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Fungal Pathogens of Oat Roots and Tomato Leaves Employ Closely Related Enzymes to Detoxify Different Host Plant Saponins

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Antifungal saponins are produced by many plants and have been implicated as preformed determinants of resistance to fungal attack. The importance of saponin detoxification in fungal pathogenesis has recently been demonstrated for the fungus *Gaeumannomyces graminis* var. *avenae*, which produces the enzyme avenacinase. Avenacinase detoxifies the triterpenoid oat root saponin avenacin A-1, and is essential for pathogenicity of *G. graminis* var. *avenae* to oats. Here we demonstrate an unexpected relatedness between avenacinase and the tomatinase enzyme produced by *Septoria lycopersici* (a tomato leaf-infecting fungus), which acts on the steroidal glycoalkaloid α-tomatine. The two enzymes share common physicochemical properties and are immunologically cross-reactive; however, there are critical differences in their substrate specificities which reflect the host preferences of the fungi from which the enzymes were purified. The DNA encoding tomatinase was isolated from a *S. lycopersici* cDNA library using avenacinase DNA as a probe. Comparison of the predicted amino acid sequences of avenacinase and tomatinase revealed that the enzymes are clearly similar.

Saponins are a diverse and chemically complex group of plant secondary metabolites which derive their name from the ability to form stable soaplike foams in aqueous solution (Price et al. 1987; Fenwick et al. 1992). They are glycosylated molecules which can be separated into two distinct classes depending on whether the aglycone is triterpenoid or steroidal. Many saponins are able to disrupt the membrane integrity of higher organisms by complexing with sterols (Schönbeck and Schlösser 1976; Mahato et al. 1982; Steel and Drysdale 1988). Saponins have been identified in over 100 plant families, including many major food crops (Price et al. 1987). Because of the potent antifungal properties associated with many saponins, there has been considerable speculation about their possible function as preformed determinants of resistance of plants to attack by saponin-sensitive fungi (Tschesche 1971; Schönbeck and Schlösser 1976; Fenwick et al. 1992; Osbourn et al 1994; Bowyer et al. 1995). Correlations have been made between the ability of various fungi to infect saponin-containing plants, and the resistance of these fungi to the relevant saponins in vitro (Arneson and Durbin 1968; Steel and Drysdale 1988; Osbourn et al. 1991).

Two major mechanisms of resistance of fungi to saponins exist. Some fungi (such as the oomycetes *Pythium* and *Phytophthora*) are resistant to the toxic effects of saponins because they have little or no sterols in their membranes (Arneson and Durbin 1968), while others produce enzymes which specifically detoxify the saponins of their host plant (Schönbeck and Schlösser 1976). Although saponins are widespread in the plant kingdom, the literature relating to enzymatic detoxification of saponins by phytopathogenic fungi is restricted primarily to fungi which encounter the triterpenoid oat root saponin avenacin A-1, or the tomato steroidal glycoalkaloid saponin α-tomatine. The structures of these molecules are shown in Figure 1. The importance of saponin detoxifying enzymes in fungal pathogenesis has recently been demonstrated for the fungus *Gaeumannomyces graminis* var. *avenae*, which infects oat roots. *G. graminis* var. *avenae* produces the enzyme avenacinase, which removes β,1-2 and β,1-4 linked D-glucose molecules from avenacin A-1 to give products which are less toxic to fungal growth (Turner 1961; Crombie et al. 1986a). Avenacinase-minus mutants of *G. graminis* var. *avenae* have been generated by targeted gene disruption using the cloned avenacinase gene (Bowyer et al. 1995). The mutants were no longer able to infect oats but retained full pathogenicity to wheat (which is not known to

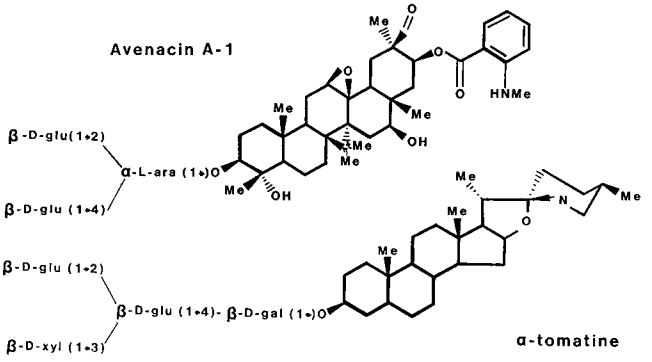

Fig. 1. Structural representations of the triterpenoid oat root saponin avenacin A-1 and the tomato steroidal alkaloid saponin α-tomatine.

Sequence data: GenBank accession numbers U35462 and U35463.

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contain saponins). These experiments indicate that avenacinase is essential for *G. graminis* var. *avenae* to infect oats, but that it is not required for the fundamental ability of the fungus to cause disease on alternative host plants which do not contain avenacin. *G. graminis* var. *avenae* also elaborates other β-glucosidases active on PNPG, a standard β-glucosidase substrate. However, these enzymes have very different properties to avenacinase, are not recognized by anti-avenacinase antisera, and have no activity towards avenacin A-1 (A. E. Osbourn, unpublished results).

Saponin detoxifying enzymes have also been described for a number of fungal pathogens of tomato, and so may have more general significance in fungal phytopathogenicity. The enzymes produced by tomato-attacking fungi detoxify the steroidal glycoalkaloid saponin α-tomatine (Fig. 1), and are known as tomatinases. The tomatinases of *Septoria lycopersici* and *Verticillium albo-atrum* both remove a single glucose molecule from α-tomatine (Arneson and Durbin 1967; Pegg and Woodward 1986), while those of *Fusarium oxysporum* f. sp. *lycopersici* (Ford et al. 1977) and *Botrytis cinerea* (Verhoeff and Liem 1975) are reported to release the intact lycotetraose group from α-tomatine to give the aglycone, tomatidine. The tomato pathogen *Alternaria solani* also degrades α-tomatine to tomatidine but does so by release of monosaccharides rather than a tetrasaccharide (Schönbeck and Schlösser 1976). The importance of these tomatinase enzymes in determining pathogenicity of fungi to tomato has not yet been tested genetically, as to date the enzymes have not been fully purified nor the cognate genes cloned.

Here we report the cloning and characterisation of the DNA encoding tomatinase from the foliar pathogen of tomato, *S. lycopersici*. We have found that the two saponin detoxifying enzymes avenacinase from *G. graminis* var. *avenae*, and tomatinase from *S. lycopersici*, have hitherto unsuspected relatedness and share common physicochemical and immunological properties. Furthermore we were able to use an avenacinase cDNA probe to isolate the DNA encoding tomatinase, indicating the degree of homology between the two genes. While avenacinase and tomatinase are clearly related, their relative activities towards the saponins avenacin A-1 and I-tomatine reflect the host specificity of the fungi from which the enzymes originate.

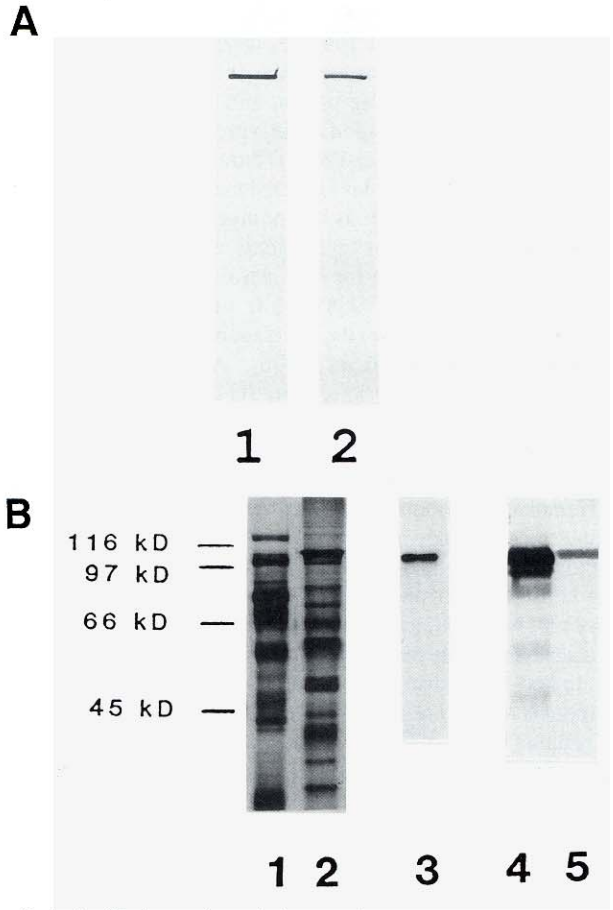

Fig. 2. A, Purified tomatinase is immunologically cross-reactive with avenacinase. Lane 1, silver-stained SDS-PAGE of purified tomatinase (113 kDA); lane 2: Western blot showing recognition of purified tomatinase by anti-avenacinase antisera. **B,** Anti-avenacinase antisera recognize a discrete protein of the same molecular weight as tomatinase in culture filtrates of *Septoria lycopersici*. Lanes 1 and 2, Silver-stained SDS-PAGE of ammonium sulphate concentrated proteins precipitated from culture filtrates of *Gaeumannomyces graminis* var. *avenae* and *S. lycopersici* respectively. Lane 3, Western blot of *G. graminis* var. *avenae* proteins shown in lane 1, showing recognition of avenacinase by the anti-avenacinase antisera. Lanes 4 and 5, Western blot of *G. graminis* var. *avenae* and *S. lycopersici* proteins shown in lanes 1 and 2, respectively, after prolonged development. The major band in lane 4 represents avenacinase, while the lower molecular weight bands are likely to be degradation products visible only after prolonged development of the Western blot. In lane 5 the anti-avenacinase antisera recognizes a single band in crude culture filtrates of *S. lycopersici* of approximately the same molecular mass as tomatinase (113 kDa).

RESULTS

Purification of tomatinase from *S. lycopersici* and comparison with avenacinase.

Proteins from filtrates of *S. lycopersici* cultures were concentrated with ammonium sulphate and dialyzed as described in Methods. The dialysate was then fractionated by free-flow isoelectric focusing, and a single peak of tomatinase activity with a pI of 4.6 was identified. Active fractions were pooled and subjected to high-performance anion exchange chromatography on DEAE-5PW at pH 6.2; all the tomatinase activity bound to the column. A gradient of 0 to 1 M sodium chloride was applied to the column and tomatinase was eluted as a single peak at a salt concentration of 0.15 M sodium chloride. Fractions containing tomatinase activity were then pooled and further fractionated by high-performance size exclusion chromatography. Again, tomatinase activity eluted as a single peak. SDS-PAGE analysis, shown in Figure 2A (lane 1), indicated that the enzyme had been purified to homogeneity and was a large protein of approximately 113 kDa. The properties of tomatinase assessed during this purification procedure are very similar to those of avenacinase (Osbourn et al. 1991; Bowyer et al. 1995) (Table 1). The two enzymes have identical pIs (4.6) and very similar molecular masses (110 and 113 kDa for avenacinase and tomatinase, respectively). Furthermore, the purified protein was immunologically related to avenacinase. Polyclonal antisera raised against avenacinase from *G. graminis* var. *avenae* recognized purified tomatinase (Fig. 2A, lane 2), and a single protein species of the same molecular mass as tomatinase in crude protein preparations from culture filtrates of *S. lycopersici* (Fig. 2B, lane 5). No bands were seen when Western blots were screened with

preimmune serum alone (data not shown). Estimation of the relative amounts of tomatinase activity and of the degree of immunological reactivity in samples from different stages of the purification were consistent with tomatinase being the only immunologically reactive protein present in the original culture filtrate. There was no evidence to indicate the existence of other *S. lycopersici* proteins which were immunologically cross-reactive with avenacinase.

Substrate specificities of avenacinase and tomatinase reflect host range.

Although the physicochemical and immunological similarities between tomatinase and avenacinase are evident, there are clear differences between these enzymes in their abilities to deglucosylate the saponins avenacin A-1 and α-tomatine (Table 1). Avenacinase deglucosylates α-tomatine with a relative activity of approximately 2% of its activity towards avenacin A-1, while tomatinase has a relative activity towards avenacin A-1 of less than 0.01% when compared to its activity towards α-tomatine. The substrate preference of tomatinase for α-tomatine and of avenacinase for avenacin A-1 is consistent with a requirement for *G. graminis* var. *avenae* and *S. lycopersici* to be able to effectively detoxify the saponins of their respective hosts. Although the specific activity of tomatinase for α-tomatine is 64 times greater than that of avenacinase for avenacin A-1, this is not reflected in substantially different Km values (the Km values of avenacinase for avenacin A-1 and of tomatinase for α-tomatine are 0.13 [Osbourn et al. 1991] and 0.06 mM [Durbin and Uchytil 1969], respectively). These differences must therefore arise through differences in turnover number, rather than in the affinity of the two enzymes for their respective substrates. Both enzymes have comparable specific activities on the standard β-glucosidase substrate para-nitrophenyl-β,-D-glucopyranoside (PNPG) (Table 1). Avenacinase is equally effective on avenacin A-1 and PNPG, while tomatinase deglucosylates α-tomatine much more effectively than it does PNPG (a difference in specific activity of 230-fold). The high specific activity of tomatinase for α-tomatine may explain why other workers have regarded the activity of this enzyme towards PNPG as insignificant (Durbin and Uchytil 1969).

Isolation of the *S. lycopersici* tomatinase gene using DNA encoding avenacinase as a probe.

A cDNA library was made in the lambda ZAP II vector (Stratagene) from RNA preparations from *S. lycopersici* cultures producing tomatinase (see Methods). This library was probed with a 1.1-kb avenacinase cDNA fragment isolated from the clone pA312 (Bowyer et al. 1995). Six cDNA clones with insert DNA sizes of up to 2.5 kb were isolated. The *S. lycopersici* DNA in these clones cross-hybridized and contained common restriction fragments, indicating that the cDNA clones were related. Southern blot analysis of *S. lycopersici* genomic DNA digested with a range of restriction enzymes and probed with the longest cDNA (insert from pCTOM6) indicated only a single cognate gene. No other cross-hybridizing DNA was detected in the *S. lycopersici* genome. The shorter cDNA clones were assumed to be truncated forms of pCTOM6, and this was later confirmed by DNA sequence analysis of the ends of these clones.

A number of lines of evidence suggest that this cDNA encodes tomatinase. N-terminal amino acid sequence information was obtained for peptides derived from purified tomatinase by proteolytic cleavage. The N-terminal sequences of two of these peptides were determined to be EDQSKHFTTIPTFPTPD and DVTEGLTFTGD. The full DNA sequence of pCTOM6 was determined (GenBank accession number U35462), and the amino acid sequence of the predicted product deduced (Fig. 3). The two experimentally determined amino acid sequences were found to be exactly as predicted for the protein encoded by pCTOM6 (amino acids 21-38 and 436-446 in Fig. 3). Amino acid sequence information obtained from tomatinase from another isolate of *S. lycopersici* by R. Sandrock also matches the predicted product of pCTOM6 (KRTESVGGRVQYLLS corresponding to amino acids 481 to 495 in Fig. 3) (R. Sandrock and H. VanEtten, personal communication). The first 19 amino acids of the predicted protein are predominantly hydrophobic (Fig. 3) and may comprise a signal sequence, suggesting that the product of pCTOM6 is an extracellular secreted protein. Attempts to obtain amino acid sequence from the N-terminus of the mature uncleaved tomatinase protein were unsuccessful, presumably because of N-terminal blockage. Consequently the exact signal peptide cleavage site could not be determined but it must clearly be N-terminal to amino acid 21 (Fig. 3), since the amino acid sequence from positions 21 to 38 has been identified in the mature protein (see above). Computer analysis of the predicted amino acid sequence derived from pCTOM6 (after removal of the first 19 amino acids) predicts a protein of molecular weight of 85 kDa, and with a pI of 4.9. Although the pI is close to that of tomatinase, the molecular weight is clearly different from that estimated by SDS-PAGE. However, it is clear that tomatinase is a glycoprotein; treatment of tomatinase with *N*-glycanase (which hydrolyzes all common classes of asparagine-linked oligosaccharides) gives a product of approximately 90 kDa (unpublished data). This evidence that tomatinase is glycosylated is consistent with the presence of 11 potential N-glycosylation sites (Asn X Ser/Thr where X is not proline [Gavel and von Heijne 1990]) in the predicted protein. Overall these results strongly suggest that pCTOM6 does indeed encode tomatinase.

The predicted product of pCTOM6 is clearly related to avenacinase (68% similar and 53% identical amino acids) (Fig. 3), as expected from the similar characteristics of the proteins. The DNA sequences encoding these enzymes are 60% homologous. Comparison of the amino acid sequences of avenacinase and tomatinase with other sequences in computer databases revealed homology with β-D-glucosidases belonging to the family 3 group of glycosyl hydrolases defined by Henrissat (Henrissat 1991; Henrissat and Bairoch

Table 1. Comparison of the properties of the purified avenacinase and tomatinase enzymes

	pI	Mol. mass[a]	Cross-reaction with anti-avenacinase antisera	Specific activity (nmoles glucose released/min/mg protein) Avenacin A-1	α-Tomatine	PNPG
Avenacinase	4.6	110	+++	132.7	2.55	134.3
Tomatinase	4.6	113	+	0.002	8,492.1	36.8

[a] Estimated by SDS-PAGE.

1993), the most closely related of which are BGL1 from *Trichoderma reesei* (Barnett et al. 1991) (60% similar and 45% identical amino acids), BGL1 (60% similarity and 41% identity), and BGL2 (59% similarity and 40% identity) from the yeast *Saccharomycopsis fibuligera* (Machida et al. 1988), and BGLS from *Candida pelliculosa* (Kohchi and Toh-e 1985) (61% similarity and 38% identity) (Fig. 3). In general these enzymes have been studied because of their importance in cellobiose degradation, and their effects (if any) on saponins have not been reported.

A highly conserved twin aspartic acid motif is implicated as the catalytic site for family 3 β-D-glucosidases by extrapolation from substrate analogue studies involving the β-D-glucosidase of *Aspergillus wentii*, although only one of these aspartic acid residues has been demonstrated directly to have a role (Bause and Legler 1980). These motifs are conserved in tomatinase and avenacinase (S/T-D-W and G-L-D-M, corresponding to amino acids 279–281 and 294–297 of tomatinase, respectively; the directly implicated aspartic acid residue is indicated by an asterisk in Fig. 3).

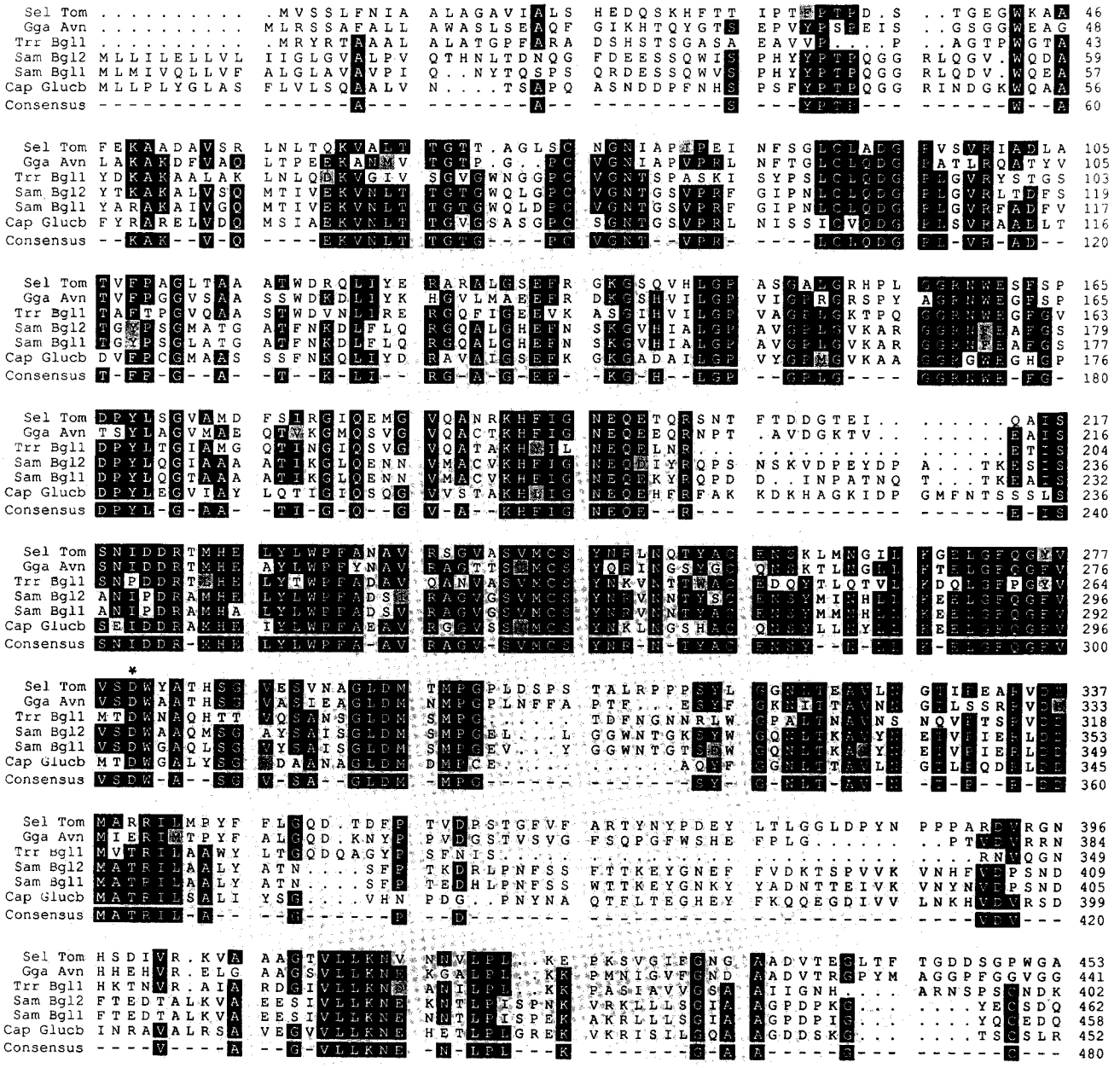

Fig. 3. Similarities between tomatinase, avenacinase, and other members of the family 3 glucosyl hydrolases. Sequences were aligned by using the algorithm PILEUP (Devereux et al. 1984). Residues found in the majority of sequences are highlighted in black. Conservative substitutions are indicated by the shaded boxes. The aspartic acid residue which has been directly implicated as the catalytic residue (Bause and Legler 1980) is marked with an asterisk. Abbreviations: Sel Tom, *S. lycopersici* tomatinase (GenBank accession number U35462); Gga Avn, *G. graminis* var. *avenae* avenacinase (GenBank accession number U35463); Trr BGL1, Sam BGL1, and BGL2 and Cap BGLS are β-glucosidases from *Trichoderma reesei*, *Saccharomycopsis fibuliger*a and *Candida pelliculosa* respectively (EMBL accession numbers U09580, M22475, M22476 and X02903). *(continued on next page)*

DISCUSSION

The saponin-detoxifying enzymes described here are produced by taxonomically distinct fungi with very different lifestyles; *G. graminis* var. *avenae* is a root-infecting pathogen of monocots, while *S. lycopersici* infects the leaves of tomato, a dicot. Also, the saponins that these fungi encounter are structurally distinct. Avenacin A-1 is a triterpenoid molecule with a trisaccharide moiety (Crombie et al. 1986b), and α-tomatine is a steroidal alkaloid with a tetrasaccharide moiety (Fontaine et al. 1951; Uhle and Moore 1954; Kühn et al. 1956) (Fig. 1). While in the past there has been considerable interest in both of these enzymes because of their ability to break down host plant saponins, a connection between the two had not been suspected. In fact, the mechanisms of action of avenacinase and tomatinase are similar, since both enzymes release a terminal β,1-2-D-glucose molecule from their respective saponin substrates (Arneson and Durbin 1967; Crombie et al. 1986a), and our experiments indicate that the two enzymes are closely related both at the DNA level, and in terms of their physicochemical and immunological properties. Detailed studies of the structure/function relationships of avenacinase and tomatinase are now under way to resolve the molecular basis of substrate specificity.

It has already been demonstrated that avenacinase is essential for *G. graminis* var. *avenae* to infect oats, and the enzyme can therefore be regarded as a determinant of host range (Bowyer et al. 1995). The results presented here indicate that a single form of tomatinase is present in culture filtrates of *S. lycopersici* and that this tomatinase is encoded by a single

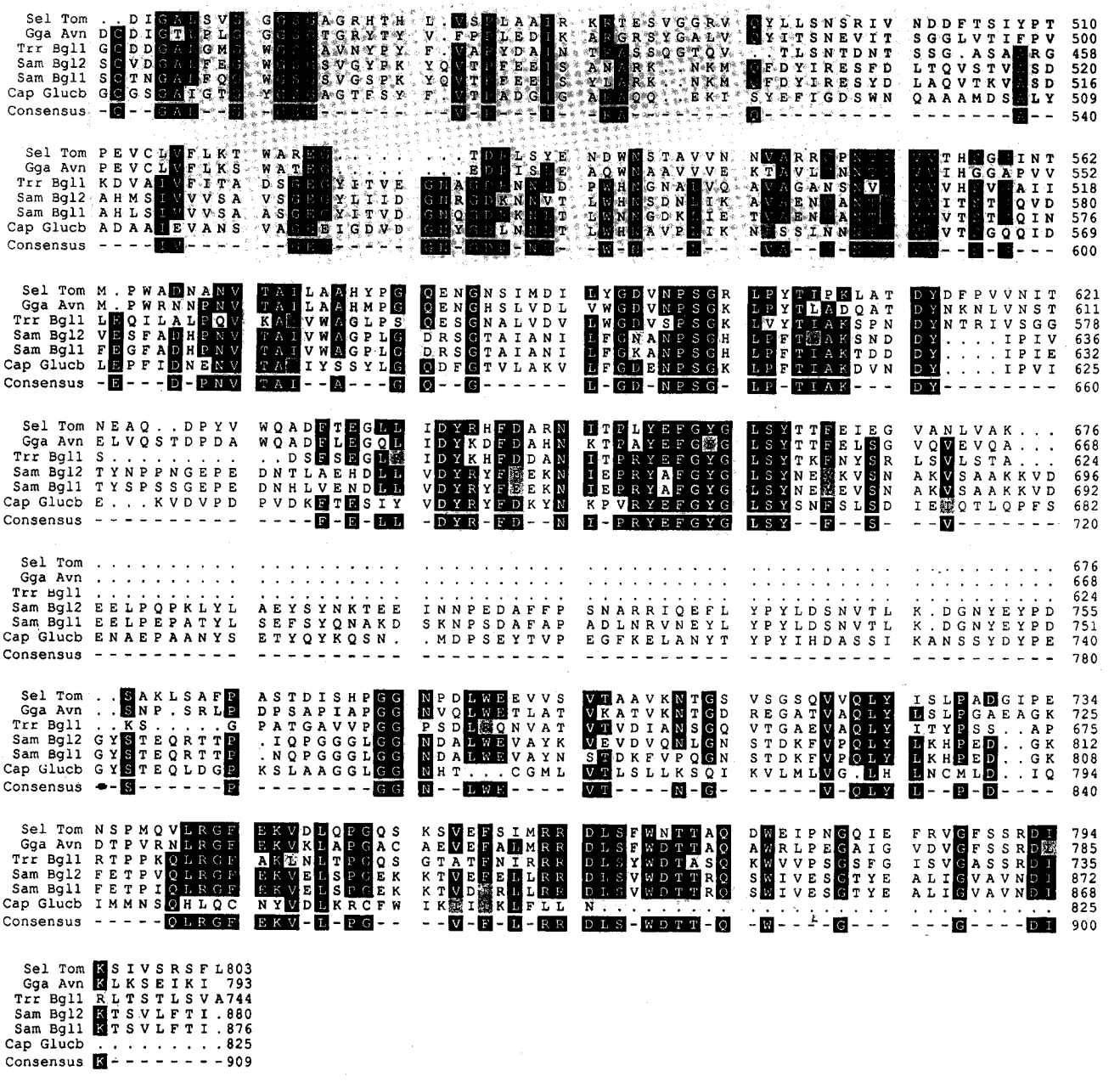

Fig. 3. *(continued from preceding page)*

gene. Now that the gene encoding tomatinase has been cloned the potential exists to address the question of the significance of tomatinase in determining the pathogenicity of *S. lycopersici* to tomato by generation of specific tomatinase-minus mutants of this fungus through targeted gene disruption (Oliver and Osbourn 1995). If studies on tomatinase suggest that saponin-degrading enzymes may have a more general role in pathogenicity, then these enzymes may become attractive targets for disease control strategies involving inhibitors. The extracellular location of the enzymes should facilitate approaches involving the use of chemicals or the expression of saponinase inhibitors in genetically engineered plants, since there should be no requirement for the inhibitors to penetrate the fungal hyphae.

Southern blot analysis indicates that DNA sequences which cross-hybridize to the cloned avenacinase and tomatinase genes are present in a diverse range of other fungi (P. Bowyer, G. Bryan, and A. Osbourn, unpublished results). These fungi include both plant pathogens and fungi which do not infect plants. The widespread distribution of such DNA sequences is probably not surprising, given that avenacinase and tomatinase are β-D-glucosidases belonging to the family 3 group of glycosyl hydrolases defined by Henrissat (Henrissat 1991; Henrissat and Bairoch 1993). However, avenacinase and tomatinase are more closely related to each other than either is to any of the other members of this family. It remains to be seen whether the two enzymes represent a subgroup within the family 3 β-D-glucosidases with the additional ability to deglucosylate saponins, or whether the other members of this family may also have saponin detoxifying activity.

The significance of related DNA sequences in other phytopathogenic fungi is unclear. It is likely that many of these sequences will encode family 3 β-D-glucosidases required solely for nutritional purposes and with no obvious role in saponin detoxification. However we have demonstrated here that, at least for *S. lycopersici*, the cross-hybridizing DNA sequences do encode a saponin-detoxifying enzyme (in this case, tomatinase). The possibility exists that the DNA probes described here could be used to clone genes encoding enzymes required for saponin detoxification from other plant pathogenic fungi. These may include not only those fungi which are known to detoxify α-tomatine and avenacin A-1, but also fungi for which the role of saponin detoxification in the disease process has not yet been addressed.

MATERIALS AND METHODS

Culture methods for *S. lycopersici*.

The *S. lycopersici* isolate used in this study was 353.49, provided by the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands. Colonies of *S. lycopersici* were stored on potato dextrose agar (PDA) slopes under mineral oil at 4°C, and were recovered by inoculating blocks of mycelium onto PDA and incubating in the dark at 22°C for approximately 14 days. Inoculum for liquid culture was prepared by scraping mycelium from fresh fungal colonies, and homogenizing in sterile water. Homogenate from one colony was used to inoculate 100 ml of liquid medium. Liquid cultures were incubated for 5 days at 22°C with shaking at 300 rpm. Cultures for DNA preparations were grown in potato dextrose broth (PDB), while those for mRNA preparation and tomatinase protein purification were grown in Czapek Dox liquid medium containing Casamino Acids (10 g/l^{-1}) (Durbin and Uchytil 1969).

Protein purification.

Liquid cultures of *S. lycopersici* were filtered through Miracloth (Calbiochem, La Jolla, CA). The following protease inhibitors were added to the filtrate to the indicated concentrations: phenylmethylsulphonyl fluoride (0.05 mM), EDTA (2 mM), benzamidine hydrochloride (1 μM), phenanthroline (0.5 μM), aprotinin (0.5 μM), leupeptin (2 μM) and pepstatin A (1.5 μM). The preparation was chilled to 4°C, and after 1 h proteins were precipitated by the addition of ammonium sulphate to 80% saturation as described in Osbourn et al. (1991). The dialyzed protein concentrate was then subjected to free-flow isoelectric focusing in the pH range 4 to 6.5 (Bowyer et al. 1995). A single peak of tomatinase activity with a pI of 4.6 was identified. Active fractions were pooled and exchanged into 20 mM sodium phosphate buffer (pH 6.2) (buffer A) using Centricon C-30 spin columns (Amicon, Beverly, MA). No tomatinase activity was lost through the column. The preparation was then applied to a TSK DEAE-5PW HPLC anion-exchange column (Tosohass, Montgomeryville, PA), which had previously been equilibrated with the same buffer. After application of the sample the column was washed with buffer A. All the activity bound to the column under these conditions. The tomatinase activity was then eluted with a linear gradient of 0 to 1 M sodium chloride in buffer A with a flow rate of 1 ml/min, and 1-ml fractions were collected. The tomatinase activity was eluted as a single peak at a salt concentration of 0.15 M. Active fractions were pooled and then fractionated by gel filtration using a 7.8 × 250 mm TSK G3000 SWXL HPLC column (Tosohass, Montgomeryville, PA) in buffer A containing 0.2 M sodium chloride. The flow rate was 1 ml/min and 0.2 ml fractions were collected. Tomatinase activity again eluted as a single peak. SDS-PAGE analysis of the active fractions indicated the presence of a single protein of molecular mass 113 kDa. Avenacinase was prepared from cultures of *G. graminis* var. *avenae* isolate A3 by essentially the same procedure (Bowyer et al. 1995).

Enzyme assays.

A 10 mM stock solution of α-tomatine was prepared by dissolving α-tomatine (Sigma) in 5% acetic acid, adjusting the pH to 5 with sodium hydroxide, and making up to a final volume with water. Tomatinase activity was routinely assayed by incubation of 2 to 50 μl of fractions with 250 μM α-tomatine in 100 mM sodium acetate buffer (pH 5) in a total volume of 1 ml. Assays were incubated at 37°C for 15 min to 1 h. Reactions were stopped by boiling for 10 min, and glucose release measured using the glucose oxidase assay (Sigma). Controls without α-tomatine were also included.

The specific activities of tomatinase and avenacinase towards α-tomatine and PNPG were measured using 5 mM substrate, and towards avenacin A-1 using 2 mM substrate. Tomatinase activity was assayed as described above, and avenacinase activity as described in Osbourn et al. (1991). Activity towards PNPG was assessed by release of *p*-nitrophenol (monitored at OD_{420nm}), after making the assays alkaline with potassium hydroxide. All assays were carried out in 100 mM sodium acetate buffer pH 5. Incubation times

ranged from 15 min to 6 h depending on activity, and activities were calculated as nmoles glucose released/min/mg protein.

Protein analysis.

Protein concentrations were determined using the Bio-Rad Protein assay, with lysozyme as a standard. SDS-PAGE was performed on 10 to 20% gradient gels (Bio-Rad, Richmond, CA) with molecular markers ranging from 29 to 205 kDa (Sigma MW-SDS-200 kit), and gels were stained with Coomassie blue or silver. Proteolytic cleavage of tomatinase was carried out using endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions, and peptides were transferred to Problott membrane (Applied Biosystems, Foster, City, CA) by electroblotting prior to determination of amino acid sequences. For Western blot analysis proteins were transferred to cellulose nitrate membrane (Schleicher & Schuell, Keene, NH). The filters were blocked with 5% bovine serum albumin in Tris-HCl buffered saline pH 7.2 before incubation with polyclonal antisera raised in rats against avenacinase (Bowyer et al. 1995) (diluted 1:2,000) or with preimmune serum (diluted 1:500). Rabbit antibody to rat immunoglobin G alkaline phosphatase conjugate (Sigma) was used as the secondary antibody.

cDNA library construction and screening.

RNA was prepared from mycelium from liquid cultures (200 ml) of *S. lycopersici* by the method of Chirgwin et al. (1979). Polyadenylated RNA was isolated from 1.5 mg of total RNA using the Pharmacia mRNA Purification Kit. cDNA was synthesized with the Pharmacia Timesaver cDNA Synthesis Kit using oligo (dT) primers, and ligated into the vector lambda ZAP II (predigested with *Eco*RI and phosphatase-treated) following the manufacturer's instructions (Stratagene, La Jolla, CA). The ligated DNA was packaged using Gigapack II Gold packaging extract, and plated on *Escherichia coli* strain XL1 Blue (Stratagene). Plaques were transferred to Hybond N filters (Amersham, UK), UV-cross-linked, and screened with a 1.1-kb fragment of avenacinase cDNA from pA312 (Bowyer et al. 1995), which was made radioactive using the Megaprime DNA Labeling System (Amersham, UK). Hybridisations were carried out at 55°C in 6×SSC, 0.5% SDS, 5× Denhardt's solution, and filters were washed in 2×SSC/0.1% SDS at 60°C. 1×SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0. Positive plaques were purified by replating and a second round of screening, and phagemids were excised from lambda ZAP II following the Stratagene instructions.

Nucleic acid methods.

Fungal DNA was isolated from *S. lycopersici* and *G. graminis* var. *avenae* by the methods of Dyer et al (1993) and Raeder and Broda (1985), respectively. Isolation of plasmid DNA, cloning, transformation and gel analysis of plasmids were by established procedures as described in Sambrook et al. (1989). The *E. coli* strain used for plasmid maintenance and DNA preparation was XL1-Blue (Stratagene, La Jolla, CA). Plasmid DNA for nucleotide sequence analysis was prepared using Plasmid Mini Kits (Qiagen, Chatsworth, CA). The complete nucleotide sequences of both strands of pCTOM6 were obtained by sequencing restriction fragments that had been subcloned into pBluescript II SK(+) (Stratagene) or pGEM-5Zf(–) (Promega). The nucleotide sequences spanning restriction enzyme sites used for subcloning were determined by sequence analysis of overlapping restriction fragments or by the use of specific primers. Dyedeoxy terminator cycle sequencing and the Model 373A DNA sequencing system (Applied Biosystems) were used. DNA and amino acid sequence data were analyzed by algorithms of the University of Wisconsin Computer Group package 7.2 (Devereux et al. 1984).

For Southern blot analysis DNA was transferred to Hybond N membrane (Amersham) according to the manufacturer's instructions. Hybridizations with homologous probes were carried out at 65°C, and the filters washed in 1× SSC/0.1% SDS with the final wash at 65°C, while hybridization with heterologous probes was carried out at 55°C and washed at 55 to 60°C with 2× SSC/0.1% SDS.

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