E. coli* strain XL1 Blue MRF' (Stratagene). Plaques were transferred to a Nitrocellulose membrane (Sigma), and screened with antiserum anti-tomatinate (Lairini and Ruiz-Rubio 1997) as described (Hurst 1997). The antisera were preabsorbed with an XL1 Blue MRF' crude extract to eliminate antibodies reacting with bacterial or phage proteins (Hurst 1997). Positive plaques were purified and submitted to a second round of screening, and the phagemids were excised from lambda ZAP Express following the Stratagene instructions. Plasmid DNA was purified with a mini-plasmid purification kit (Qiagen, Hilden, Germany) and the corresponding cDNA sequenced on both strands.

Nucleic acid isolation and analysis.

Genomic DNA was extracted from mycelium as described previously (Aljanabi and Martinez 1997). DNA was digested

with *Hind*III restriction enzyme and subjected to Southern hybridization analysis as described in standard protocols (Sambrook et al. 1989) with the non-isotopic digoxigenin labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer. Total RNA was prepared from mycelium of *F. oxysporum* grown from 10 min to 72 h in the presence of α -tomatine (final concentration 20 μ g/ml) as described (Farrell 1993). Five micrograms of total RNA was separated on a formaldehyde-1% agarose gel and transferred to positively charged Nylon membranes (Boehringer Mannheim) by capillary transfer. For quantification, transferred RNA was stained 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 with the non-isotopic digoxigenin-labeling kit. A single-stranded, antisense DNA probe of 1,008 bp corresponding to complete *FoTom1* cDNA was generated by a standard protocol (Konat et al. 1994).

Isolation and characterization of the *FoTom1* gene.

A lambda EMBL3 genomic library of strain 42-87 from *F. oxysporum* f. sp. *lycopersici* was screened with the 1,008 bp *FoTom1* cDNA as a probe. Library screening and subcloning were performed as described in standard protocols (Sambrook et al. 1989). DNA sequence analysis was performed with the Dye-deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA) on an ABI Prism 310 Genetic Analyzer apparatus (Applied Biosystems, Foster City, CA).

DNA and predicted amino acid sequence comparisons were carried out with the BLAST (Altschul et al. 1990) network service at the National Center for Biotechnology Information (NCBI). Contiguous peptide sequences in the non-redundant protein data base were scored against the inquiry sequence with the BLOSUM62 homology matrix (Henikoff and Henikoff 1992). Sequences retrieved from the BLAST search were analyzed with multiple sequence alignments (Feng and Doolittle 1987).

Tomatinase overexpression and purification.

Plasmid DNA containing the tomatinate cDNA was purified with a mini-plasmid purification kit (Qiagen) and added as a template in a PCR. The oligonucleotides 5'-CCATCCCTCATATGGTCAAAATCCCCTGCACC-3' and 5'-CTATCTGGATCCTACTCTCGTGCCGATTTCG-3' were used to engineer *Nde*I and *Bam*HI restriction sites at the beginning and the end of the *FoTom1* cDNA, respectively. Forty amplification cycles were carried out with PFU DNA polymerase (Stratagene). PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. An initial denaturation step of 2 min at 94°C and a final elongation step at 72°C for 1 min were performed. In order to add a 10 histidine-tag at the amino terminal end of the tomatinate protein, the amplified fragment was inserted into the pET-16b vector by digestion with *Nde*I and *Bam*HI restriction enzymes and ligation. The construct, called pFoTom1, was used to transform *E. coli* expression strain BL21(DE3) (Studier et al. 1990) and a single transformant colony was inoculated into 0.5 liters of LB medium containing carbenicillin (50 μ g/ml). The culture was incubated overnight at 30°C without shaking and then placed on a shaker at 30°C for 24 h; the cells were then collected by centrifugation at $4,000 \times g$ for 30 min and the pellet frozen at -80°C.

The stored pellet was thawed and resuspended in 5 ml of sonication buffer (TSB: 20 mM Tris-HCl pH 7.8, 0.1 M NaCl, 5 mM imidazole). Cells were disrupted by sonication and the lysate was clarified by centrifugation. The supernatant was mixed with 0.7 ml of Ni²⁺-NTA resin (Qiagen) preequilibrated with TSB buffer and stirred gently for 1 h. The resin was then packed into a column and washed with 12 ml of TSB followed by a wash with 16 ml of TSB supplemented with 60 mM imidazole. The inclusion of 5 mM imidazole in the sonication buffer (TSB) reduced nonspecific binding. Washes of the Ni²⁺-NTA column with TSB and TSB containing 60 mM imidazole removed non-specifically bound proteins. Histidine-tagged protein was eluted with 3 ml of TSB supplemented with 500 mM imidazole, and collected in 0.5-ml fractions. An aliquot of each fraction was analyzed by SDS-PAGE, and those fractions containing a single band of the overexpressed protein were pooled. The protein preparation was aliquoted, frozen, and stored at -80°C. All steps were carried out at 4°C or on ice. Protein concentrations were determined by the Bradford assay. Denatured proteins were analyzed by (SDS)-PAGE (12%) with broad-range molecular weight standards (Bio-Rad, Richmond, CA).

For immunoblotting, proteins were separated by SDS-PAGE, transferred to nitrocellulose, incubated in the presence of polyclonal antisera raised against tomatinase (Lairini and Ruiz-Rubio 1997), and detected by reaction with an antibody-HRP-conjugate (Sigma Immunochemicals, Spain), in the presence of hydrogen peroxide and 4-chloro-1-naphthol (Lairini and Ruiz-Rubio 1997).

RT-PCR of RNA from infected tomato plants.

Ten-day-old tomato seedlings (cv. Moneymaker) were inoculated with *F. oxysporum* f. sp. *lycopersici* strain 42-87 by dipping the roots for 30 min in a suspension containing 5 × 10⁶ microconidia ml⁻¹ water. Control plants were immersed in water. Seedlings were planted in minipots containing vermiculite and grown in a growth chamber at 25°C with 14 h light and 10 h dark. At varying intervals, five plants from each treatment were removed and washed carefully under running water to remove adhering vermiculite particles. The roots and the lower parts of the stems were separated with a sterile scalpel and frozen at -80°C. Total RNA was isolated from the tissue as described above. RT-PCR was performed as recommended in the Retrotools cDNA/DNA kit (Biotools, Madrid, Spain), with gene-specific primers for *FoTom1*. Total RNA was treated twice with RNase-free DNase (Pharmacia Biotech, Spain) and reverse-transcribed into cDNA with Tth recombinant DNA polymerase (Retrotools cDNA/DNA kit from Biotools) following the manufacturer's instructions, and using the specific antisense primer TRT-R1: 5'-CCTGAGATAAGC CCATGTCC-3'. The entire RT reaction was used for PCR amplification on a Perkin Elmer GeneAmp System 2400, with the sense primers TRT-F1: 5'-CTCGTATAACGCTCTCCCC G-3' or TRT-F2: 5'-TGAAGGCCACTATCATCATC-3' and the antisense primer (TRT-R1) described above. PCR conditions were as follows: denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step of 2 min at 94°C and a final elongation step at 72°C for 1 min were performed. Aliquots of the PCR products were analyzed on 2% agarose gels. For Southern analysis, amplified fragments were transferred to positively charged

Nylon membranes (Boehringer Mannheim) by capillary transfer and hybridized with the 1,008 bp *FoTom1* cDNA probe described above.

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