The Disruption of a $G\alpha$ Subunit Sheds New Light on the Pathogenicity of *Stagonospora nodorum* on Wheat

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***Gna1*, a gene encoding a $G\alpha$ subunit, a key component of signal transduction pathways, has been cloned and characterized in the wheat pathogen *Stagonospora nodorum*. Analysis of *Gna1* expression during infection revealed a slight decrease in transcript levels shortly after germination, after which levels steadily increased until sporulation. Inactivation of *Gna1* had a pleiotropic effect on phenotype. The *Gna1* mutants were less pathogenic, attributed to coinciding with a defect in direct penetration. Also, *Gna1* mutants were unable to sporulate, showed an albino phenotype, and secreted one or more brown pigments into growth media. Analysis of growth medium identified tyrosine, phenylalanine, and dihydroxyphenylalanine (L-DOPA) were excreted by the *gna1* strains but not by wild type. The presence of these compounds, and the insensitivity of melanization to tricyclazole suggest that *S. nodorum* synthesizes melanin via the L-DOPA pathway, the first fungal phytopathogen described to do so. Decreases in protease (and several other depolymerases) activities and sensitivity to osmotic stress were other phenotypes identified in the *Gna1* mutants. *Gna1* is the first signal transduction gene to be cloned and characterized from *S. nodorum* and its inactivation has uncovered several previously unknown facets of pathogenicity.**

Additional keyword: G protein.

Signal transduction pathways are used by eukaryotic organisms to sense and to react to the external environment. The heterotrimeric G proteins are a key component of these signal transduction pathways. These proteins consist of α , β , and γ subunits and are coupled to the cytoplasmic side of a membrane-bound G protein-coupled receptor. G proteins are activated by the interaction of an extracellular ligand with a G protein-coupled receptor, triggering a conformational change within the β and γ subunits. This causes the displacement of GDP by GTP on the α subunit, resulting in its release from the parental trimer and allowing interaction with intracellular effectors. Once the ligand is no longer present, the $G\alpha$ subunit catalyzes GTP hydrolysis, permitting reassociation of the α subunit with the $\beta\gamma$ subunits (Neer 1995; Pennington 1994). In mammals, two of the best-studied $G\alpha$ protein families are the stimulatory ($G\alpha_s$) and inhibitory ($G\alpha_i$) groups. The $G\alpha_s$ family stimulate adenylyl cyclase to produce cAMP, while the $G\alpha_i$ family inhibits adenylyl cyclase activity, thus preventing cAMP production.

A number of genes encoding for different $G\alpha$ protein subunits have been cloned from phytopathogenic fungi and have been subjected to intense study (Table 1). Many of these have been disrupted, and the effects on pathogenicity have been examined. A deletion of *MagB* from *Magnaporthe grisea* results in a severely reduced pathogenic strain, while deleting the homologue in *Cochliobolus heterostrophus* has no effect on pathogenicity (Horwitz et al. 1999; Liu and Dean 1997). Other phenotypes affected in different strains include germination, sporulation, reduced protease activity, reduced osmotic tolerance, and melanization. Clearly, the role of the $G\alpha$ subunit and its requirement for pathogenicity appears unique for each phytopathogen (references are discussed in Table 1).

The ascomycete *Stagonospora* (syn. *Septoria*) *nodorum* (Berk.) Castell. & Germano [teleomorph *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (Müll.) Hedjar.] is a major pathogen on wheat, causing leaf and glume blotch diseases (Weber 1922). In Western Australia alone, *S. nodorum* causes 5 to 20% crop losses per annum (Murray and Brown 1987). The mechanisms by which *S. nodorum* infects wheat leaves are poorly understood. Furthermore, there has been no research undertaken to date investigating signal transduction in this pathogen. During the sequencing of an expressed sequence tag library, an open reading frame was identified as a $G\alpha$ protein. We have used this $G\alpha$ protein cDNA to initiate investigations into signal transduction and to shed new light on the pathogenicity of *S. nodorum*.

RESULTS

Cloning and sequencing of *Gna1*.

A genomic library was screened using *Gna1* cDNA as a probe. Four genomic clones were isolated that hybridized to the probe, with one, named pGAP4g, selected for further analysis. Sequencing of pGAP4g identified an open reading frame of 1,215 bp encoding a protein of 353 amino acids (GenBank accession number AY327542). A comparison of the genomic and cDNA sequences revealed the presence of three introns at bases 118 to 169, 511 to 564, and 1121 to 1171. A BLASTX (Altschul et al. 1990) search of the NCBI nonredundant database revealed that *Gna1* has nearly complete amino acid identity with $G\alpha$ proteins from the fungal phytopathogens *Cochliobolus heterostrophus*, *Colletotrichum trifolii*, *Cryphonectria parasitica*, and *Magnaporthe grisea*. *Gna1* also contained motifs common to the $G\alpha$ protein family, such sites for myristoylation (M-G-C-G-M-S) at the N-terminus (residue 1 to 6) (Mumby et al. 1990), ADP-ribosylation by pertussis toxin (C-G-L-I) at the C-terminus (residue 350 to 353) (Fields and Casey 1997), a phosphate-binding loop or P-loop site (G-A-G-E-S-G-K-S) (Saraste et al. 1990) at residue 40 to 48, and

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a G'' region (T-C-A-T-D-T) (Pennington 1994) at residue 323 to 328. Southern analysis of digested genomic DNA revealed *Gna1* to exist as a single copy within the *S. nodorum* genome (Fig. 1A).

Fig. 1. A, Diagram outlining the construction of the knockout vector pGPSH-Gna1. Note the transposon inserted at 671 bp downstream of the predicted start site, as determined by sequencing, using primers homologous to the transposon ends, TnsL and TnsR. B, Southern analysis of SN15 (lane 1), SNGa17 (lane 2), SNGa33 (lane 3), and SNGa35 (lane 4) hybridized with *GNAI* probe (i) and a hygromycin-specific probe (ii). C, Polymerase chain reaction analysis on using actin, *Gna1*-5', and *Gna1*-3' primer sets on cDNA samples generated from SN15 (lanes 1 through 3), SNGa17 (lanes 4 through 6), SNGa33 (lanes 7 through 9), and SNGa35 (lanes 10 through 12).

In planta reverse transcription-polymerase chain reaction (RT-PCR).

In order to determine whether *Gna1* was expressed in planta, transcript levels were measured during infection using quantitative RT-PCR. Figure 2 shows the expression of *Gna1* normalized against actin. The data shown are the results of the experiment repeated three times on two independent RNA populations. The pattern of expression showed *Gna1* transcription decreased slightly but significantly with respect to actin after germination at 4 h postinfection. Between 1 and 2 days postinfection (dpi), the expression increased to levels observed at 4 h postinfection and then continued to rise throughout the remainder of the infection. These results demonstrated that not only was *Gna1* expressed during infection but also that the expression varied significantly. Consequently, the role that *Gna1* plays during infection was investigated further by gene disruption.

Gna1 gene inactivation.

The insertional inactivation construct pGPSH-Gna1 was developed by transposon mutagenesis as described below and outlined in Figure 1A. Sequencing from the transposon ends revealed the transposon to have inserted 671 bp downstream of the predicted translation start site. pGPSH-Gna1 was then used to transform *S. nodorum*. Approximately 50 colonies, of which two appeared to be nonsporulating, were obtained; all were selected for further analysis. Screening by colony PCR indicated the two nonsporulating transformants had undergone homologous recombination with pGPSH-Gna1. For further confirmation, genomic DNA from the two potential disruptants as well as an ectopic transformant was digested with *ApaI* and was analyzed by Southern analysis (Fig. 1B). As previously determined, only one band was evident for the wild-type strain when hybridized with the *Gna1* probe. The two potential mutants showed a band shift consistent with the size of the transformation construct, indicating insertion of the construct into the *Gna1* gene. This was further confirmed by hybridizing with a hygromycin-specific probe. No band was evident from the wild type, while single bands were observed in the potential disruptants of the same size as when hybridized

Fig. 2. Normalized *Gna1* transcript levels during infection.

Table 1. A summary of *Ga* genes characterized in phytopathogenic fungi

Gene	Organism	Effect of disruption on pathogenicity	Reference
<i>Bcg1</i>	<i>Botrytis cinerea</i>	Reduced	Gronover et al. 2001
<i>Cga1</i>	<i>Cochliobolus heterostrophus</i>	No effect	Horwitz et al. 1999
<i>Cpg1</i>	<i>Cryphonectria parasitica</i>	Nonpathogenic	Gao and Nuss 1996
<i>Ctg1</i>	<i>Colletotrichum trifolii</i>	Reduced	Truesdell et al. 2000
<i>Gpa1</i>	<i>Cryptococcus neoformans</i>	Reduced	Alspaugh et al. 1997
<i>Gpa3</i>	<i>Ustilago maydis</i>	Nonpathogenic	Regenfelder et al. 1997
<i>MagB</i>	<i>Magnaporthe grisea</i>	Reduced	Liu and Dean 1997
<i>Fga1</i>	<i>Fusarium oxysporum</i>	Reduced	Jain et al. 2002

with the *GnaI* probe. Characterization of the ectopic transformant revealed *GnaI* to be intact, with the construct appearing to have recombined elsewhere in the genome. On the basis of this, the mutants were subsequently named *S. nodorum* SNGna33 and SNGna35, and the ectopic transformant was named *S. nodorum* SNGna17.

As the impairment of *GnaI* was due to an insertion rather than a deletion, it was important to determine that no part of *GnaI* was transcribed and, therefore, possibly active. To do this, RNA was collected from the strains used in this study and in reverse transcription reactions. PCR was then performed on

the resulting cDNA to ascertain the status of the *GnaI* transcript in the impaired strains (Fig. 1C). Amplification using primers homologous to actin ensured the presence and quality of the cDNA with strong PCR bands present for all samples. Using primer sets homologous to regions within *GnaI* that were 5' (*GnaI*-5') and 3' (*GnaI*-3') of the transposon insertion site, products at the expected size were present for the cDNA samples from *S. nodorum* SN15 and the ectopic SNGa17. However, when using these same primer sets on cDNA generated from *S. nodorum* SNGa33 and SNGa35, there was no evidence of any amplification, implying the absence of any intact *GnaI* transcript.

Phenotypic analysis of *S. nodorum* *GnaI* strains.

The growth of the mutants and ectopic transformant was compared with that of wild type in vitro. The most immediate difference between the wild-type and ectopic strains and the *Ga*-impaired strains was that the mutants were unable to sporulate. Consequently, all inoculations and subculturing were performed by excising small agar plugs of mycelial growth and placing them either on plates or into flasks. Growth rate was determined by measuring the diameter of the growth on plates. All four strains grew at a similar rate on all types of media tested (minimal medium, CzV8CS, and wheat meal agar). However, in minimal medium SNGna33 and SNGna35 secreted a brownish pigment that was not evident for either SN15 or SNGna17 (Fig. 3). Furthermore, by growing the strains on CzV8CS, both mutant strains produced an almost albino phenotype compared with wild-type and ectopic strains. A secreted pigment was also evident from the mutants on this medium. Note that SNGna17 appears identical to SN15 when grown on CzV8CS (data not shown).

The brown pigment was further analyzed by inoculating flasks of minimal medium with agar plugs of the wild-type, ectopic, and mutant strains. The growth medium was harvested after 4 days and was analyzed by UV-visible spectral analysis (Fig. 4). The absorption spectra of the growth media of SN15 and SNGna17 clearly differed from that of the *GnaI*-inactivated strains. The spectra from SNGna33 and SNGna35 contained a broad peak present in the near visible region (approximately 370 nm) that was absent from both the wild-type and ectopic strains. The wild-type and ectopic extracts exhibited a peak and a shoulder at 265 nm that were absent from the mutant strains. A further shoulder was also detected at 343 nm in SN15 and SNGna17 that was not evident in the mutants; however, the broad peak exhibited by the mutants at 370 nm may have hidden this shoulder. The growth media of *S. nodorum* SN15 and SNGna35 were further analyzed by mass spectrometry (Fig. 5). Three peaks were identified in the growth media of the *GnaI* mutants that were not present in either the wild-type or the ectopic strains. These peaks were assigned the mass to charge ratio of 19.8816, 180.9073, and 164.9346. Allowing for a small internal shift within the instrument, these mass to charge ratios correspond to those of dihydroxyphenylalanine (L-DOPA), tyrosine, and phenylalanine.

The effect of tricyclazole on pigmentation.

The lack of melanization and the finding of L-DOPA secreted during growth in the *gnaI* mutants raises questions regarding the synthesis of melanin in *S. nodorum*. To examine this further, the ability of *S. nodorum* to synthesize melanin was examined in the presence of tricyclazole (5-methyl-1,2,4-triazole (3,4-*a*) benzothiazole). Tricyclazole is a fungicide that targets the dihydroxynaphthalene (DHN) melanin synthetic pathway (Hewitt 1998). Spores of *S. nodorum* and *Colletotrichum lagenarium*, a fungus known to produce melanin via the DHN synthetic pathway, were used to inoculate wheat meal agar. Tricyclazole was

Fig. 3. Photographs of SN15 and transformants growing on **A**, minimal media and **B**, CzV8CS. SNGna33 (i and vi), SNGna35 (ii and vii), SNGna17 (iii), and SN15 (iv and v).

Fig. 4. The absorption spectra of the extracellular growth media of SN15 (—), SNG17 (---), SNGna33 (....), and SNGna35 (-.-.).

applied after one day's growth, and the fungi were examined after a further 6-day incubation period (Fig. 6.). Without tricyclazole, *Colletotrichum lagenarium* appeared a dark gray in color. The gray pigment, assumed to be melanin, decreased to insignificant levels with increasing concentrations of tricyclazole. In contrast to *Colletotrichum lagenarium*, even at the highest levels of tricyclazole used, there was only a very slight change in the color of *S. nodorum*, suggesting that the fungicide does not significantly affect melanin production.

Osmotic sensitivity.

The mutants were examined for their ability to withstand osmotic stress. The four strains were grown on minimal medium plates, each containing a known concentration of NaCl. These were incubated for two weeks, and the diameter of the growth was measured daily (Fig. 7). As previously mentioned, the growth rate of the four strains was not significantly different when grown on normal minimal medium (i.e., not osmotically challenged). When grown in the presence of NaCl however, the growth rate of both *Gna1*-disrupted strains was much lower than that of either the wild type or the mutant. This lower growth rate occurred for concentrations

of NaCl ≤ 1 M NaCl, above which growth was virtually inhibited for all strains.

Extracellular protease and other depolymerase activities.

A further phenotypic test was to examine the secreted protease activity of the transformants. Each of the four strains (wild type, ectopic, and mutants) was grown on casein agar plates for two weeks. The distance from the edge of mycelial growth to that of the halo that resulted from protease degradation of casein was measured after two weeks, as an indication of secreted protease activity (Fig. 8). The ectopic and the wild-type strains produced similar amounts of casein degradation, with over 5 mm of cleared halo measured after two weeks, but growth of the mutants resulted only in approximately 1 mm of degraded casein.

A trypsin SNP1 and a subtilisin SNP3 are the main alkaline proteases produced by *S. nodorum* (Bindschedler et al. 2003), and both were markedly reduced in the two *Ga* mutants. Significant activities only occurred in the absence of nitrate in the medium (Fig. 9A and B.). The effect of the mutation was less evident with general protease activities; although still substantially reduced at pH 8, the effect was not significant at pH 5 or pH 3.2 (Fig. 9 C and E). In these instances, repression of synthesis by nitrate was much less severe.

Xylanase, glucosidase, galactosidase, and butyrate esterase were also significantly reduced in the *Ga* mutants in the absence of nitrate but not in its presence (data not shown).

Pathogenicity assay.

The ability of the *Gna1* disrupted strains to infect wheat leaves was investigated using a detached leaf assay. Equal size plugs of growth from agar plates of the four strains were placed on wheat leaves and were allowed to grow. The ability of the strains to infect leaves was determined by measuring the

Fig. 5. Matrix-assisted laser desorption-ionization time of flight mass spectra of extracellular growth media of **A**, SN15 and **B**, SNGna35.

Fig. 6. The effect of tricyclazole on the melanization of **A**, *Stagonospora nodorum* and **B**, *Colletotrichum lagenarium*. Active concentrations of tricyclazole used were as follows: (1) 20 ppm, (2) 2 ppm, (3) 0.2 ppm; (4) 0.02 ppm; and (5) 0.

Fig. 7. Growth of SN15 (●), SNGna17 (□), SNGna33 (○), and SNGna35 (■) at varying NaCl concentrations.

lesion size (Fig. 10A, B, and C). The lesions of the four strains did not appear significantly different throughout the first five days of infection. After 5 days, the wild-type and ectopic strains continued rapid growth to produce large typical lesions, while the two mutant strains produced lesions less than half the size of the wild-type and ectopic strains. At 14 dpi, sporulation was clearly apparent for both *S. nodorum* SN15 and SNGna17, while no pycnidia were evident for either of the *Gna1*-disrupted strains.

The infection at 24 h postinfection for *S. nodorum* SN15, SNGna17, and SNGna35 was examined using both trypan blue-stained material under a light microscope and also by scanning electron microscopy (SEM) (Fig. 10D). Trypan blue staining showed many obvious penetration attempts by SN15 on both epidermal cells and periclinal cell walls. A similar pattern of penetration was also observed for the ectopic strain. In contrast, essentially no penetration attempts were detected for the *S. nodorum* SNGna35 at either site. Although difficult to observe at the magnification shown in Figure 10D, stomatal penetration was apparent in all three strains, suggesting that a functional *Ga* subunit is not required for entry through stomata.

A closer examination of penetration at 24 h postinfection using SEM revealed all three strains produced what appeared to be penetration structures that branched off mycelium sporadically. The number of these putative penetration structures did not appear significantly different between strains, implying that the effectiveness of these structures may be reliant upon a functional *Ga* protein.

Examining the effect of cholera toxin and cAMP.

The effect of adding cholera toxin and cAMP to detached leaf assays was examined. In the absence of any treatment, the infections progressed as described above for all strains (Fig. 11). In the presence of cAMP, there was no significant effect on lesion size for any of the strains tested. The effect of cholera toxin on lesion development was also examined. Lesion size was unaffected in the *Ga* mutant in the presence of cholera toxin. However, in both *S. nodorum* SN15 and the ectopic strain SNGap17, the lesions were significantly larger in the cholera-toxin-treated samples than were those caused by the untreated samples or those treated with cAMP. A statistical comparison using Student's *t*-test of the untreated samples and those treated with cholera toxin in both the wild type and the ectopic mutant determined $p = 0.001$.

Fig. 8. Diameter of the cleared 'halo' on casein plates as an indication of extracellular protease activity.

DISCUSSION

This study describes the characterization and role, through targeted gene disruption, of the *Ga* subunit *Gna1* from *S. nodorum*.

The putative amino acid sequence of the *Gna1* open reading frame revealed several consensus sites commonly associated with the *Ga* subunit family. The site for myristoylation at the amino terminus (Mumby et al. 1990) is important for attach-

Fig. 9. Activity of A, Snp1 and B, Snp3 protease. C, through E, General protease activity at pH 8, pH 5, and pH 3.2, respectively. Black bars represent activity when grown on wheat cell walls, and the clear bars represent that on wheat cell walls plus 2 g of NaNO₃ per liter.

ment to the membrane (Jones et al. 1990). The presence of a P-loop and G' region within Gna1 indicates GTP/GDP binding properties (Kaziro et al. 1991). The P-loop is common to many ATP/GTP-binding proteins, such as elongation factors, various kinase families, ATP synthase β -subunits, the nonheterotrimeric RAS G-proteins, and myosin heavy chains (Saraste et al. 1990). The carboxyl terminus of the polypeptide has a pertussis toxin-labeling site. ADP ribosylation prevents the α subunit coupling with the $\beta\gamma$ dimer, resulting in constitutive activation (Fields and Casey 1997).

In mammalian systems, it is possible to differentiate between the inhibitory or stimulatory $G\alpha$ subunits on the basis of amino acid sequence. In fungal systems however, the assignment of $G\alpha$ class does not appear this simple, as the addition of exogenous cAMP has been found to restore wild-type phenotypes to apparent $G\alpha_i$ mutants, indicating a positive effect on adenylate cyclase activity (Ivey et al. 1999). This complementation of phenotype would suggest that the $G\alpha$ subunit was stimulatory rather than inhibitory. On the basis of amino acid sequence, Gna1 appears to be an inhibitory subunit. To examine this further, we applied cholera toxin, which ribosylates $G\alpha_s$ subunits, resulting in a permanently activated state (West et al. 1985), to the strains used in this study. The addition of cholera toxin to the wild-type strain resulted in increased pathogenicity, though no effect was observed when applied to the *Gna1* mutants, suggesting that *Gna1* could be stimulatory. BlastP analysis revealed Gna1 shows homology to many fungal $G\alpha$ proteins, most of which are predicted to encode $G\alpha_i$ subunits, although many have not been functionally characterized. A phylogenetic tree was constructed based on the differences in amino acid residues between the putative fungal $G\alpha_i$ proteins, with Gna1 appearing most closely related to the $G\alpha$ proteins of

the dothideomycetes *Leptosphaeria maculans* and *Cochliobolus heterostrophus* (Fig. 12). Consequently, on the basis of sequence analysis, Gna1 appears to be inhibitory. However, experimental evidence has been collected demonstrating that the gene does encode the stimulatory subunit, proving again that risk is involved in classifying $G\alpha$ subunits into classes on the basis of sequence.

The inactivation of $G\alpha$ genes in the plant pathogens *Fusarium oxysporum* (Jain et al. 2002), *Botrytis cinerea* (Gronover et al. 2001), *Colletotrichum trifolii* (Truesdall et al. 2000), *Cochliobolus heterostrophus* (Horwitz et al. 1999), *Magnaporthe grisea* (Liu and Dean 1997), and *Cryphonectria parasitica* (Gao and Nuss 1996) has shown that the gene varies in its contribution to pathogenicity of these diverse pathogens. To determine whether *Gna1* in *S. nodorum* was a suitable candidate for gene disruption, the expression of the gene in planta was analyzed. The expression throughout the infection increased steadily apart from a slight decrease at 12 h postinfection, which corresponds approximately to the time when the fungus penetrates the leaf surface.

Ga-disrupted *S. nodorum* strains produced lesions considerably smaller than those produced by either the wild-type or ectopic strains. Furthermore, after 14 dpi, the *Ga*-disrupted strains completely failed to sporulate. In contrast to wild type, which attempted to penetrate the wheat leaf via several points, including stomata, epidermal cells, and periclinal cell walls, the mutants displayed no evidence of attempted penetration of either the epidermal cells or the periclinal cell walls. Thus disruption of *Gna1* has affected the ability of the fungus to penetrate the leaf directly other than through natural openings. In contrast to many other fungal phytopathogens, there remains conjecture on how *S. nodorum* penetrates the leaf. Indeed

Fig. 10. Wheat leaves at **A**, 9 and **B**, 16 days postinfection with SN15 (i), SNGna17 (ii), SNGna33 (iii), and SNGna35 (iv). **C**, Graph showing the size of the lesions formed by SN15 (○), SNGna17 (□), SNGna33 (●), and SNGna35 (■). **D**, Trypan blue-stained (i to iii) and scanning electron micrographs (iv to vi) of leaves 24 h postinfection with SN15, SNGna35, and SNGna17, respectively. No blue halos (penetration attempts) were observed when uninfected wheat leaves were stained with trypan blue (data not shown). Note that both the *Gna1*-disrupted mutants appeared identical under the microscope and, consequently, only *Stagonospora nodorum* SNGap35 is shown.

much of the previous literature is contradictory as to the presence of a penetration structure and also regarding the ability of the fungus to gain entry through natural openings (Bird and Ride 1981; Karjalainen and Louatmaa 1986; O'Reilly and Downes 1986). Evidence obtained in this study conclusively demonstrates that *S. nodorum* is able to penetrate through natural openings and also directly through the cuticle, though the nature of the intercalary penetration structure revealed here by SEM is unclear.

The *Gna1*-disrupted strain secretes a brown pigment in vitro comprising tyrosine, phenylalanine, and L-DOPA. Until recently, it was thought that fungi synthesized melanin via the polyketide biosynthetic pathway leading to the production of DHN melanin. Recently, it has been shown that several fungi previously reported to produce DHN melanin in fact produce DOPA melanin (Butler and Day 1998). The synthesis of DOPA melanin, which has been well studied in humans, occurs via the oxidation of tyrosine with phenylalanine, tyrosine, and L-DOPA as key intermediates in the pathway. The accumulation of these intermediates in the culture medium is the first evidence to date that *S. nodorum* or any phytopathogenic fungus may synthesize melanin via the L-DOPA pathway. Tricyclazole is a well-known fungicide that inhibits DHN melanin biosynthesis and is particularly effective at controlling *Magnaporthe grisea* (Wheeler and Greenblatt 1988; Woloshuk et al. 1980) and other pathogens with melanized appressoria, such as *Colletotrichum lagenarium* (Kubo et al. 1982). The chemical had only a very slight effect on the melanization of *S. nodorum* and only at very high concentrations. In contrast, the pigmentation of *Colletotrichum lagenarium* was significantly reduced by very small amounts of tricyclazole. This further implies that *S. nodorum* is able to synthesize melanin via a pathway independent of DHN melanin. Nevertheless, the slight reduction of pigmentation in *S. nodorum* at higher tricyclazole concentrations suggests the possibility that two melanin synthetic pathways may exist in this pathogen. Furthermore, tricyclazole had no effect on *S. nodorum* pathogenicity (data not shown), which concurs with the lack of evidence to date that melanization is required for disease.

The involvement of *Ga* genes on melanin synthesis has been previously demonstrated or implicated in several fungal phytopathogens, including *Cochliobolus heterostrophus* (Horwitz et al. 1999), *Magnaporthe grisea* (Liu and Dean 1997), *Ustilago maydis* (Regenfelder et al. 1997), *Colletotrichum lagenarium* (Kubo et al. 1991; Perpetua et al. 1996), and *Cryphonectria parasitica* (Gao and Nuss 1996). The effect on pathogenicity of this impairment in melanin biosynthesis does, however, differ in each pathogen. While loss of melanization in *Magnaporthe grisea* and *Colletotrichum lagenarium* results in

drastically reduced pathogenicity, *Cochliobolus heterostrophus* and *Alternaria alternata* remain fully pathogenic in the absence of complete melanization (Horwitz et al. 1998; Tanabe et al. 1995).

The pleiotropic nature of the *Gna1* disruption prevents any conclusions relating pathogenicity reduction and loss of melanization. The putative penetration structures produced by SNGna33 and SNGna35 may be ineffective because of a lack of melanization. However, *S. nodorum* appears versatile and is able to penetrate through natural openings and, consequently, the loss of melanin does not entirely prevent pathogenicity.

Further phenotypic testing of the *Gna1* strains revealed they both secreted lower activities of proteases than the wild-type strain and were also more susceptible to increasing osmotic stress. Two major alkaline proteases, trypsin SNP1 and subtilisin SNP3, have been linked with pathogenicity of *S. nodorum* (Bindschedler et al. 2003), and both were drastically reduced in SNGna33 and SNGna35. This was evident with specific peptide substrates and to a lesser extent with a general protease assay at pH 8. Their synthesis is highly sensitive to metabolite repression by nitrogen and, in wild type and mutants, both enzymes remained nitrate repressed. With general protease assays at pH 8, 5, and 3.2, the influence of the *Ga* mutation decreased respectively, but the activities were less affected by nitrate repression. Other depolymerases (xylanase, two glycosidases, and an esterase), which are usu-

Fig. 11. Lesion sizes caused by *Stagonospora nodorum* SN15, SNGna17, and SNGna35 in the presence of 10 mM cAMP (gray bars) or 50 ng cholera toxin per ml (black bars). Negative controls inoculated with only 0.02% Tween 20 are represented by clear bars.

Fig. 12. Cladogram of *Ga* subunit sequences from fungal sources. Gene name when known and GenBank accession numbers are as follows: *Aspergillus fumigatus* *GpaA* (CAC81704), *Blumeria graminis* (CAC10177), *Botrytis fuckeliana* (CAC19871), *Cochliobolus heterostrophus* *Gga1* (O74227), *Colletotrichum trifolii* *Ctg1* (O42874), *Cryphonectria parasitica* *Gba1* (Q00580), *Emericella nidulans* *FdaA* (Q00743), *Giberella fujikuroi* (CAC45042), *Hypocrea virens* *TgaA* (AAO18695), *Leptosphaeria maculans* (AAM18375), *Magnaporthe grisea* *MagB* (O13315), *Neurospora crassa* *Bna1* (Z05425), *Penicillium marneffeii* *BasA* (AAM64110), *Rosellinia necatrix* *Rga2* (JC7661), *Sporothrix schenckii* *Ssg1* (O74259), *Stagonospora nodorum* *Gna1* (AY327542), *Tapesia yallundae* *Tyg1* (CAC35694), *Trichoderma atroviridae* *Tga1* (AAK74191). Tree constructed using Treeview, based on a ClustalX alignment.

ally subject to severe carbon catabolite repression but not to N repression (Carlile et al. 2000), were also significantly reduced in the *Ga* mutants in the absence of nitrate but not in its presence. It appears that nitrogen has an overriding influence in subsequent signal transduction in the *Ga* mutants. Proteases have been implicated by gene deletion in fungal infection of animals (by *Candida albicans*) and of insects (by *Metarhizium anisopliae*). Although the role of pathogen proteases in plant diseases remains equivocal, *S. nodorum* SNP1 and SNP3 are produced at the crucial stages of penetration and infection (Bindschedler et al. 2003).

To summarize, as in some but not all plant pathogenic fungi, the *S. nodorum* *Ga* protein appears to be a major upstream regulator of many processes involved in pathogenicity, such as secretion, melanization, penetration, osmoregulation, and sporulation. For the first time, we have unequivocally demonstrated that *S. nodorum* forms an epidermal penetration structure. We have also shown for the first time that a plant pathogenic fungus produces DOPA melanin.

Further study of the *Gna1* gene will allow the detailed definition of the downstream targets of the *Ga* subunit as well as the identification of the presumed upstream receptor that is required for activating the signal transduction pathway that is critical for pathogenicity.

MATERIALS AND METHODS

Fungal strains and media.

S. nodorum SN15 was provided by the Department of Agriculture, Western Australia. The fungus was routinely grown on CzV8CS (Czapek Dox agar (Oxoid), 45.4 g/liter; agar, 10.0 g/liter; CaCO₃, 3.0 g/liter; Campbell's V8 juice, 200 ml/liter; casamino acids, 20.0 g/liter; peptone 20, g/liter; yeast extract, 20 g/liter; adenine, 3 g/liter; biotin, 0.02 g/liter; nicotinic acid, 0.02 g/liter; *p*-aminobenzoic acid, 0.02 g/liter; pyridoxine, 0.02 g/liter; thiamine, 0.02 g/liter) containing 1.5% agar. Plates were incubated at 22°C in 12-h cycles of darkness and near-UV light (Phillips TL 40W/05). Liquid cultures were started with the addition of 10⁷ spores to 100 ml of CzV8CS and were also grown at 22°C and shaking at 130 rpm in the dark. For experiments that required defined growth conditions, *S. nodorum* SN15 was used to inoculate minimal medium, which consisted of 30 g of sucrose, 2 g of NaNO₃, 1.0 g of K₂HPO₄, 0.5 g of KCl, 0.5 g of MgSO₄·7H₂O, 0.01 g of ZnSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O per liter, and 0.0025 g of CuSO₄·5H₂O per liter. of Agarose (15 g/liter) was added if plates were required.

Cloning of insertional inactivation construct.

A modified form of the vector pGPS3 (New England Biolabs, Beverly, MA, U.S.A.) was used to create insertional inactivation constructs. A hygromycin cassette from *pAN7.1* was cloned into the *NotI* site within the transprimer of pGPS3, creating pGPS-hyg. The transposition reaction was then undertaken as per the pGPS3 protocol. Briefly, 20 ng of pGPS-hyg and 80 ng of *Gna1* cDNA were incubated together in the presence of the TnsABC transposase complex (New England Biolabs). The reaction was followed by a *PI-SceI* restriction enzyme digest that destroyed any remaining pGPS-hyg vector. The products of the transposon reaction were transformed into *E. coli* XL1Blue (Stratagene, La Jolla, CA, U.S.A.), and the resulting colonies screened by restriction enzyme digests to determine whether the transposon had inserted into the cDNA or the pBluescript backbone. Those that appeared to have inserted within the cDNA were then sequenced, using primers homologous to the ends of the transposon.

Preparation of *S. nodorum* protoplasts.

Preparation of *S. nodorum* protoplasts was essentially as described elsewhere (Cooley et al. 1988). Mycelium grown by inoculating 100 ml of CzV8CS liquid medium with 5×10^7 spore suspension and shaking it at 140 rpm overnight at 22°C was harvested by centrifuging at $10,000 \times g$ for 10 min at 4°C. The supernatant was discarded, and the cells were washed once with 600 mM MgSO₄ before resuspension in 20 ml of filter-sterilized 1.2 M MgSO₄, buffered to pH 5.8 with 10 mM phosphate buffer and containing 15 mg of glucanex per ml (Novo Nordisk). The solution was incubated without agitation in a glass petri dish for 2 h at 28°C, was transferred to a sterile centrifuge tube, and was gently overlaid with 5 ml of 600 mM sorbitol in 10 mM Tris (pH 7.5). After centrifugation at $4,000 \times g$ for 25 min at 4°C, the protoplasts, which settled at the interface, were removed and mixed with an equal volume of 1 M sorbitol in 10 mM Tris (pH 7.5). The protoplasts were centrifuged at $1,500 \times g$ for 10 min at 4°C, were washed with 3 ml of STC (1.2 M sorbitol, 10 mM calcium chloride, 10 mM Tris, pH 7.5) buffer, and were gently resuspended in 0.5 ml of the same buffer. The concentration of the protoplast suspension was estimated using a haemocytometer and was adjusted as required. The protoplasts were now ready for use in transformation experiments. All solutions used for the preparation of *S. nodorum* protoplasts were made the day prior to use and were filter-sterilized.

Transformation of *S. nodorum* to hygromycin resistance.

Transformation was essentially as previously described (Cooley et al. 1988). Up to 7.5 µg of transforming DNA was dissolved in a maximum volume of 25 µl STC buffer and was added to 100 µl of protoplast suspension. For reliable transformation, a concentration of at least 5×10^8 protoplasts per ml was required. A negative control was also prepared, in which protoplasts received an equivalent volume of STC buffer. Mixtures were incubated at room temperature for 20 min before the addition of 200 µl of filter-sterilized 60% (wt/vol) polyethylene glycol 4000, 10 mM calcium chloride in 10 mM Tris (pH 7.5). The solution was mixed by gentle inversion, and a further two additions (200 and 800 µl) of the same solution were made, followed by gentle mixing after each addition. After another incubation of 20 min at room temperature, the mixture was centrifuged at $12,000 \times g$ for 5 min, the supernatant was discarded, and the protoplasts were gently resuspended in 1 ml of STC buffer. Selection of hygromycin-resistant transformants was accomplished by adding 250 µl of the transformation mixture to 5 ml soft Cz-top proto (Czapek-Dox liquid, 45.4 g/liter; Oxoid agar, 7.5 g/liter; sorbitol, 182.2 g/liter; filtered V8 juice, 200 ml/liter, pH 6.0) and pouring the solution onto Cz plates (Czapek-Dox agar 45.4 g/l, Oxoid agar 10 g/l, sorbitol 182.2 g/l, filtered V8 juice 200 ml/l, pH 6.0). This was repeated four times using the full 1 ml of the transformed protoplasts. After 40 h, the protoplasts were overlaid with a further 5 ml of soft Cz containing enough hygromycin to give a final concentration of 250 µg ml⁻¹ for the whole plate. All incubations were at 22°C in the dark. Approximately nine days after the hygromycin overlay, colonies were cut from the agar and were placed on fresh CzV8CS + hygromycin for three weeks. Spores from these were collected and were diluted to provide single spores. After one week's growth, pycnidia from single-spore growth was plated out and incubated for a further three weeks. The resulting pycnidia were used for further analysis.

Pathogenicity assay.

The pathogenicity of the mutants was assayed on detached leaves from 2-week-old wheat seedlings, using a method

modified from those described by Benedikz and associates (1981). The detached wheat leaves distal ends (2 cm) were removed. The next 4- to 5-cm portions were embedded onto benzimidazole agar adaxial side up. Instead of challenging the detached wheat leaves with spores as described by Benedikz and associates (1981), small mycelial plugs were used as inoculum, since the *Gna1* mutants do not sporulate. The mycelial plugs were placed on the leaves in the presence of 5 μ l of 0.02% Tween 20, and the leaves then were incubated with 12-h light and 12-h dark periods at 25°C to enable disease development. The size of lesions was measured with a calliper.

Tricyclazole growth experiments.

Wheat meal agar (1% blended wheat meal, 1.5% agar; 1 ml) was pipetted into each well of a sterile 24-well plate (Greiner Bio-One Inc., Monroe, NC, U.S.A.). The wells were inoculated with 10^4 spores of either *S. nodorum* or *Colletotrichum lagenarium*, and the plate was placed at 22°C for 24 h. The appropriate concentration of 10 $\times$ tricyclazole (51 μ l) (Dow AgroSciences, NSW, Australia) was mixed with 249 μ l of soft wheat meal agar (1% blended wheat meal, 0.5% agar) prewarmed to 50°C, and 150 μ l of this mixture was then pipetted onto the top of each fungal culture and was allowed to set. The plate was then covered, wrapped in parafilm and placed at 22°C for a further six days prior to examination.

Liquid chromatography mass spectrometry.

Liquid minimal media was inoculated with *S. nodorum* SN15 and SNGna35 and was incubated at 22°C for three days. An aliquot of growth media was then taken and passed through a 0.2- μ m filter. For analysis, the samples were diluted to a 5 or 10% concentration in 2% formic acid solution and were analyzed by electrospray mass spectrometry using a Q-Star Pulsar i (Applied Biosystems, Inc. Foster City, CA, U.S.A.) in direct injection mode.

Leaf clearing and staining.

Two- to four-day old necrotic lesions on wheat leaves caused by *S. nodorum* SN15 and the transformants were examined by light microscopy. Diseased leaf tissue was cleared and stained with trypan blue, using a method modified from those described by Bruzzese and Hasan (1983) and Shipton and Brown (1962). The tissue was boiled with trypan blue stain for 5 min and then destained and stored in a 2.5 g ml⁻¹ chloral hydrate solution.

SEM.

The process of fungal infection on the surface of wheat leaf was examined by SEM. Four-day-old fungal lesions were excised from the detached wheat leaf and were fixed overnight in 25 mM phosphate buffer containing 2.5% glutaraldehyde. This was then rinsed, and the tissue was fixed in phosphate buffer containing 2% osmium tetroxide. The fixed tissue was dehydrated in 30, 50, 75, 95, and 100% ethanol washings and then transferred to an ethanol/amyl acetate mixture and further washed by two rounds of 100% amyl acetate. The tissue was dried with a critical point dryer and then mounted, coated with gold particles, and viewed with a Philips XL Series SEM, using an accelerating voltage of 5.00 kV.

Osmotolerance assay.

The *S. nodorum* strains developed within this study were tested for their ability to tolerate hyperosmolarity. Mycelial plugs no greater than 6 mm³ were placed onto minimal medium supplemented with 0, 250, or 500 mM, or 1 M NaCl and were incubated at 22°C under near-UV light for 16 days.

Measurements of colony diameter were taken with a caliper from day 4 and every 2 days thereafter until day 16. The experiment was performed with a sample size of eight from two independent experiments.

Extracellular protease (and other depolymerase) activities.

The putative disruptants were tested for extracellular protease activity on casein agar onto which mycelial plugs were placed. Plates were incubated for 5 days at 22°C under near-UV light. A caliper was used to measure the diameter of the fungal colony and cleared halo; the difference in diameter size indicated the level of extracellular protease activity. The experiment was performed in duplicate.

Proteases and other depolymerases were induced in vitro according to Bindschedler et al. (2003). Briefly, 44 h mycelial cultures were established in CzV8CS liquid medium, then were washed and resuspended in minimal medium lacking carbon and nitrogen sources for 6- to 8-h starvation. The mycelium was washed in 50 mM MES, pH 6.0, then was suspended in basal salts medium containing wheat cell walls as sole source of C (1% wt/vol). Protease production was monitored over 24 h in the absence and presence of N (NaNO₃, 2 g per liter).

Protease assays were as described by Bindschedler and associates (2003) and Carlile and associates (2000) and are summarized here. Trypsin-like protease SNP1 was measured with the specific substrate benz-Phe-Val-Arg-pNa, and the subtilisin SNP3 was assayed with suc-Ala-Ala-Pro-Phe-pNA. The absorbance at 405 nm (A_{405}) of nitroanilide was measured over 15 min. General protease activity was determined with azo-casein at pH 5 and pH 8, and released red azo dye was monitored at A_{340} . Acidic proteases were assayed against bovine *N,N*-dimethylated hemoglobin at pH 3.2; after precipitation with trichloroacetic acid, A_{280} of the soluble fraction was measured. Xylanase was assayed with Remazol brilliant blue xylan (RBB). A solution of 180 μ l of 0.1% RBB in 50 mM citrate buffer, pH 5, was added to 20 μ l of culture fluid and incubated at 37°C for 1 h, and then, reactions were stopped with 500 μ l of 96% ethanol. After 30 min, centrifugation was performed at 12,000 $\times$ g for 2 min, and A_{595} was read. Glucosidase, galactosidase, and butyrate esterase were assayed by measuring the increase in A_{410} from released *p*-nitrophenol from *p*-nitrophenyl β -D-glucoside, β -D-galactoside, and butyrate, respectively. Reactions were made up of 180 μ l of citrate (pH 5.0) buffered substrates and 20 μ l of culture fluids incubated at 37°C for 30 min. All assays were performed in triplicate.

Cholera toxin and cAMP addition experiments.

The effect of adding the known signal transduction effectors was examined. Detached-leaf assays were set up as described above, with the exception that either 10 mM cAMP or 50 ng of cholera toxin per ml was included in the 5- μ l 0.02% Tween 20. Negative controls with neither compound added were also included. Necrotic lesion size was measured nine days postinoculation. The experiment was repeated twice with six replicates used for each treatment. The results were statistically verified using Student's *t*-test.

Molecular techniques.

Genomic DNA was isolated using a Nucleon Phytopure genomic DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For Southern analysis, 5 μ g of genomic DNA was digested with the appropriate restriction enzyme in a volume of 300 μ l at 37°C for 16 h. Upon completion, the digest was ethanol-precipitated and was resuspended in 20 μ l of sterile water. The digested genomic DNA was then transferred to Hybond N+ membrane, as previously described (Sambrook et al. 1989). The blot was air-dried, and the DNA

was cross-linked using a UV GS Genelinker (BioRad, Hercules, CA, U.S.A.). Hybridization and detection was performed using the DIG system (Roche Molecular Systems, Branchburg, NJ, U.S.A.). The *Gna1* probe was generated by excising the cDNA from pBluescript using *XhoI* and *PstI* and was labeled as outlined in the manufacturer's instructions.

Total RNA was isolated from fungal mycelium and plant material using TRIzol Reagent (Invitrogen Corp., San Diego, CA, U.S.A.). RNA (5 µg) was reverse transcribed using Superscript II RNase H⁻ reverse transcriptase, using an oligo dT₍₁₈₎ primer and performed as per the manufacturer's protocol. The resulting cDNA was then quantified using an ABI PRISM770 Sequence detector (Applied Biosystems). The PCR was as follows: 0.5 µl cDNA, 1.5 mM MgCl₂, 0.4 mM dNTPs, 2 µM of each primer, 2 U *Taq* polymerase, 0.5 µl ROX reference dye (Invitrogen), and 0.5 µl of 1× SYBR green nucleic acid stain (Molecular Probes Inc., Eugene, OR, U.S.A.), with the reaction made up to 25 µl with sterile H₂O. The conditions used were 95°C for 2 min, then 95°C for 10 s, 50°C for 20 s, and 72°C for 30 s for 40 cycles, and 72°C for 5 min. The primers used for *Gna1* cDNA amplification were 5'-CCGACTACATCCCCAATGAC-3' (GapcrF) and 5'-GGCAGCTTCTCCTTGAAAC-3' (GapcrR). Amplification of an actin control fragment was performed as per *Gna1*. The primers used were as follows: 5'-CTGCTTTGAGATCCACAT-3' (actinF) and 5'-GTCACCACTTTCAACTCC-3' (actinR). For quantification of gene expression, the expression of *Gna1* was normalized against the actin expression for that particular time-point or growth condition. To ensure statistical confidence, each sample was assayed five times and the data was processed by the Student's *t*-test.

The PCR primers used for examining the *Gna1* transcript 5' and 3' of the transposon insertion site were *Gna1*-5f and *Gna1*-5r (5'-ATTTTCATGCAACCCGCAC-3' and 5'-CGATTGAGTCGAAGTAGCTAGG-3') and *Gna1*-3f and *Gna1*-3r (5'-CGTCAAGACATCCATCATCC-3' and 5'-CATACCGTTTAC TGCTGCC-3'). RNA extraction, reverse transcription, and PCR conditions were as mentioned above.

All sequencing was performed using a Perkin Elmer PCR sequencing premix and were analyzed on an ABI 373 or 377 automatic DNA sequencer.

***S. nodorum* spore PCR.**

Preliminary screening of fungal transformants was performed by spore PCR. A full loopful of transformant growth (including spores and mycelia) was scraped into 500 µl of DNA extraction buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS). The suspension was then placed at -20°C and was allowed to freeze before being thawed at 37°C. After repeating the freeze and thaw cycle, the suspension was centrifuged at 12,000 × *g* for 15 min to remove cellular debris, and the supernatant was added to an equal volume of isopropanol. The precipitated DNA was collected by centrifugation at 12,000 × *g* for 10 min and then was washed once with 70% ethanol before being air-dried and resuspended in 10 µl of sterile water. DNA solution (2 µl) was used in the resulting PCR, using the conditions listed above.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

SeqTool Treeview and ClustalX alignment software: www.seqtools.dk