Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* Is Required for Full Virulence on Tomato Plants

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Submitted 14 January 2008. Accepted 3 March 2008.

Saponin detoxification enzymes from pathogenic fungi are involved in the infection process of their host plants. *Fusarium oxysporum* f. sp. *lycopersici*, a tomato pathogen, produces the tomatinase enzyme Tom1, which degrades α -tomatine to less toxic derivatives. To study the role of the *tom1* gene in the virulence of *F. oxysporum*, we performed targeted disruption and overexpression of the gene. The infection process of tomato plants inoculated with transformants constitutively producing Tom1 resulted in an increase of symptom development. By contrast, tomato plants infected with the knockout mutants showed a delay in the disease process, indicating that Tom1, although not essential for pathogenicity, is required for the full virulence of *F. oxysporum*. Total tomatinase activity in the disrupted strains was reduced only 25%, leading to β_2 -tomatine as the main hydrolysis product of the saponin in vitro. In silico analysis of the *F. oxysporum* genome revealed the existence of four additional putative tomatinase genes with identities to tomatinases from family 3 of glycosyl hydrolases. These might be responsible for the remaining tomatinase activity in the $\Delta tom1$ mutants. Our results indicate that detoxification of α -tomatine in *F. oxysporum* is carried out by several tomatinase activities, suggesting the importance of these enzymes during the infection process.

Additional keywords: fungal pathogen, saponins.

Plants produce, as a consequence of their normal metabolism, a set of secondary metabolites with antimicrobial properties that have been classified as phytoanticipins (Vanetten et al. 1994). Therefore, plant pathogens must combat these compounds in order to perform a successful interaction. Tomato plants (*Lycopersicon esculentum*) produce the antifungal compound α -tomatine, a steroidal glycoalkaloid consisting of an aglycone moiety (tomatidine) and an oligosaccharide moiety (β -lycotetraose) composed of four sugars (one molecule of galactose, one of xylose, and two of glucose), considered to be a saponin. The molecule is present in roots, stems, leaves, and green fruit, reaching concentrations up to 1 mM in some areas (Roddick 1977). Similarly to other saponins, the toxic effect of α -tomatine is attributed to its ability to form complexes with the 3β -hydroxy sterols of the fungal membrane, causing pores and leakage of the cellular content (Keukens et al. 1992, 1995; Roddick 1979).

Fusarium oxysporum f. sp. *lycopersici* is a soilborne pathogen that causes vascular wilt disease on tomato plants (Di Pietro et al. 2003). During the infection process, the fungus copes with

inhibitory levels of the saponin. Tomato pathogens are less sensitive to α -tomatine than those nonpathogenic on this plant species (Arneson and Durbin 1967a and b; Steel and Drysdale 1988; Suleman et al. 1996); therefore, these microbes must display mechanisms to circumvent the effects of the molecule (Morrissey and Osbourn 1999; Osbourn 1996; Vanetten et al. 2001). Some pathogens tolerate saponins through passive mechanisms such as lacking sterols in their cell membranes, resulting in and inability to cause electrolyte leakage (Arneson and Durbin 1967a and b; Defago and Kern 1983; Keukens et al. 1992). Others produce specific enzymes, collectively designated tomatinases, that remove at least one sugar from the tetrasaccharide moiety, which seems to be sufficient to detoxify the molecule. This is the case of *F. oxysporum* f. sp. *lycopersici*, which expresses at least one extracellular protein with tomatinase activity (Ford et al. 1977; Roldán-Arjona et al. 1999). Tomatinases have been identified in other tomato pathogens, such as *Septoria lycopersici* (Arneson and Durbin 1967a and b; Durbin and Uchtyl 1969), *Verticillium albo-atrum* (Sandrock and VanEtten 1998), *Botrytis cinerea* (Quidde et al. 1998), *F. solani* (Lairini and Ruiz-Rubio 1998), or *Alternaria solani* (Schönbeck and Schlösser 1976); however, they differ in their mechanism of action and seem to belong to different protein families. Whereas tomatinases from *S. lycopersici* and *V. albo-atrum*, both members of family 3 of β -glycosyl hydrolases, remove the β -1,2-linked glucose to produce β_2 -tomatine (Sandrock and VanEtten 1998), *B. cinerea* seems to catalyze the hydrolysis of the β -1,3-xylose to release β_1 -tomatine (Quidde et al. 1998). In contrast, tomatinase from *A. solani* degrades the saponin to tomatidine by releasing the four monosaccharide (Schönbeck and Schlösser 1976). Tomatinase from *F. oxysporum* f. sp. *lycopersici*, which belongs to family 10 of β -glycosyl hydrolases, cleaves the β -1-galactose, releasing the entire oligosaccharide chain and tomatidine (Roldán-Arjona et al. 1999). Several tomatinases belonging to family 10 of glycosyl hydrolases have been reported in the weak tomato pathogen *Gibberella pulicaris* (Weltring et al. 1998) and in the pathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis* (Kaup et al. 2005) and *Streptomyces turgidiscabies* (Kers et al. 2005). The role of saponinases in fungal pathogenicity to plants remains controversial. Detoxification of saponins has been associated with pathogenicity, although this has been clearly confirmed only for the interaction between *Gaeumannomyces graminis* var. *avenae* and oat plants (Bowyer et al. 1995). Targeted disrupted mutants deficient in avenacinase activity from *G. graminis* var. *avenae* revealed that the enzyme was essential for pathogenicity to oat (Bowyer et al. 1995). Despite this finding, tomatinase deficient mutants from *Septoria lycopersici* (Martin-Hernandez et al. 2000) and from *Colletotrichum coccodes* (Sandrock and Vanetten 2001) showed unaltered virulent phenotypes. On the other hand, het-

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erologous expression of the tomatinase gene from *S. lycopersici* in the nontomatinate-producing pathogens *Cladosporium fulvum* and *Nectria haematococca* resulted in increased sporulation rates on tomato plants (Melton et al. 1998; Sandrock and Vanetten 2001), thus demonstrating their implication in virulence. It also has been reported that tomatinases might play a role in suppression of fundamental disease resistance processes (Bouarab et al. 2002; Ito et al. 2004; Martin-Hernandez et al. 2000), being present and expressed even in nonpathogens of tomato during symptomless infection of the plant (Ito et al. 2005; Lairini et al. 1997). Recently, in a failed attempt to disrupt the tomatinase gene from *F. oxysporum* f. sp. *lycopersici*, partially silenced transformants of this gene showed reduced virulence, revealing a possible role of tomatinase from *F. oxysporum* in pathogenicity (Ito et al. 2002).

The aim of this report is to clarify the role of the tomatinase gene *tom1* (Roldan-Arjona et al. 1999) in the pathogenic behavior of *F. oxysporum* f. sp. *lycopersici*. We have generated fungal strains of *F. oxysporum* f. sp. *lycopersici* that do not express the Tom1 enzyme through targeted gene disruption, as well as a strain that constitutively expresses Tom1. Our results indicate that tomatinase contributes to virulence of this fungal species on tomato plants.

RESULTS

Targeted disruption and overexpression of the *tom1* gene in the wild-type strain.

To determine the function of the *tom1* gene (Roldan-Arjona et al. 1999), mutants carrying a disrupted copy of the gene were generated by gene replacement. A linear fragment con-

taining an internal region of the *tom1* allele interrupted by the hygromycin resistance (*Hyg^r*) cassette (Fig. 1A) was introduced into protoplasts of *F. oxysporum* f. sp. *lycopersici* wild-type strain 4287. *Hyg^r* selection allowed the isolation of 131 transformants which initially were screened by polymerase chain reaction (PCR) amplification and analysis of the pattern consistent with homologous replacement of the *tom1* gene. According to PCR analysis, only eight transformants showed amplification bands consistent with the disrupted *tom1* allele (named transformants no. 1 to 8) (data not shown). The homologous recombination events in transformants no. 1 and 2 were confirmed by Southern analysis using an internal fragment of the *tom1* coding region as a probe (Fig. 1B, probe a). The wild-type strain showed a 1.5-kb *XhoI* hybridizing fragment corresponding to the *tom1* allele (Fig. 1B). This fragment was replaced by a 4.3-kb band in transformants that had undergone disruption of the tomatinase gene (Fig. 1B). Transformant #9 displayed the original 1.5-kb *XhoI* band and additional hybridizing fragments, indicating ectopic insertions of the disrupted allele (Fig. 1B).

F. oxysporum strains carrying a constitutively overexpressed *tom1^C* allele were produced by fusing the *tom1* coding region to the *Aspergillus nidulans* *gpdA* promoter (*PgpdA*) (Fig. 2A). The entire construct was amplified by PCR and introduced into protoplasts of the wild-type strain by co-transformation together with the phleomycin resistance (*Phleo^r*) cassette. A number of *Phleo^r* transformants were selected, and the presence of the complete *tom1^C* allele was confirmed by Southern analysis using an internal *tom1* gene fragment as a probe (Fig. 2B, probe b). The presence of the wild-type allele (2 kb) was detected in the wild-type strain as well as in all the analyzed transformants. One additional hybridizing band was observed in each transformant consistent with the ectopic insertion of the *tom1^C* construct into the genome (Fig. 2B).

Fig. 1. Targeted disruption of the *Fusarium oxysporum* *tom1* gene. **A**, Restriction map of the genomic region and gene disruption vector. The *tom1* coding region is shown as a black arrow indicating the orientation of the open reading frame. Small arrows indicate the primers used for polymerase chain reaction amplification of the gene disruption vector. The hygromycin resistance (*Hyg^r*) cassette is indicated by a gray box. Restriction enzymes are X = *XhoI* and B = *BamHI*. **B**, Southern analysis of *Fusarium* strains: lane 1, wild-type strain; lanes 2 and 3, *Atom1#1* and *Atom1#2* transformants; and lane 4, ectopic integration transformant no. 9. DNAs were *XhoI* digested and hybridized with the indicated probe. Size markers are in kilobases.

Fig. 2. Overexpression of the *tom1* gene. **A**, Physical map of the overexpression vector. The *tom1* gene is shown as a black arrow fused to the *Aspergillus nidulans* *gpdA* promoter. Small arrows indicate the primers used for polymerase chain reaction amplification. **B**, Southern analysis of lane 1, the wild-type strain; and lanes 2, 3, and 4, three different *tom1^C* transformants. Genomic DNAs were digested with *HindIII* and hybridized with the indicated probe. Size markers are in kilobases.

Northern analysis was used to confirm the absence of *tom1* transcripts in the knockout mutants and its overexpression in the *tom1^C* strains (Fig. 3). It has been reported that the *tom1* gene is induced by α -tomatine (Roldan-Arjona et al. 1999). Total RNA obtained from mycelia of the different strains grown for 3 h in the absence or presence of α -tomatine at 30 μ g ml⁻¹ was hybridized to an antisense *tom1* probe amplified with primer tom1-16 (Table 1). The *tom1* transcripts were detected only in the wild-type strain grown in the presence of α -tomatine (Fig. 3), whereas no signal was detected in the knockout mutants in any condition. By contrast, in the constitutive *tom1^C#1* transformant, *tom1* transcription was independent of the presence of α -tomatine. One transformant harboring an ectopic insertion of the disruption construct showed the same expression pattern as the wild-type strain. These results confirmed that transformants no. 1 and 2 were targeted mutants lacking a functional copy of the *tom1* gene, whereas transformant *tom1^C#1* constitutively transcribed the *tom1* gene.

Tomatinase activity in Δ *tom1* and *tom1^C* transformants.

The effect of the disruption or the overexpression of the *tom1* gene on total extracellular tomatinase activity from *F. oxysporum* was determined. Total protein was extracted from culture filtrates by acetone precipitation from the different strains grown for 48 h in liquid CA (casamino acids) in the absence or presence of α -tomatine. Tomatinase activity was assayed by visualizing the presence of reducing sugars spectrophotometrically at 525 nm with the dinitrosalicylic acid reagent (DNS) (Fig. 4). As expected, the wild-type strain showed a significant increase in the levels of tomatinase activity under inducing conditions, whereas no significant activity was observed in the absence of α -tomatine. A reduction of approximately 25% in tomatinase activity was detected in the filtrates of the disrupted mutants Δ *tom1*#1 and Δ *tom1*#2 in the presence of α -tomatine compared with the wild-type strain. By contrast, high levels of activity were observed in filtrates from the constitutive *tom1^C* transformants independently of the presence of α -tomatine in the culture medium.

The degradation products of α -tomatine produced by the different strains were analyzed by thin-layer chromatography (TLC) under both inducing and noninducing conditions (Fig. 5). The analysis revealed culture filtrates from the wild-type strain

grown in the presence of α -tomatine caused the hydrolysis of the saponin and showed the appearance of two degradation products: a major spot corresponding to tomatidine and a minor one, which may correspond to β_2 -tomatine. This second spot was the only degradation product obtained from the induced crude extract of the disrupted strains, suggesting that α -tomatine was degraded to β_2 -tomatine by these mutant extracts, whereas tomatidine was the only product released by crude extracts from the constitutive transformants under either inducing or noninducing conditions, probably because the high amount of the Tom1 enzyme in these strains favors the degradation of the α -tomatine to tomatidine.

Sensitivity of Δ *tom1* mutants to α -tomatine.

The abilities to grow on agar medium containing α -tomatine shown by two targeted mutants, Δ *tom1*#1 and Δ *tom1*#2, and by the constitutive *tom1^C#1* transformant, were compared with the wild-type strain (Fig. 6A). The differences were quantified by measuring the colony diameters and the halos surrounding them (Fig. 6B). No differences between strains were observed in the absence of α -tomatine. Both the wild-type strain and the *tom1^C* mutant showed approximately 35% growth inhibition (GI) on plates containing 0.5 mM α -tomatine compared with their growth in its absence (Fig. 6A and C). By contrast, the

Fig. 3. Expression analysis of the *tom1* gene. Upper panels, Northern analysis of total RNA extracted from mycelia grown in the presence (+) or absence (-) of α -tomatine using a *tom1* antisense probe. Lane 1, wild-type strain; lanes 2 and 3, Δ *tom1*#1 and Δ *tom1*#2 transformants; lane 4, *tom1^C* transformant; lane 5, an ectopic integration transformant. The lower panels show RNAs stained with methylene blue.

Table 1. Oligonucleotides used in this study

Name	Sequence ^a
tom1-X1	tctagaat gaaggccactatcatcatcg
tom1-3	cttgccactggaatgaaagtc
tom1-5B	tgggttac aggatcct gagataag
tom1-8	gctgacccagacaggctgc
tom1-9B	actgagag aggatc ggccaagaa
tom1-10B	ttctggcc ggatcct ctctcagt
tom1-11X	ctcgagct caagcagtcgaaatccct
tom1-12H	aagcttc gacgagttgtgctaccatct
tom1-16	aacatcaagggtcgtcc
tom2-1	ccacttctgcgactgcta
tom2-2	ctgtgttctgtgtgttcc
tom3-1	ctatcattggtcttgccgtt
tom3-2	tgttgaggatactgcgtcta
tom4-1	atccccctctatccctccc
tom4-2	aagagactccagaatcggttg
tom5-1	tgtgtgtctcgggtctttt
tom5-2	tagtctctccatcgcaatac
gpdA-6H	aagcttc agttcatggcggtt
gpd-2k	cagcgact ggatcct cca
trp1-2k	gattaccttaaca agggtacc
act-1	atgaccagatcgcttcgag
act-2	gagggaccgcctcgtct

^a Restriction sites introduced in the original sequences are shown in bold.

Fig. 4. Total tomatinase activity from *Fusarium oxysporum* strains, measured using protein extracts from culture medium from the different strains grown in the absence (gray bars) or presence (black bars) of α -tomatine. Tomatinase activity is expressed as the absorbance at 525 nm of the reaction mixture. Each column represents the mean of three independent experiments, and the standard error bars are indicated.

knockout mutants showed 50% GI on 0.5 mM α -tomatine. The appearance of a translucent halo surrounding the colonies was evidenced when strains were grown in the presence of α -tomatine, which might be a consequence of the saponin hydrolysis. The ratio halo/colony diameter was similar in the wild-type strain and the $\Delta tom1$ mutants, despite the fact that $\Delta tom1\#1$

and $\Delta tom1\#2$ colonies were always smaller. By contrast, this value was higher in the constitutive $tom1^C$ transformant. Similar results were observed when strains were grown on 0.1 or 1 mM α -tomatine (data not shown).

Pathotypic behavior of $\Delta tom1$ and $tom1^C$ transformants on tomato.

The role of the $tom1$ gene in virulence of *F. oxysporum* f. sp. *lycopersici* was determined by plant infection assays, performed by immersing the roots of 2-week-old tomato plants in microconidial suspensions of the wild-type strain, the deficient $\Delta tom1$ mutants, or the constitutive $tom1^C$ transformants. Plants were scored for vascular wilt symptoms at different time intervals after inoculation (Fig. 7). Severity of wilt symptoms in plants inoculated with the wild-type strain increased steadily throughout the experiment, and most of the plants were dead 20 days after inoculation. Plants inoculated with the $\Delta tom1$ mutants showed a significant delay in symptom development compared with the wild-type strain, with most of the plants dead 25 days after inoculation. By contrast, $tom1^C$ transformants showed a significant increase in symptom development, because most of the plants were dead 15 days after inoculation (Fig. 7). Virulence experiments were performed three times with similar results.

Identification of additional tomatinase genes in the *F. oxysporum* genome.

The genome of *F. oxysporum* was explored in silico to further investigate the existence of additional tomatinase-encoding genes. No genes showing high similarity to $tom1$ (Roldan-Arjona et al. 1999) were found, whereas at least four genes encoding proteins with putative tomatinase activity were iden-

Fig. 5. Thin-layer chromatography analysis of tomatinase reaction products. Tomatine (5 mM) was incubated overnight at room temperature with noninduced (N) or induced (I) crude extracts from the wild type (wt), disrupted $\Delta tom1\#1$, and constitutive $tom1^C\#1$ strains. α -Tomatine and tomatidine were loaded in lanes 1 and 2, respectively, and used as markers. The assay was repeated twice with similar results. Figure shows the results from a representative experiment.

Fig. 6. Effect of α -tomatine on *Fusarium oxysporum* growth. **A**, Wild-type strain (wt), two disrupted transformants $\Delta tom1\#1$ and $\Delta tom1\#2$, and a constitutive transformant ($tom1^C$) grown onto solid CA (casamino acid) medium in the absence (–) or presence (+) of 0.5 mM α -tomatine for 7 days. **B**, Diameter of the colonies in the presence of 0.5 mM α -tomatine (C) and of the surrounding halos (H). **C**, Measurement of the colony diameter (cm) in the absence of α -tomatine (C_0), in the presence of 0.5 mM α -tomatine ($C_{0.5}$), and the halos surrounding them ($H_{0.5}$). Growth inhibition (GI) indicates the percentage of growth inhibition for each strain ($C_{0.5}$ values compared with C_0). Ratio ($H_{0.5}/C_{0.5}$) represents the relation between halo size and colony size observed for each strain in the presence of α -tomatine.

tified showing identity to the β_2 -tomatinase from *S. lycopersici* (Durbin and Uchytel 1969; Osbourn et al. 1995; Sandrock et al. 1995). These genes were named *tom2* (FOXG_09849), *tom3* (FOXG_02734), *tom4* (FOXG_13018), and *tom5* (FOXG_09125). The deduced amino acid sequences of these genes presented high identity degrees with family 3 glycosyl hydrolases: 51.8% identity between Tom2 and *B. cinerea sap1* avenacinase, 53.5% between Tom3 and *S. lycopersici* tomatinase, 50.0% identity between Tom4 and *G. graminis* avenacinase, and 56.8% identity was found between Tom5 and *G. graminis* avenacinase. To establish the relationship between these proteins, the amino acid sequence from *F. oxysporum* tomatinases was aligned together with *S. lycopersici* β_2 -tomatinase (Osbourn et al. 1995; Sandrock et al. 1995), *G. graminis* avenacinase (Bowyer et al. 1995), *B. cinerea sap1* avenacinase (Quidde et al. 1999), *A. fumigatus* glycosyl hydrolase family protein (Nierman et al. 2005), *Streptomyces turgidiscabies* tomatinase (Kers et al. 2005), and *Clavibacter michiganensis* tomatinase (Kaup et al. 2005) (data not shown). The resulting phylogenetic tree demonstrated that *F. oxysporum* tomatinases cluster into two different groups: Tom1 belongs to family 10

Fig. 7. Virulence of *Fusarium oxysporum* strains on tomato. Wilt disease incidence of the different strains on tomato plants (cv. Monika). Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain (■), $\Delta tom1\#1$ transformant (Δ), $\Delta tom1\#2$ transformant ($\blacktriangle$), constitutive *tom1C*#1 transformant ($\circ$), constitutive *tom1C*#8 transformant ($\blacklozenge$), and the uninoculated control ($\square$). Error bars indicate the standard deviations from 15 plants for each treatment. The data shown are from one representative experiment.

Fig. 8. Phylogenetic relationship between tomatinases from *Fusarium oxysporum*. Phylogram of a consensus phylogenetic tree obtained by maximum likelihood method of *F. oxysporum* predicted tomatinases Tom1, Tom2, Tom3, Tom4, and Tom5; β_2 -tomatinase from *Septoria lycopersici* (Sttom) (AAB08446); avenacinase from *Gaeumannomyces graminis* (Ggave) (AAA63146); tomatinase from *Streptomyces turgidiscabies* (Sttom) (AAW49296); tomatinase from *Clavibacter michiganensis* (Cmtom) (YP_001220829); a glycosyl hydrosase family protein from *Aspergillus fumigatus* (Afgh) (XP_751064); and avenacinase from *Botrytis cinerea* (Bcsap1) (CAB61489). Amino acid sequences were aligned using clustal W. Numbers at the branch points represent the bootstrap value based on 1,000 replicates. A scale bar indicating the genetic distance (substitutions per site) is shown.

glycosyl hydrolases, whereas Tom2, Tom3, Tom4, and Tom5 are members of family 3 glycosyl hydrolases (Fig. 8).

Reverse-transcription (RT)-PCR analysis using specific primers for each gene was performed in order to determine the expression pattern of tomatinase genes in the wild-type, $\Delta tom1$, and *tom1C* strains grown in the absence or presence of α -tomatine. In order to discriminate the amplicons obtained from genomic DNA, primer pairs were designed located within exons of the corresponding gene, except for the *tom1* gene, which lacks intron sequences. The results indicated low expression levels of *tom2* and *tom5* in comparison with *tom1* gene, while expression of *tom3* and *tom4* was not detected in any condition (Fig. 9). Expression of *tom2* gene appeared to be induced by α -

Fig. 9. Expression analysis of the *tom1*, *tom2*, and *tom5* genes by reverse-transcription polymerase chain reaction (RT-PCR). Transcript levels of the predicted tomatinase genes *tom1*, *tom2*, and *tom5* were determined by RT-PCR in the wild-type strain (wt), disrupted $\Delta tom1$ transformant, and a constitutive *tom1C* mutant, grown in the absence (N) or presence (I) of α -tomatine. The actin gene was used as an internal control. Amplification of genomic DNA (gDNA) was used as positive PCR control.

tomatine, whereas expression of *tom5* was independent of the presence of the saponin in the culture medium. Inactivation or overexpression of the *tom1* gene did not alter the expression pattern of any of the genes. Expression of the *tom1* gene, also included in this analysis, showed a pattern similar to that in the Northern expression analysis.

DISCUSSION

The infection process of pathogenic fungi into plants involves a confrontation between two mechanisms: the attack of the pathogen and the plant defense response. Among the tomato plant defense weapons is the production of the saponin α -tomatine, which is detoxified by a tomatinase enzyme secreted by the pathogen *F. oxysporum* f. sp. *lycopersici* (Lairini et al. 1996). This protein, encoded by the *tom1* gene, degrades the α -tomatine, releasing tomatidine and the tetrasaccharide β -lycotetraose (Lairini et al. 1996; Roldan-Arjona et al. 1999).

Detoxification of plant saponins is considered to be a virulence determinant for pathogenic fungi and, therefore, several efforts have been made to establish the importance of saponinases in pathogenicity. Interestingly, an attempt to disrupt the *tom1* gene in *F. oxysporum* resulted in the isolation of partial post-transcriptional silenced transformants with reduced pathogenicity on tomato plants (Ito et al. 2002). However, the authors could not conclude whether the *tom1* gene played an essential role in pathogenicity. Here, we report the effects of the targeted disruption and overexpression of the *tom1* gene in *F. oxysporum* f. sp. *lycopersici*.

Northern analysis of the constitutive *tom1*^C strains reveals the presence of *tom1* transcripts under both inducing and non-inducing conditions, confirming the overexpression of this gene. In agreement with this, tomatinase activity in *tom1*^C transformants was shown to be independent of the presence of α -tomatine in the culture medium. By contrast, although *tom1* transcripts were absent in Δ *tom1* mutants grown in the presence of the saponin, confirming *tom1* gene inactivation, a reduction of only 25% in the total tomatinase activity level was detected compared with the wild-type strain.

Further characterization of Δ *tom1* and *tom1*^C transformants revealed increased growth inhibition of the disrupted strains on solid media containing α -tomatine, whereas no changes were observed in the constitutive transformants compared with the wild-type strain. These results indicate higher sensitivity to α -tomatine displayed by the Δ *tom1* transformants due to the reduction in total tomatinase activity. However, comparable degradation halos surrounding the colonies were observed in the presence of high concentrations of the saponin for the Δ *tom1* and the wild-type strain, whereas the halos in the constitutive *tom1*^C transformants were significantly larger, suggesting a stronger hydrolytic activity of α -tomatine displayed by these transformants as a consequence of the overexpression of the *tom1* gene.

Pathogenicity tests on tomato plants indicated that *tom1* disruption produces a delay in the progression of the disease symptoms compared with the wild-type strain, whereas the overexpression of the *tom1* gene results in a faster development of the symptoms. These results suggest that, although the *tom1* gene is not an essential factor, it does contribute to full virulence of *F. oxysporum* on tomato plants.

Taken together, all these observations (the high tomatinase activity remaining in the Δ *tom1* strains, the similar degradation halo produced by the Δ *tom1* and the wild-type strains, and the reduced virulence of Δ *tom1*) suggest that additional enzyme activities present in *F. oxysporum* might be contributing to the detoxification of the α -tomatine, thus playing redundant functions. In silico analysis of the recently released complete ge-

nome sequence of *F. oxysporum* revealed the existence of at least five independent tomatinase-encoding genes: the *tom1* gene which detoxifies the molecule into tomatidine and β -lycotetraose and belongs to family 10 of glycosyl hydrolases (Roldan-Arjona et al. 1999), and four additional genes with high identity to the avenacinase gene from *G. graminis* and the β_2 -tomatinase gene from *Septoria lycopersici*, which belong to family 3 of glycosyl hydrolases (Henrissat and Bairoch 1993; Osbourn et al. 1995). We speculate that the proteins encoded by these four genes might be responsible for the remaining tomatinase activity and the degradation halos displayed by the Δ *tom1* strains, in spite of the low expression levels of these genes. This hypothesis is supported by the results obtained from the TLC analysis of tomatine degradation products released by induced crude extracts from Δ *tom1* mutants where β_2 -tomatine was the only detectable hydrolysis product, confirming the degradation of α -tomatine by additional enzymes with a mechanism of action different from Tom1.

The existence of various genes encoding for tomatinase enzymes has been proposed previously in the tomato pathogen *Colletotrichum coccodes* (Sandrock and Vanetten 2001), where mutants lacking a functional copy of a β_2 -tomatinase gene still were able to degrade α -tomatine to tomatidine. In the fungal pathogen *B. cinerea*, at least three different saponin-degrading enzymatic activities have been described: *sap1* that detoxifies the avenacin, another one active on the digitonine and on the α -tomatine, and a third one proposed as an avenacosidase (Quidde et al. 1999). The existence of five different tomatinase genes in the genome of *F. oxysporum* f. sp. *lycopersici* supports the idea that detoxification of α -tomatine plays an important role during the infection of tomato plants. In *S. lycopersici*, mutants deficient in a β_2 -tomatinase gene showed no changes in pathogenicity (Martin-Hernandez et al. 2000). The authors proposed that additional nondegradative mechanisms of resistance to α -tomatine might exist, because no other hydrolysis products of the saponin were detected in the knockout mutants. Additionally, *tomA* genes, orthologous to *F. oxysporum tom1*, recently have been targeted inactivated in the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* and in the plant-pathogenic bacteria *Streptomyces turgidiscabies*, although the resulting mutants were not reduced in virulence (Kaup et al. 2005; Kers et al. 2005). By contrast, disruption of the avenacinase-encoding gene in the cereal-infecting fungus *G. graminis* var. *avenae* revealed that detoxification of avenacin A-1 is a determinant of host range (Bowyer et al. 1995; Osbourn et al. 1994). A similar role has been proposed for *B. cinerea* and *Corynespora cassiicola* tomatinases, where nonpathogenic strains lack the corresponding tomatinase-encoding genes (Oka et al. 2006; Quidde et al. 1998). On the other hand, heterologous expression of the *Septoria lycopersici* β_2 -tomatinase gene in *N. haematococca* and *Cladosporium fulvum* strains unable to degrade α -tomatine resulted in acquisition of the capacity to infect green tomato fruit and increase sporulation and infection extension on cotyledons, respectively (Melton et al. 1998; Sandrock and Vanetten 2001).

We conclude that, in *F. oxysporum* f. sp. *lycopersici*, detoxification of α -tomatine plays an essential role during the infection of tomato plants, likely being the result of the combined action of different tomatinase activities. Therefore, simultaneous inactivation of the five tomatinase-encoding genes present in the genome of *F. oxysporum* would clarify this hypothesis.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

F. oxysporum f. sp. *lycopersici* wild-type strain 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Spain, and

stored at -80°C with 30% glycerol as a microconidial suspension. The pathotype of the strain was confirmed periodically by plant infection assays. For DNA extraction and microconidia production, cultures were grown in potato dextrose broth (Difco Laboratories, Detroit) at 28°C with shaking at 170 rpm during 3 days as described previously (Di Pietro and Roncero 1998). For expression analysis, 5×10^8 microconidia from each strain were grown with shaking for 12 h at 28°C in 50 ml of CA medium (CA at 10 g/liter, 10 mM ammonium sulfate, and yeast nitrogen base at 0.5 g/liter) before the addition of α -tomatine at $30 \mu\text{g ml}^{-1}$ (Extrasynthèse, Genay, France), dissolved in 50 mM sodium acetate buffer, pH 4.5, and followed by additional incubation for 3 h. The mycelia then were collected by filtration through nylon cloth (Monodur, mesh size 10 μm), washed with sterile water, and stored at -80°C . For tomatinase activity determination and TLC analysis, 2.5×10^6 microconidia were grown with shaking for 48 h at 28°C in 5 ml of CA medium containing α -tomatine at $30 \mu\text{g ml}^{-1}$, previously dissolved in 50 mM sodium acetate buffer, pH 4.5. The cultures were filtered to separate mycelia and spores, and the supernatants were used to determine the tomatinase activity or the degradation products in TLC analysis.

Nucleic acid isolation and analysis.

Genomic DNA was extracted from fungal mycelia as described previously (Aljanabi and Martinez 1997). DNA was treated with the corresponding restriction enzyme and subjected to Southern analysis as described in standard protocols (Sambrook et al. 1989) with the nonisotopic digoxigenin-labeling kit (Roche Diagnostics SL). Probe labeling was carried out as previously reported (Di Pietro and Roncero 1998) using the primers tom1-11X and tom1-12H (Table 1) for probe a or tom1-X1 and tom1-5B (Table 1) for probe b. Total RNA was isolated from powdered mycelia using Tripure Isolation Reagent (Roche Diagnostics SL, Mannheim, Germany), according to the manufacturer's recommendations, except for all incubation steps that were developed on ice. Total RNA (20 μg) was separated on a formaldehyde-1% agarose gel and transferred to positively charged Nylon membranes (Roche Diagnostics SL) by capillarity. For quantification, the 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, the filters were subjected to Northern analysis with the nonisotopic digoxigenin-labeling kit. Probe labeling was carried out as previously reported (Di Pietro and Roncero 1998) using the antisense primer tom1-16 (Table 1).

Construction of plasmid vectors and fungal transformation.

Gene replacement vector pDtom1 was constructed as follows. A *Bam*HI site was introduced at position 90 of the *tom1* coding region, using the overlapping method (Sambrook et al. 1989) with the primers tom1-9B and tom1-10B (Table 1), and the final product was cloned into pGEM-T (Promega, Madison, WI, U.S.A.). A 2.75-kb *Bam*HI fragment containing the hygromycin B resistance gene under the control of an *A. nidulans* *gpdA* promoter (Punt et al. 1990) and *trpC* terminator (Mullaney et al. 1985) (*Hyg*^r cassette) was introduced at the new *Bam*HI site in the *tom1* coding region. A linear fragment containing the interrupted *tom1* allele was amplified with primers tom1-8 and tom1-3 (Table 1) and used to transform protoplasts of *F. oxysporum* f. sp. *lycopersici* strain 4287, according to a protocol described previously (Di Pietro and Roncero 1998; Kistler and Benny 1988). *Hyg*^r transformants routinely were subjected to two consecutive rounds of single sporing and stored as microconidia at -80°C . To generate a highly expressed allele of *tom1*, the coding region of the gene

was fused to the strong constitutive *A. nidulans* *gpdA* promoter (Punt et al. 1990) as follows. A 1.16-kb fragment containing the complete open reading frame (ORF) of *tom1* was amplified by PCR from *F. oxysporum* genomic DNA, using primers tom1-X1 and tom1-3 (Table 1), and cloned into pGEM-T to generate plasmid ptomX. An *Xba*I-*Spe*I fragment containing the *tom1* ORF then was isolated from plasmid and cloned in frame with the *gpdA* promoter contained in plasmid pPgpdA. The complete fusion construct was amplified with primers gpdA-6H and tom1-3 (Table 1) and used to co-transform protoplasts of *F. oxysporum* f. sp. *lycopersici* strain 4287 together with a 3-kb PCR fragment containing the *Phleo*^r amplified from the plasmid pAN8-1 with the primers gpd-2k and trp-2k (Table 1).

Tomatinase activity assays.

Proteins contained in supernatants obtained from induced cultures were concentrated by acetone precipitation and resuspended in sterile water. Tomatinase activity was measured spectrophotometrically at 525 nm with the DNS for determination of reducing sugars (Miller 1959). A 200- μl volume from a reaction mixture containing 50 mM sodium acetate buffer (pH 4.5), α -tomatine at 2 mg ml^{-1} , and 6.5 μg of total protein was incubated overnight at room temperature. Reaction mixtures containing α -tomatine-free buffer and protein extracts were used as control. After incubation, 200 μl of 3,5-dinitrosalicylic acid solution was added, the mixture was boiled for 5 min, and the final volume was brought to 1 ml with water. Negative controls were prepared with protein extracts incubated in the absence of α -tomatine.

TLC analysis.

Supernatants obtained from culture filtrates from the different strains grown in the absence or presence of α -tomatine were incubated overnight with 5 mM α -tomatine in 50 mM sodium acetate buffer (pH 4.5) at room temperature. Incubation mixtures were vacuum-dried, resuspended into an appropriate volume 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, by volume). Spots were visualized after TLC plates were sprayed with 50% sulphuric acid and heated at 110°C . The metabolites were identified by comparison with standards (α -tomatine and tomatidine, both 5 mM) which were treated in the same way as the incubation mixtures. α -Tomatine appeared as a black spot near the origin, whereas tomatidine was localized as a dark green spot near the solvent front.

α -Tomatine sensitivity tests.

For phenotypic analysis of colonies, fungal strains were grown on plates of CA medium containing agar at 20 g/liter, pH 7, supplemented with different concentrations of α -tomatine previously dissolved in 50 mM sodium acetate buffer (pH 4.5). Aliquots of 2.5×10^6 freshly obtained microconidia were transferred to the center of each plate and incubated at 28°C for 7 days. Growth was examined by measurement of the colony diameter and the halo surrounding it.

Pathogenicity assays.

Infection of tomato plants was performed as reported previously (Di Pietro and Roncero 1998). Briefly, 2-week-old tomato seedlings were inoculated with *F. oxysporum* strains by immersing the roots in a suspension of 5×10^6 microconidia ml^{-1} for 30 min, then planted in vermiculite and maintained in a growth chamber. Fifteen plants were used for each treatment. Severity of disease symptoms was recorded at different times after inoculation using an index ranging from 1 (healthy plant)

to 5 (dead plant) (Huertas-Gonzalez et al. 1999). Virulence experiments were performed three times with similar results. Seed from tomato cv. Monika were kindly provided by Novartis Seeds.

Identification of additional tomatinase genes in the *F. oxysporum* genome.

Genes showing identity to tomatinases belonging to family 3 of β -glycosyl hydrolases were searched in the *F. oxysporum* genome database available at the Broad Institute database using the blast algorithm (Altschul et al. 1990). Clustal-W analysis was used to compare amino acid sequences of these genes.

Phylogenetic analysis.

Amino acid sequences were aligned with the CLUSTAL W algorithm (Thompson et al. 1994). The phylml v2.4.4 program (Guindon and Gascuel 2003) was used to perform a 1,000-nonparametric bootstrap phylogenetic analysis of the resulting alignment with the maximum likelihood method after optimization of the settings by the modelgenerator v84 program (Keane et al. 2006). The analysis was performed using the WAG substitution model (Goldman and Whelan 2000) with a gamma distribution parameter α of 3.19. The phylogenetic relationships between sequences were depicted in a phylogenetic tree constructed using the TreeView v1.6.6 program (Page 1996).

RT-PCR.

Total RNA isolated from the different strains was treated with RNase-free DNase (Roche Diagnostics SL) and subjected to reverse transcription into cDNA with M-MLV reverse transcriptase (Invitrogen SA, San Diego, CA, U.S.A.) using a poli-dT antisense primer. The cDNA then was used for PCR amplification using the Expand High-Fidelity PCR System (Roche Diagnostics SL) with the primers tom2-1 and tom2-2 for *tom2*, tom3-1 and tom3-2 for *tom3*, tom4-1 and tom4-2 for *tom4*, tom5-1 and tom5-2 for *tom5*, or tom1-11X and tom1-12H for *tom1* (Table 1). The actin gene was used as the internal control using the primers act-1 and act-2 (Table 1).

ACKNOWLEDGMENTS

This research was supported by the Ministerio de Educación y Ciencia of Spain (BIO2004-0276) and Junta de Andalucía (CVI-138). Y. Pareja-Jamie was supported by a fellowship from Ministerio de Educación y Ciencia of Spain. We thank N. Rispail for valuable help with phylogenetic analysis, A. Di Pietro for helpful discussions, and E. Martínez for technical assistance (all from the University of Córdoba).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute *F. oxysporum* genome database:
www.broad.mit.edu/annotation/genome/fusarium_group/