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Analysis of a secreted aspartic peptidase disruption mutant of *Glomerella cingulata*

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Abstract

Peptidases have been implicated in the pathogenicity of fungi that cause disease in plants. Expression of the secreted aspartic peptidase gene (*gcsap*), of a *Glomerella cingulata* isolate pathogenic on apples, is induced during appressorium formation. To determine whether the secreted aspartic peptidase (GcSAP) is essential to pathogenicity, *gcsap* was disrupted using a vector containing a 637 bp fragment of genomic DNA that encodes the sequence spanning the two active site aspartic acid (Asp) residues. To ensure that the truncated *gcsap* gene products could not have residual peptidase activity the codons for the active site residues Asp^{112} and Asp^{297} were both mutated to histidine residues. Both PCR and Southern analysis confirmed disruption of *gcsap*. Neither *gcsap* mRNA nor GcSAP activity was detected in the disruption mutant. Pathogenicity tests on fruit from three apple cultivars showed that GcSAP was not required for pathogenicity. The disruption mutant grew on medium containing protein as the sole source of nitrogen because *G. cingulata* secretes a previously undetected peptidase(s). A serine peptidase that had a pH optimum between pH 7.0 and 8.0 and a K_m of 0.25 mM for the synthetic substrate succinyl-Ala–Ala–Pro–Phe-*p*-nitroanilide was identified.

Abbreviations: DIG – Digoxigenin; GcSAP – the *Glomerella cingulata* secreted aspartic peptidase; *gcsap* – the gene encoding GcSAP.

Introduction

Several lines of evidence suggest a possible role for fungal peptidases in the pathogenicity of phytopathogenic fungi. Proteins are structural and functional components of plant cell walls (Showalter, 1993) and plasma membranes (Lebrun-Garcia et al., 1999). Phytopathogenic fungi secrete peptidases *in vitro* and *in planta* (Kuć and Williams, 1962; Movahedi and Heale, 1990a; Carlile et al., 2000; Poussereau et al., 2001) and there is a correlation between levels of peptidase activity and virulence of *Botrytis cinerea* (Movahedi and Heale, 1990b). Many plants produce peptidase inhibitors (Ryan, 1990; Christeller et al., 1998; Joshi et al., 1998) that are actively expressed in response to fungal infection (Roby et al., 1987; Cordero et al., 1994; Pernas et al., 2000). In cucurbits there are high levels of peptidase inhibitors in unripe fruit. The levels of these inhibitors decrease as the fruit

ripen and this correlates with an increased susceptibility to fungal attack. Complementation of a cysteine peptidase mutant of *Pyrenopeziza brassicae* with a single cosmid clone encoding the peptidase from a genomic library of *P. brassicae* restored pathogenicity to the non-pathogenic mutant (Ball et al., 1991). A non-pathogenic peptidase deficient mutant of *Colletotrichum coccodes* has been generated by chemical mutagenesis (Redman and Rodriguez, 2002) but complementation of this mutation has not been attempted.

Gene disruption has been used to analyze the role of several different putative pathogenicity genes in a number of fungi (e.g. Froeliger and Carpenter, 1996; van Kan et al., 1997; Scott-Craig et al., 1998; Redman et al., 1999; Rogers et al., 2000; Yakoby et al., 2001). However, analysis of single gene disruption mutants is often complicated by the existence of a family of enzymes all having the same enzymatic activity. Disruption of one member of the family results in a reduction of the total measurable activity but often no detectable difference in pathogenicity (Gorlach et al., 1998).

Among plant pathogens, peptidase gene families have been demonstrated in *Magnaporthe poae* (Sreedhar et al., 1999), *Cryphonectria parasitica* (Jara et al., 1996; Razanamparany et al., 1992) and *Cochliobolus carbonum* (Murphy and Walton, 1996). The requirement for peptidase activity in pathogenicity was examined using gene disruption in *C. carbonum* (Murphy and Walton, 1996) and *Fusarium oxysporum* (Di Pietro et al., 2001) and using gene deletion in *Stagonospora nodorum* (Bindschedler et al., 2003). *C. carbonum* secretes three serine peptidases and disruption of the single gene encoding two of these enzymes (Alp1a and Alp1b, both products of the gene *ALP1*) showed that this gene was not required for pathogenicity. However, unaltered secretion of the third peptidase prevented assessment of the significance of peptidase activity *per se* in pathogenicity. Similar results were reported for both *S. nodorum* and *F. oxysporum* and therefore the role of peptidases in pathogenicity remains unresolved.

The plant pathogenic fungus *Glomerella cingulata* (anamorph. *Colletotrichum gloeosporioides*) causes anthracnose disease of a variety of crops (e.g. apple, tomato, capsicum and pumpkin) (Mordue, 1971; Irwin and Cameron, 1978). Once the cuticle has been penetrated *G. cingulata* secretes an array of plant cell wall degrading enzymes which function synergistically in the extraction of nutrients and to facilitate ramification of fungal hyphae through host tissue. In liquid culture containing protein as the sole nitrogen source this fungus secretes a single aspartic peptidase (GcSAP) (Clark et al., 1997). Furthermore, no other peptidase activity was detected during the purification of the GcSAP and Southern blot analysis demonstrated that no closely related genes were present (Clark et al., 1997). *G. cingulata* appeared, therefore, to be a suitable organism in which to test, by a single gene disruption, the role of peptidase activity in pathogenicity. However, we report here that the disruption of *gcsap* revealed a previously unknown secreted serine peptidase. The pathogenicity of the *gcsap* disruption mutant is indistinguishable from the wild type.

Materials and methods

Fungal isolates

Gomerella cingulata strain ICMP11016, isolated from apple (cv. Granny Smith, single spore #824) (Templeton et al., 1992), was used to create the disruption mutant. Both the wild type and the disruption mutant were cultured as described in Rikkerink et al. (1994). For analyses of growth and peptidase production, cultures were grown as described in Clark et al. (1997) with either NH_4Cl (0.5% w/v), bovine serum albumin (0.2% w/v) or casein hydrolysate (2% w/v) as the nitrogen source. Cultures (50 ml) were inoculated with 8×10^6 conidia and incubated with shaking at 28 °C for 48 h.

Vector construction and fungal transformation

To create the truncation vector (pSJ9) an internal fragment of the *gcsap* gene (residues 331–967) was amplified by PCR using the primers 5′-CGCGGATCC-GCGCTCGAGTTCCACACCGGATCTTCCG-3′ and 5′-CGCGCTCGAGGCGGATCCTCCGGTGTGAG-CGATGCCG-3′ and a cloned genomic DNA fragment as template (Clark et al., 1997). These primers each contain a point mutation in one of the two triplets encoding catalytically essential aspartic acid residues and introduce flanking *Bam*HI restriction enzyme recognition sites. The *Bam*HI digested PCR product was cloned into the *Bgl*II site of pAN7-1 (Punt et al., 1987) to create pSJ9 using standard protocols (Sambrook et al., 1989).

The production of competent *G. cingulata* protoplasts and their subsequent transformation has been

described previously (Rikkerink et al., 1994). Genetic homogeneity of transformants was ensured by isolation of single conidia. Retention of antibiotic resistance in transformants was monitored after repeated subculturing of transformants on a non-selective medium (potato dextrose agar).

DNA isolation and Southern analysis

DNA was isolated as described previously (Al-Samarrai and Schmid, 2000) and digested with *Sal*I. Standard protocols were followed for gel electrophoresis and Southern blotting (Sambrook et al., 1989). A DIG-labeled 1.0 kb *gcsap* fragment was used as the probe. The PCR-based cloning of this *gcsap* fragment, using primers SAP-primer-1 and SAP-primer-3, was described previously (Clark et al., 1997). The plasmid containing this clone was digested with *Kpn*I and the probe was then gel purified and labeled (DIG–High Prime DNA Labeling and Detection Kit, Roche). Hybridization and washing (0.5× SSC, 0.1% SDS) of the membrane was performed at 56 °C according to the DIG System User's Guide (Roche) and the chemiluminescence detected using X-ray film. The 1 kb DNA Ladder (GibcoBRL) was used as a size standard and in addition, the unlabeled 1.0 kb gene fragment and two linear plasmids (3.9 and 7.4 kb), both containing *gcsap*, were blotted as standards onto the membrane.

PCR analysis of the G. cingulata *transformants*

DNA for the PCR analysis was prepared from lyophilized mycelium (Rodriguez, 1993). Two primer sets were used to analyze the transformants. The primers SAP-primer-1 and SAP-primer-3 (Clark et al., 1997) were designed to amplify a 1.0 kb fragment of the wild type *gcsap*. Homologous integration at the *gcsap* locus with disruption of *gcsap* would result in amplification of a 9 kb product. Ectopic integration events, that did not disrupt *gcsap*, would result in amplification of the 1.0 kb fragment. Reaction mixtures contained 1.5 units of the Expand Long Template PCR (Roche) enzyme mix (containing *Taq* and *Pwo* DNA polymerases), 2.25 mM $MgCl_2$, 1 pmol of each primer, 200 μM each of dATP, dGTP, dCTP and dTTP, 50 ng genomic DNA and reaction buffer in a total volume of 25 μl. The reaction was initiated with denaturation for 1 min at 92 °C followed by 35 cycles of denaturation for 10 s at 92 °C, annealing at 63 °C for 30 s, elongation at 68 °C for 8 min and an additional incubation at 68 °C for 7 min at cycle 35.

The primers SAP-primer-1 and #39/1 (5′-GTTTGATGATTTCAGTAACG-3′), complementary to the *Aspergillus nidulans trpC* transcription termination sequence flanking the hygromycin resistance gene in pSJ9, were designed to amplify a 4 kb fragment, including the recombination junction, in transformants where homologous integration had disrupted *gcsap*. No products would be expected in the wild type or transformants resulting from ectopic integration events. Reaction mixtures contained 1 unit of *Taq* polymerase (Roche), 1 pmol of each primer, 200 μM each of dATP, dGTP, dCTP and dTTP, 50 ng genomic DNA and reaction buffer in a total volume of 20 μl. The reaction was initiated with denaturation for 3 min at 92 °C followed by 34 cycles of denaturation for 1 min at 92 °C, annealing at 65 °C for 90 s, elongation at 72 °C for 2 min and an additional incubation at 72 °C for 7 min at cycle 34.

Appressorium induction

Apple wax and spore suspensions were prepared as described previously (Al-Samarrai et al., 2002). About 7×10^7 spores in 70 ml of sterile water were applied to wax covered glass Petri dishes (10 × 150 mm) and incubated at 27 °C. Under these conditions the spores germinated and formed an appressorium. Appressoria began to appear after 3 h incubation. At 5.5 h, 90% of the spores had germinated and 70% formed an appressorium. By 24 h, 90% of the spores had germinated and formed a melanised appressorium. Germination without appressorium formation was induced in the same manner except that the spores were resuspended in 1% (w/v) yeast extract instead of water (Al-Samarrai et al., 2002).

RNA isolation and Northern blot analysis

Washed spores were resuspended in 300 μl of 4 M guanidine isothiocyanate containing 25 mM sodium citrate, 0.5% (w/v) sodium lauryl sarcosine and 0.3% (v/v) β-mercaptoethanol. Glass beads (200 μl), 2 M sodium acetate pH 4.0 (25 μl), water equilibrated phenol (150 μl) and chloroform/isoamyl alcohol (24 : 1) (150 μl) were added and the mixture vortexed for 2 min to disrupt the spores. Cellular debris was removed by centrifugation and the aqueous phase extracted twice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) (300 μl) and once with chloroform/isoamyl alcohol

(24 : 1) (300 µl). The RNA was precipitated with an equal volume of isopropanol, washed with 70% ethanol and dissolved in 20 µl of sterile water.

Germinating or appressorium forming spores on wax covered Petri dishes were washed with sterile water and covered with 5 M guanidine isothiocyanate containing 50 mM sodium citrate pH 7.0, 1% (w/v) sodium lauryl sarcosine and 1% (v/v) β-mercaptoethanol (6 ml) for 2–4 min. The buffer was removed and the Petri dish flooded with liquid nitrogen. After this had evaporated, the frozen wax was transferred to an Eppendorf tube, thawed and incubated on ice for 2 min. After the addition of glass beads (200 µl), 2 M sodium acetate pH 4.0 (50 µl), water equilibrated phenol (250 µl) and chloroform/isoamyl alcohol (24 : 1) (100 µl), the mixture was vortexed for 2 min to disrupt the cells. Cell debris was removed, the aqueous phase extracted, RNA precipitated and redissolved in sterile water as described above. Mycelial RNA was prepared as described previously (Clark et al., 1997).

For Northern blot analysis the RNA (about 10 µg) was fractionated in formaldehyde agarose gels and transferred to Hybond N+ membranes using standard protocols (Sambrook et al., 1989). The probe, a 1.2 kb *gcsap* fragment, was prepared by *Kpn*I digestion of pSJ1 (Clark et al., 1997), gel purification of the 1.2 kb fragment and labeling with [α-^{32}P]-dCTP using standard protocols (Sambrook et al., 1989). Membranes were hybridized with the probe in Church and Gilbert solution (Church and Gilbert, 1984) at 65 °C for 16 h, washed twice with 2× SSC containing 0.5% (w/v) SDS at room temperature for 15 min and then twice at 65 °C for 30 min. Northern blots were quantified and signals normalized to the ethidium bromide stained 18S rRNA band using a BioRad model GS-670 densitometer and the Molecular Analyst software package.

Peptidase assays

The GcSAP was assayed, as described in Clark et al. (1997), at pH 3.2 with haemoglobin as the substrate. The haemoglobin assay was also adapted, by using 50 mM sodium phosphate buffer pH 7.0 and extending the incubation time to 120 min, for determination of the secreted serine peptidase activity. However, routine assays of the secreted serine peptidase were performed using succinyl–Ala–Ala–Pro–Phe-*p*-nitroanilide (DelMar et al., 1979) as substrate. These assays contained 1.6 mM substrate (10 µl in DMSO) in 1 ml of 0.1 M Tris/HCl buffer pH 8.0. The absorbance at 410 nm was monitored continuously and one unit of peptidase activity was defined as the amount of enzyme that released 1 µmol of *p*-nitroanilide min^{-1}. The peptidase inhibitors pepstatin and phenylmethylsulphonylfluoride (PMSF) were prepared as stock solutions in ethanol and used at final concentrations of 1 µM and 5 mM, respectively.

Pathogenicity assays

The pathogenicity of the disruption mutant was compared with that of the wild type isolate on ripe fruit of three cultivars of apple (*Malus* X *domestica*, cv.'s Granny Smith, Royal Gala and Splendour). Apple fruit were surface sterilized by immersion in 5% sodium hypochlorite for 10 min and were then rinsed five times in sterile distilled water. The sides of fruit of each cultivar were inoculated with 2 µl of a spore suspension (10^6 spores ml^{-1}). Sterile water was used for control inoculations. Positioning of the inoculum was randomized. The surface of the fruit was then pierced through the droplet to a depth of 2 mm using a sterile 0.9 mm gauge needle. The inoculated fruit were incubated at 22 °C in the dark in a humid atmosphere. At the end of the incubation period the apples were assessed visually for symptom development and lesion diameters were measured. As lesions were sometimes slightly asymmetrical, the largest diameter was recorded. Data were analyzed by analysis of variance (ANOVA) or by Student's *t*-test.

Results

Expression of gcsap *in wild type* G. cingulata

Expression of *gcsap* was examined in conidia, during germination of conidia and during appressorium formation induced on apple wax. No *gcsap* transcript was detectable in RNA prepared from *G. cingulata* conidia (Figure 1 (lane 1)). The *gcsap* gene was expressed during appressorium formation (Figure 1 (lanes 6–9)) but not in control incubations in which conidia were allowed to germinate but not differentiate to form appressoria (Figure 1 (lanes 2–5)). In this *in vitro* induction system germination began at about 2 h, by 4 h about 60% of the conidia had formed an appressorium and melanisation of the appressoria was evident at 5.5 h. After 24 h incubation 90% of the conidia had germinated and formed a melanised appressorium. A longer *gcsap* transcript was evident in conidia

differentiating to form appressoria and this is currently under investigation (P.C. Farley, unpubl. data).

Disruption of gcsap

An established disruption strategy was adopted (Bowen et al., 1995). A heterologous truncation vector was constructed in which a 637 bp fragment from the coding sequence of *gcsap* was placed adjacent to the hygromycin B resistance gene from *E. coli*, the expression of which was controlled by heterologous (*A. nidulans*) promoter and terminator sequences. Homologous recombination at the *gcsap* locus would generate two truncated copies of *gcsap* (Figure 2). These two truncated genes correspond to residues Met[1]–Gly[298] and Phe[111]–Ser[407], respectively. Therefore, as an additional precaution, to ensure that any protein produced from the truncated *gcsap* genes would be inactive, the *gcsap* fragment was altered by site-directed mutagenesis so that the codons encoding the two essential catalytic residues (Asp[112] and Asp[297]) were replaced by codons encoding His residues. PCR analysis of 21 transformants identified one transformant (#1) in which a single homologous recombination event had occurred at the *gcsap* locus. A number of ectopic transformants were detected and one of these (#7) was retained for use as a control in the pathogenicity analysis.

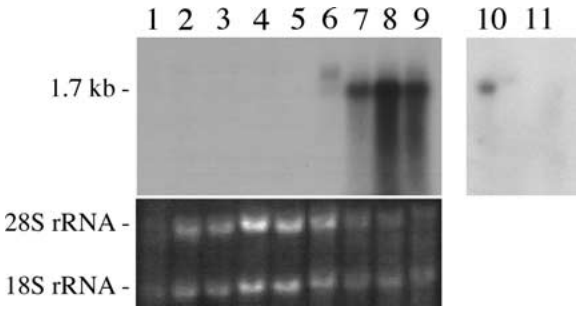

Figure 1. Northern analysis of *gcsap* expression. Total RNA, extracted from conidia (lane 1), conidia forming germ-tubes (for 2, 4, 7.5 and 16 h; lanes 2–5), conidia forming appressoria (for 2, 4, 7.5 and 16 h; lanes 6–9) or mycelium (lanes 10–11), was fractionated in formaldehyde agarose gels, transferred to a Hybond N+ membrane and probed with the 1.2 kb fragment of *gcsap* (Clark et al., 1997). The bottom panel shows the ethidium bromide stained rRNA. Mycelium of the wild type (lane 10) and *gcsap* disruption mutant (lane 11) was grown on medium containing protein as the sole nitrogen source. Approximately equal amounts of RNA were loaded (lanes 10 and 11) based on ethidium bromide staining of the rRNA (not shown).

Southern blot analysis (Figure 3) of the *G. cingulata* wild type DNA was consistent with the restriction map of *gcsap* and known flanking sequences and with previous analysis of wild type genomic DNA (Clark et al., 1997). The single hybridizing band was 4.0 kb in size. In the disruption mutant (#1) two bands consistent with the expected values (2.7 and 8.5 kb) hybridized to the probe indicating integration of the disruption vector by homologous recombination at the *gcsap* locus. The absence of other hybridizing bands indicated that neither tandem integration (which would be expected to generate a hybridizing band of 7.4 kb) nor secondary ectopic integration events had occurred. PCR

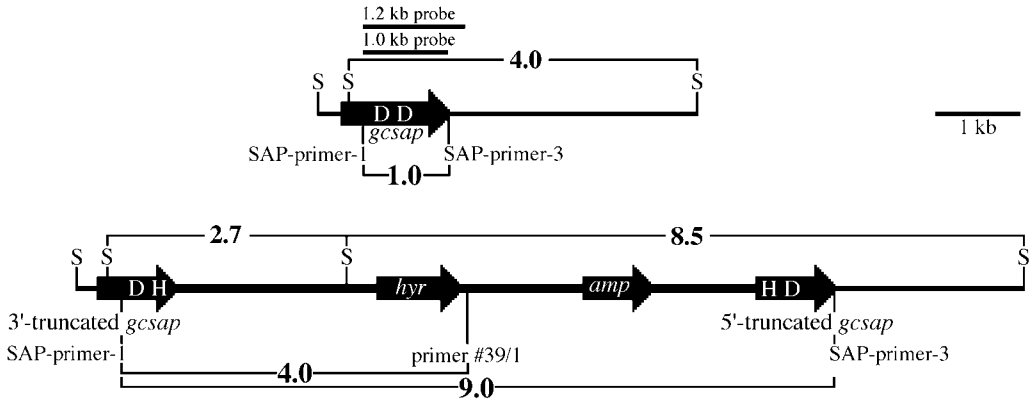

Figure 2. Genomic DNA maps. The *gcsap* locus of the wild type (top map) and the *gcsap* disruption mutant (bottom map) showing the location of the *Sal*I recognition sites (S), primer annealing sites, fragments used as probes, open reading frames (*hyr*, hygromycin phosphotransferase; *amp*, β-lactamase) and the catalytic Asp residues (D) and the His residues (H) that replace these in the truncated *gcsap* sequences. The sizes of the *Sal*I fragments and the PCR products are shown (in kb).

analysis using either primers to *gcsap* and the vector-derived *A. nidulans* translation termination sequence or *gcsap* specific primers confirmed this result (data not shown).

Further evidence that *gcsap* was disrupted comes from Northern analysis of RNA and analysis of GcSAP activity in the culture supernatants of the wild type and the disruption mutant (#1). Whereas aspartic peptidase activity was present in culture supernatants of the wild type, no aspartic peptidase activity was detected in culture supernatants from the *gcsap* disruption mutant (Table 1 (compare activity at pH 3.2)). In agreement with this result no *gcsap* mRNA was detected in the disruption mutant (#1) grown under conditions that induced *gcsap* expression in the wild type (Figure 1, lanes 10 and 11). No truncated transcript was detected on the Northern blot of RNA from the disruption mutant (Figure 1, lane 11).

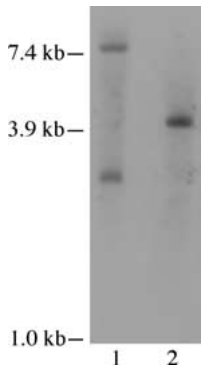

Figure 3. Southern blot analysis of the *gcsap* disruption mutant (#1) and wild type *G. cingulata*. *Sal*I digests of genomic DNA (5 μg) from the disruption mutant (lane 1) and wild type strain (lane 2) were probed with the 1.0 kb *gcsap* fragment (Clark et al., 1997).

Pathogenicity of the gcsap *disruption mutant*

The *gcsap* disruption mutant was able to infect undamaged fruit but the variation between replicates was too large for a quantitative comparison of pathogenicity (data not shown). The fruit were therefore pierced at the inoculation site in subsequent tests. No statistically significant difference ($P > 0.05$ ANOVA) in pathogenicity was observed between the conidia of the wild type, the *gcsap* disruption mutant (#1) and an ectopic transformant (#7) (Table 2, Experiment 1). An identical result was obtained in a second experiment with fruit from three different cultivars of apple. In this experiment, however, inoculation did not always result in lesion formation. This was true for all strains, that is, for wild type and both transformants, and resulted in large standard deviations for some data points (Table 2, Experiment 2). Nevertheless, Splendour apples were significantly less susceptible ($P < 0.01$ Student's *t*-test) than either the Granny Smith or Royal Gala cultivars. The susceptibility of Granny Smith apples to infection was expected since the wild type was originally isolated from Granny Smith fruit. Lesions had a similar appearance for all inoculation sites. No difference in extent of sporulation was observed and the disruption mutant formed appressoria.

Growth and peptidase secretion by the gcsap *disruption mutant*

Growth rates of the disruption mutant and the wild type were comparable on both liquid minimal medium (Figure 4) and on minimal agar medium containing ammonium chloride as nitrogen source (0.14 ± 0.01 and $0.12 \pm 0.02\,\text{mm}\,\text{h}^{-1}$, respectively; $n = 5$, $P > 0.05$ Student's *t*-test). Growth rates were also comparable on medium containing casein hydrolysate

Table 1. Peptidase activity in culture supernatants after growth in medium containing bovine serum albumin as the sole source of nitrogen

Strain	Peptidase activity[1] using haemoglobin (units g^{-1} mycelium)					Peptidase activity[2] using succinyl-Ala–Ala–Pro–Phe-*p*-nitroanilide (units g^{-1} mycelium)
	pH 3.2		pH 7.0			
	No addition	Pepstatin	No addition	Pepstatin	PMSF	
Wild type	1.9	0.2	1.2	ND[3]	1.0	0.30 ± 0.03 (6)
gcsap disruption mutant	0	ND	0.7	0.7	0.1	1.83 ± 0.83 (7)

[1]Values are the means of duplicate assays using the supernatants from 48 h cultures, with or without the addition of specific peptidase inhibitors.
[2]Values are means (±standard error of the mean) of the number of experiments shown in parenthesis. The difference is not statistically significant ($P > 0.05$ Student's *t*-test).
[3]Not determined.

Table 2. Pathogenicity analysis on apple fruit

Treatment	Lesion diameter (mm)[1]			
	Experiment 1[2]	Experiment 2[2]		
	cv. Granny Smith	cv. Granny Smith	cv. Royal Gala	cv. Splendour
Wild type	40.5 (±3.3, 6)	29.2 (±14.4, 6)	19.5 (±9.6, 4)	10.2 (±5.4, 6)
gcsap disruption mutant (#1)	39.0 (±4.6, 6)	34.3 (±5.6, 6)	30.5 (±8.9, 4)	4.8 (± 7.5, 6)
Ectopic transformant (#7)	39.3 (±3.3, 6)	27.2 (±13.7, 6)	24.3 (±16.5, 4)	10.2 (±7.9, 6)
Water	0	0	0	0

[1]Average diameter (±standard deviation, number of replicates).
[2]Symptoms were measured 12 days post-inoculation in Experiment 1 and 11 days post-inoculation in Experiment 2.

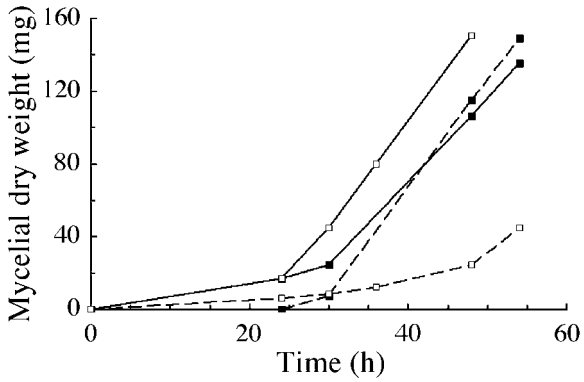

Figure 4. Growth of the wild type and *gcsap* disruption mutant (#1). The wild type (■) and *gcsap* disruption mutant (□) were grown on either minimal medium containing NH_4Cl as nitrogen source (—) or on minimal medium containing bovine serum albumin as sole nitrogen source (- - -). Growth of mycelium is expressed as dry weight (mg) per culture (50 ml).

as the nitrogen source, a semi-synthetic medium and potato dextrose agar (data not shown). On medium containing bovine serum albumin as the sole nitrogen source, growth of the mutant was slower than the wild type in both liquid medium (Figure 4) and on agar (0.35 ± 0.02 and 0.46 ± 0.02 mm h^{-1}, respectively; $n = 5$, $P < 0.001$ Student's t-test). Growth of the *gcsap* disruption mutant (#1) on medium containing protein as the sole source of nitrogen suggested that it was able to secrete peptidase(s) into the growth medium. That the secreted peptidase(s) were able to hydrolyze bovine serum albumin was demonstrated by the production of a clear halo around the colony growing on agar plates containing bovine serum albumin as the sole source of nitrogen.

Peptidase activity in the culture supernatants was assessed at pH 3.2 (the pH optimum for the GcSAP) and pH 7.0 using denatured haemoglobin as substrate (Table 1). Using pepstatin, which specifically inhibits aspartic peptidases, and PMSF, which specifically inhibits serine peptidases, the disruption mutant was shown to secrete a serine peptidase. The data obtained with the wild type were consistent with the production of GcSAP. The activity was higher at pH 3.2 than pH 7.0 and was inhibited by pepstatin. Furthermore, the activity detected at pH 7.0 was 63% of that measured at pH 3.2 which is consistent with the known pH activity profile of this enzyme (Clark et al., 1997). In contrast, the *gcsap* disruption mutant secreted peptidase that was active at pH 7.0 but not pH 3.2 and that was inhibited by PMSF but not pepstatin. The *gcsap* disruption mutant was therefore secreting a serine peptidase that, with haemoglobin as substrate, was not detectable in wild type cultures. Similar results were obtained with samples taken at other time points (data not shown).

Characterization of the serine peptidase activity

The serine peptidase activity could be detected with azocoll as substrate and activity could be quantitated using either haemoglobin or the synthetic substrate succinyl-Ala–Ala–Pro–Phe-*p*-nitroanilide. In each case the activity was inhibited by PMSF but not by pepstatin. The activity was detected in culture supernatants from mycelium grown on bovine serum albumin as the sole nitrogen source but not in culture supernatants from mycelium grown on minimal salts medium. On medium containing protein as sole nitrogen source, the specific activity of the serine peptidase produced by the *gcsap* disruption mutant (#1) was not statistically different to that of the wild type strain (Table 1).

The serine peptidase from culture supernatants of the disruption mutant was stable at 4 °C and pH 6.0 for a month and at 40 °C and pH 6.0 for 4 h, at higher

temperatures the enzyme was rapidly inactivated. It had optimal activity against succinyl-Ala–Ala–Pro–Phe–*p*-nitroanilide between pH 7.0 and 8.0 and a K_m for this substrate of 0.25 mM. The pH optimum is similar to that reported for the serine peptidase from *C. coccodes* (Redman and Rodriguez, 2002) whereas the K_m is lower than values reported for other fungal serine peptidases with this synthetic substrate (Iida et al., 1988; Apodaca and McKerrow, 1989; Larcher et al., 1992; 1996; Pekkarinen et al., 2002). The activity of the serine peptidase against a number of synthetic substrates was determined. Enzyme activity relative to succinyl-Ala–Ala–Pro–Phe-*p*-nitroanilide (100%) was 52% with Ala–Ala–Val–Ala-*p*-nitroanilide, 13% with succinyl-Ala–Ala–Ala-*p*-nitroanilide, 8% with Lys–*p*-nitroanilide and 8% with Ala-*p*-nitroanilide. In addition, the following substrates were not hydrolyzed: Glu-*p*-nitroanilide, Leu-*p*-nitroanilide, carbobenzoxy-Gly–Gly–Leu-*p*-nitroanilide, Arg-*p*-nitroanilide, succinyl-Ala–Ala–Val-*p*-nitroanilide.

Discussion

Glomerella cingulata was considered an appropriate candidate for the analysis of the role of secreted peptidases during infection because the organism appeared to secrete a single aspartic peptidase (Clark et al., 1997). Furthermore, the gene encoding the aspartic peptidase was transcribed during appressorium formation but not during germination of conidia in the absence of appressorium development. A gene disruption strategy was adopted because, in *G. cingulata*, gene deletion was invariably accompanied by ectopic integration events (Bowen et al., 1995).

Disruption of *gcsap* was achieved as evidenced by Southern and Northern blot analysis, PCR analysis and peptidase activity assays. However, only one of the 21 transformants analyzed (less than 5%) arose from a homologous recombination event at the *gcsap* locus. Higher frequencies have been observed for homologous recombination at other loci: 95% at the *gpdA* promoter (Rikkerink et al., 1994) and 55% at the *pnlA* locus (Bowen et al., 1995). The results obtained with the *gcsap* disruption are further evidence for a strong positional effect on the frequency of homologous recombination in *G. cingulata* (Bowen et al., 1995).

GcSAP was not required for pathogenicity on a variety of apple hosts. When spores were punched through the skin, this may be because appressorium formation was not induced and therefore *gcsap* was not expressed. However, the *gcsap* disruption mutant was able to infect undamaged fruit and therefore GcSAP was not required for penetration of the apple epidermis. Neither was GcSAP required for appressorium formation *in vitro*. Surprisingly, the *gcsap* disruption mutant was able to grow on medium containing protein as sole nitrogen source. This suggested that the mutant was secreting peptidase(s) into the medium. Analysis of the culture filtrates confirmed the absence of any GcSAP activity but revealed the presence of a secreted serine peptidase that was most active at slightly alkaline pH and towards a synthetic chymotrypsin substrate (DelMar et al., 1979). The serine peptidase was secreted by the wild type strain but its presence had not been detected earlier because its contribution to protein degradation in culture filtrates of the wild type was not significant compared to the activity of the aspartic peptidase. That it was expressed in the wild type was confirmed using a specific synthetic substrate. The specific activity of the serine peptidase, in cultures of the *gcsap* disruption mutant, was not significantly higher than in cultures of the wild type. Therefore, whereas in *S. nodorum* it has been suggested that protoplast formation and selection of transformants might have caused the increased peptidase activity seen in ectopic integrants (Bindschedler et al., 2003), there is no evidence for a second-site mutation affecting expression of the serine peptidase in the *gcsap* disruption mutant. It remains to be established whether or not a gene family is responsible for the serine peptidase activity.

Deletion of a peptidase gene has also revealed the existence of otherwise undetected peptidase activity in *Aspergillus flavus* (Ramesh and Kolattukudy, 1996) and *S. nodorum* (Bindschedler et al., 2003). We have demonstrated that GcSAP is not required for pathogenicity in *G. cingulata*, but expression of the serine peptidase complicates analysis of the role of peptidases *per se* in pathogenicity which will now require disruption of both peptidase genes.

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