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A class Vb chitin synthase in *Colletotrichum graminicola* is localized in the growing tips of multiple cell types, in nascent septa, and during septum conversion to an end wall after hyphal breakage

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Summary. Previous complementation of a chitin synthase class Vb null mutant (*Colletotrichum graminicola chsA*) indicated that the encoded protein is responsible for approximately 30% of the conidial chitin, is essential for conidial wall strength in media with high water potential, and contributes to strength of hyphal tips. We complemented a *chsA* null mutant with *chsA* fused to the green-fluorescent protein (*sgfp*) gene driven by a heterologous constitutively expressed promoter. Comparisons of the strain with the ectopic *chsA-sgfp* to the wild type indicated that ChsA-sGFP serves the same biological functions as ChsA in that like the wild type, the *chsA*Δ *chsA*::*sgfp* (EC) had conidia that did not explode and hyphal tips that did not swell. Confocal microscopy of ChsA-sGFP (EC) cells stained with the membrane stain FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide) indicated that ChsA is localized in the plasma membrane of the following: growing apices of hyphal branches, conidiophores, and falcate and oval conidia; in nascent septa; and in septa that are being converted to an end wall after hyphal breakage. The data support the hypothesis that *chsA* either directly or indirectly encodes the information for its localization, that ChsA is localized in the plasma membrane, and that the class Vb enzyme produces chitin synthase in multiple cells and after wall breakage.

Keywords: Chitin synthase; Confocal microscopy; GFP fusion; *Glomerella graminicola*; Fungal growth; Myosin motor.

Introduction

Although only 10 to 20% of the dry weight of walls of filamentous fungi is chitin, it is critical for wall strength and fungal survival. Chitin, a polymer of β-1,4 N-acetylglucosamine, is synthesized by chitin synthase (CS) (EC 2.4.1.16). Fungi produce multiple CSs (Ruiz-Herrera et al. 2002) and the isozymes are classified on the basis of amino acid sequence. Whereas ascomycetous yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* produce three and four isozymes, respectively, in three classes (I, II, and IV), ascomycetous filamentous fungi such as *Botrytis cinerea* produce eight isozymes in seven groups, including the traditional classes one through five and two additional groups (Choquer et al. 2004). While there is apparent redundancy in CSs between some classes, analysis of mutants with disruptions in class V CS (CS V) indicate that CS V is essential in filamentous fungi.

Class V CSs can be divided into two groups on the basis of amino acid sequence (Amnuaykanjanasin and Epstein 2003, Chigira et al. 2002, Choquer et al. 2004, Nino-Vega et al. 2000), and authors have suggested divisions into either class Va (CS Va) and Vb (CS Vb) (Amnuaykanjanasin and Epstein 2003), or the construction of a new class VI (Chigira et al. 2002, Choquer et al. 2004) or a new class VII (Nino-Vega et al. 2004); the class names are problematic because homologs of *Aspergillus fumigatus* ChsD, which differ from those discussed here, have also been called class VI (Mellado et al. 2003, Roncero 2002) and class VII (Choquer et al. 2004). Regardless of nomenclature, examples of what we will refer to as class CS Vb include *Colletotrichum graminicola* ChsA (Amnuaykanjanasin and Epstein 2003), *Paracoccidioides brasiliensis* Chs4 (Nino-Vega et al. 2000), *Aspergillus oryzae* ChsZ (Chigira et al. 2002), and *Botrytis cinerea* BcChsVI (Choquer et al. 2004). Utilizing databases with whole-genome fungal sequences, putative CS Vb were also identified in *Aspergillus fumigatus*, *Aspergillus nidulans*, *Histoplasma*

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capsulatum, and *Neurospora crassa* (Choquer et al. 2004). Interestingly, of the eight fungi with a known or putative CS Vb, apparently all also produce a CS Va (Aufauvre-Brown et al. 1997, Amnuaykanjanasin and Epstein 2003, Chigira et al. 2002, Choquer et al. 2004, Horiuchi et al. 1999, Mellado et al. 1996, Nino-Vega et al. 2004, Specht et al. 1996). Identified class Va CSs include *A. fumigatus* ChsE (Aufauvre-Brown et al. 1997), *A. nidulans* CsmA and ChsD (Fujiwara et al. 1997, Specht et al. 1996), *A. oryzae* ChsY (Müller et al. 2002), *Blumeria graminis* BgChs2 (Zhang et al. 2000), *Botrytis cinerea* ChsV (Choquer et al. 2004), *Colletotrichum graminicola* ChsC (NCBI accession AY052547), *Fusarium oxysporum* class V (Madrid et al. 2003), *Magnaporthe grisea* Csm1 (Park et al. 1999), and *Wangiella* (*Exophiala*) *dermatitidis* WdCHS5 (Liu et al. 2004).

To date, all mutants with disruptions in CS Va and in CS Vb have remarkable phenotypes. Of the reported members of CS Vb, the only gene that has been disrupted is *C. graminicola chsA* (*CgchsA*). *CgchsA* is present as a single copy and *chsA* disruptants have conidia that explode in media with high water potential and hyphal tips that swell; the phenotype is annulled by addition of osmotica to the media (Epstein et al. 1998, 2001; Amnuaykanjanasin et al. 2003). Complementation of the *C. graminicola chsA* disruptant T30 with *chsA* indicates that the encoded protein is responsible for production of 29% ± 6% of the conidial chitin (95% confidence interval), is essential for conidial wall strength in media with high water potential, and contributes to strength of hyphal tips (Amnuaykanjanasin and Epstein 2003, Amnuaykanjanasin et al. 2003). Genes in CS Va have been disrupted in five taxa and results indicate that CS Va is involved in the production of 20 to 70% of the hyphal chitin (Aufauvre-Brown et al. 1997, Horiuchi et al. 1999, Mellado et al. 2003, Specht et al. 1996). A variety of phenotypes, some of which may be species specific, have been reported for fungi with disrupted CS Va: swollen hyphal tips and conidiophore vesicles (Specht et al. 1996), periodic subapical swellings along the length of the hyphae (Aufauvre-Brown et al. 1997, Madrid et al. 2003, Müller et al. 2002a, Specht et al. 1996), proliferation of intracellular new hyphae (called intrahyphal hyphae) (Horiuchi et al. 1999, Müller et al. 2002a), irregularly positioned septa (Horiuchi et al. 1999), an increased number of subapical hyphal branches (Müller et al. 2002b), excessive conidial swelling and then lysis (Specht et al. 1996), reduced conidiation (Aufauvre-Brown et al. 1997, Müller et al. 2002a), reduced virulence in plants (Madrid et al. 2003) and animals (Liu et al. 2004), and increased sensitivity to plant-produced antifungal compounds (Madrid et al. 2003).

Only filamentous fungi produce CSs V. All reported CSs V have a C-terminal chitin synthase 2 catalytic domain. Except for the one characterized basidiomycete CS V (Garcerá-Teruel et al. 2004), all class V CSs have an N-terminal myosin-like domain with homology to myosin I ("single-headed myosin") (Cheney and Mooseker 1992). On the basis of sequence analysis of the myosin-like domain in two of the CSs Va, Hodge and Cope (2000) classified the CSs V in their own class (XVII) of myosins. On the basis of sequence analysis of both the myosin-like and the CS catalytic domains, there are differences between CS Vb and CS Va; the myosin-like domain in CS Vb appears to have diverged more from myosin than CS Va. CSs V also have cytochrome b_5-like heme/steroid binding domain(s), but no ligand for this domain is known (Marchler-Bauer et al. 2003).

Little is known about the cell biology of CS V in general and CS Vb in particular. Here, we complemented *C. graminicola* T30, which does not produce CS Vb, with a DNA construct with *CgchsA* fused to the reporter green-fluorescent protein (sGFP) gene; the construct was driven by the strong heterologous promoter *toxA* from *Pyrenophora tritici-repentis*. Confocal microscopy of ChsA-sGFP (EC) cells stained with the membrane stain FM 4-64 indicated that ChsA is localized in the plasma membrane of the following: growing apices of hyphal branches, conidiophores, and falcate and oval conidia; in nascent septa; and in septa that are being converted to an end wall after hyphal breakage.

Material and methods

Strains and vectors

Colletotrichum graminicola wild-type strain M1 and its insertionally mutagenized derivative T30 with a disrupted *chsA* were described previously (Amnuaykanjanasin and Epstein 2003) (Table 1). Two plasmids were obtained: pCT74 (Lynda Ciuffetti, Oregon State University, Corvalis, Oreg.) and pCB1534 (Fungal Genetic Stock Center, Kansas City, Kans.; Sweigard et al. 1997). *Escherichia coli* strain DH5-α was used to maintain all vectors.

Construction of the CgchsA-sgfp fusion

Standard molecular techniques (Sambrook et al. 1989) were used for purification of DNA plasmids, restriction enzyme digestion, and ligation. PCR reactions were performed in 50 µl containing 1× Platinum High Fidelity PCR buffer (Invitrogen, Carlsbad, Calif.), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.12 µM of each primer, and 1.5 units of Platinum High Fidelity *Taq* DNA polymerase (Invitrogen). All PCR amplifications were performed on a GeneAmp PCR 2400 (Perkin Elmer, Norwalk, Conn).

To construct the recombinant plasmid that encodes for a ChsA-sGFP translational fusion, *chsA* was amplified from the genomic DNA of wild type M1 as a 5349 bp fragment with the first ATG codon of the *chsA* open reading frame at the 5′ end and a 678 bp noncoding sequence at the 3′ end. *chsA* was amplified with the forward primer PLE48 (5′-GTGgaa

Table 1. *Colletotrichum graminicola* strains used in this study

Strain	Genotype [a]	Derivation	Functional ChsA	Comment on strain with functional GFP and ChsA
M1	(wild type) *chsA*$^+$	none	yes	
T30	*chsA*Δ::*hyg*^{R}	M1	no	
T30-ChsA-GFP [b]	*chsA*Δ::*hyg*^{R} *sgfp*$^+$::*chsA*$^+$(EC) *bar*^{R}	T30	yes	*toxA* promoter drives *sgfp* and *chsA*
M1-GFP	*chsA*$^+$ *sgfp*$^+$::*hyg*^{R}	M1	yes	*toxA* promoter drives *sgfp*; wild-type promoter drives *chsA*
T30-BAR	*chsA*Δ::*hyg*^{R} *bar*^{R}	T30	no	

[a] *chsA*, gene encoding for *C. graminicola* class Vb chitin synthase; EC, ectopic copy; *sgfp*, gene encoding for green fluorescent protein

[b] Three isolates were used with indistinguishable results: T30-ChsA-GFP-2CD, T30-ChsA-GFP-2DC, and T30-ChsA-GFP-2GG

ttcATGGCGAACGGTCGAATGTCCATG-3′) and the reverse primer PLE32 (5′-TTTTTTcctaggCAACGTGCCAGGTGTGAC-3′); the lowercase bases are introduced *Eco*RI and *Avr*II restriction sites, respectively. The thermal cycling program was as follows: 5 min at 95 °C; 5 cycles of 1 min at 94 °C, 1 min at 55 °C, and 5 min at 72 °C; 30 cycles of 0.5 min at 94 °C, 1 min at 55 °C, and 5 min at 72 °C; and 10 min at 72 °C. The PCR product was digested with *Eco*RI and *Avr*II and ligated to pCB1534, which had been cut with the same restriction enzymes; pCB1534 contains the *bar* gene, which encodes for resistance to glufosinate ammonium. The resultant recombinant plasmid is called pCB-ChsA.

The plasmid pCT74 (Lorang et al., 2001) contains the *toxA* constitutive promoter from *Pyrenophora tritici-repentis* (Ciuffetti et al. 1997) fused to a modified version of the green-fluorescent protein gene, *sgfp* (S65T; Chiu et al. 1996). The *toxA* promoter-*sgfp* cassette was amplified from pCT74 with primers PLE51 (5′-GGGatgcatTGGAATGCATGGAGGAGT-3′) and PLE52 (5′-GTTgaattc*ACCTCC*CTTGTACAGCTCGTCCATGCC-3′); the lowercase bases are introduced *Nsi*I and *Eco*RI restriction sites, respectively. The italicized sequence in PLE52 encodes a linker for two glycines; it is inserted after the open reading frame of *sgfp* and deletes its stop codon TAA. The thermal cycling program was as follows: 5 min at 95 °C; 5 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2.5 min at 72 °C; 30 cycles of 0.5 min at 94 °C, 1 min at 55 °C, and 2.5 min at 72 °C; and 7 min at 72 °C. After digestion of the PCR product with *Nsi*I and *Eco*RI, the *toxA-sgfp* fragment was ligated to the *Nsi*I and *Eco*RI-restricted pCB-ChsA. The resultant plasmid, called pTxA-sGFP-ChsA, contains *sgfp* fused in-frame to the N terminus of *chsA* (Fig. 1). The fusion plasmid was sequenced at the *toxA* promoter, the *sgfp* coding sequence, and the *sgfp-chsA* junction to verify the sequence could produce a functional translation product.

Transformation of C. graminicola mutant T30 and the selection for expression of ChsA and sGFP

The *C. graminicola chsA*-disrupted strain T30 was transformed as described previously (Amnuaykanjanasin and Epstein 2003) with pTxA-sGFP-ChsA. Transformants were selected for resistance to 50 μg of glufosinate ammonium (Sigma, St. Louis, Mo.) per ml on a minimal medium (Epstein et al. 1998). Putative transformants were examined for complementation of the phenotype caused by a disrupted *chsA*: conidial bursting and hyphal tip swelling. Assays for the phenotype were conducted as described previously (Epstein et al. 1998). Isolates that were resistant to glufosinate ammonium and that had the wild-type ChsA phenotype were screened for fluorescence with a Nikon SMZ-U dissecting microscope with optics for GFP.

As controls for transformation, wild type M1 was transformed with pCT74 and the T30 mutant was transformed with pCB1534 (Table 1).

Preparation of cells for microscopy

Colletotrichum graminicola falcate conidia were produced and collected as indicated previously (Epstein et al. 1998, 2001). Aliquots of conidia (8×10^2 in 100 μl of 0.15× potato dextrose broth) were incubated on multiwell glass slides in moist chambers at 22 °C for the following times for observations of the indicated structures or processes: 8 h for germ tubes; 2 days for hyphae and oval conidia; 3 days for falcate conidia and septa; and 4–5 days for recycling of chitin synthase. For examination of hyphal repair, when indicated, after 48 h incubation, hyphae were cut with a blade and then allowed to incubate for an additional 1 h before visualization of the hyphae in the severed area.

To determine whether the ChsA-sGFP fusion was expressed in the plasma membrane, when indicated, cells were poststained with FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide; Molecular Probes, Eugene, Oreg.) (Fischer-Parton et al. 2000, Hickey et al. 2002). After incubation, the liquid medium was withdrawn from the slide culture and mixed with a stock solution of 1 mg of FM 4-64 per ml of dimethyl sulfoxide for final stain concentrations of 14 and 28 μg of FM 4-64 per ml. After the slide culture was covered with the stain and incubated for 2, 10, and 20 min, the excess liquid was removed, a glass cover slip was applied, and the cells were observed by either fluorescence or confocal microscopy.

Fluorescence and confocal microscopy

For preliminary observations, we used a Nikon Microphot SA fluorescence microscope (Nikon, Melville, N.Y.) equipped with either filter B-2E (480 ± 15 nm for excitation and 535 ± 20 nm for the barrier) or an Endow GFP Longpass Emission FGP(R)-LP (470 ± 20 nm for excitation and 500 nm for the barrier). The latter filter block allows simultaneous visualization of green (GFP) and red (FM 4-64) fluorescence.

For confocal laser scanning microscopy, we used model MRC-1024 (Bio-Rad, Hercules, Calif.) mounted on a Nikon Optiphot microscope. All images were recorded in 1024 by 1024 pixels. All images were taken using either a 25× oil immersion objective lens with a 3× digital zoom or a 100× oil immersion lens with either a 1.5×, 2× or 4× digital zoom. Parameters for images with the 25× objective lens were as follows: laser power, 10–30%; iris, 2.3 to 3.0; and gain, 1236–1270. Parameters for images with the 100× objective lens were as follows: laser power, 30–100%; iris, 4.2 to 4.5; and gain, 1500. For each image, the offset voltage was adjusted until the background appeared black or nearly so. Kalman filtering (n = 6) was generally used to improve the signal-to-noise ratio of images. A z-series was used to collect images at different depths of fungal specimens. For each image, ten sections were collected with a focal step of 1 μm. Images were captured in TIFF files

by Laser Sharp (Bio-Rad) software. Composite images were prepared with Adobe Photoshop version 5.5 (Adobe, San Jose, Calif.).

Results

ChsA-sGFP serves the same biological functions as ChsA in C. graminicola

The *chsA*-disrupted mutant T30 was transformed with a *chsA-sgfp* fusion driven by the heterologous promoter *toxA* (Fig. 1). The 48 putatively antibiotic-resistant transformants were scored for GFP fluorescence and the wild-type phenotype for *chsA*: nonexploding conidia and nonswollen hyphal tips. The putative transformants were divided into the following groups: 20 were positive for GFP fluorescence and had the wild-type phenotype; four were positive for GFP fluorescence but did not have the wild-type phenotype; two were negative for GFP fluorescence but had the wild-type phenotype; and the remainder had neither GFP fluorescence nor the wild-type phenotype. Three isolates that were positive for GFP fluorescence and had the wild-type phenotype were examined by epifluorescence and confocal microscopy; no differences between the isolates were ever observed. Transformants carrying the *chsA-sgfp* fusion had colony morphology and radial growth rate similar to those of the T30 isolates complemented with *chsA* alone (data not shown). A comparison of transformants with different constructs (Table 1) indicates that the *chsA* open reading frame either directly or indirectly encodes for information for its localization. When the wild type was transformed with *sgfp* driven by the *toxA* promoter, i.e., with sGFP that was not fused to ChsA, sGFP was distributed throughout the cytoplasm and was not present in the vacuoles of young cells (Fig. 2). However, when the same *sgfp* driven by the same promoter was fused to *chsA*, the GFP was localized in the cells (Figs. 3–37). However, occasionally, there was some diffuse green fluorescence in the cytoplasm e.g., in conidiophores (Figs. 23 and 24), and hyphae (Figs. 29 and 31).

ChsA localization in plasma membranes of multiple cell types

Colletotrichum graminicola transformed with the *chsA-sgfp* fusion expressed the reporter protein in tips of nascent hyphal branches (Fig. 4). Staining with the plasma membrane stain FM 4-64 (Figs. 5 and 6) indicated that ChsA-sGFP is present in the plasma membrane (Fig. 6). The ChsA-sGFP fusion was also present in small particles in the cytoplasm, e.g., as indicated in Fig. 4. The ChsA-sGFP fusion was present in the plasma membrane of conidiophores and in the plasma membrane at the apex of nascent falcate conidia (Figs. 7–9). In nearly mature conidia that were still attached to their conidiophores, the ChsA-sGFP fusion was present in the plasma membrane, particularly in the apex. The ChsA-sGFP fusion was also present in the plasma membrane in the nascent septum between the conidium and the conidiophore (Figs. 10–12).

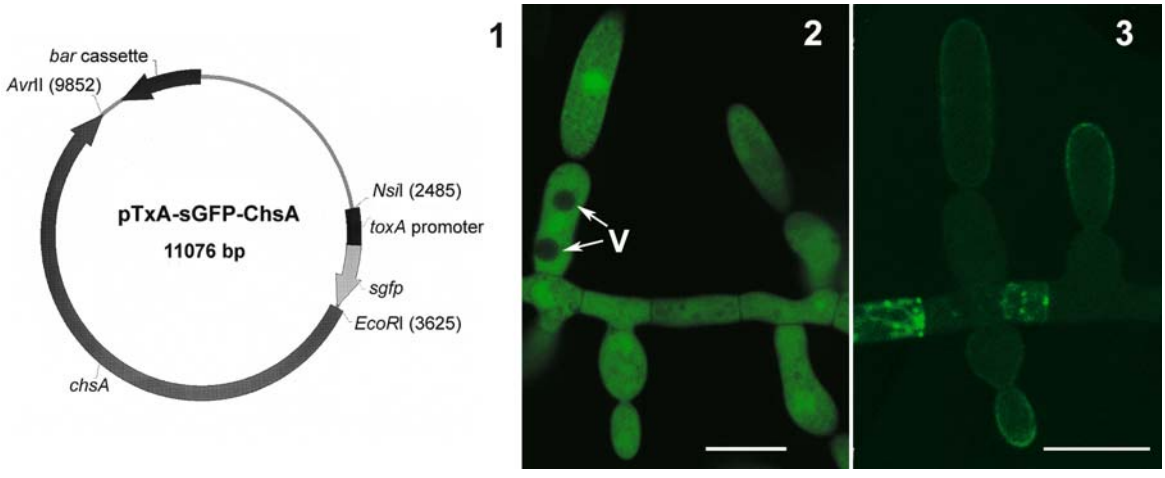
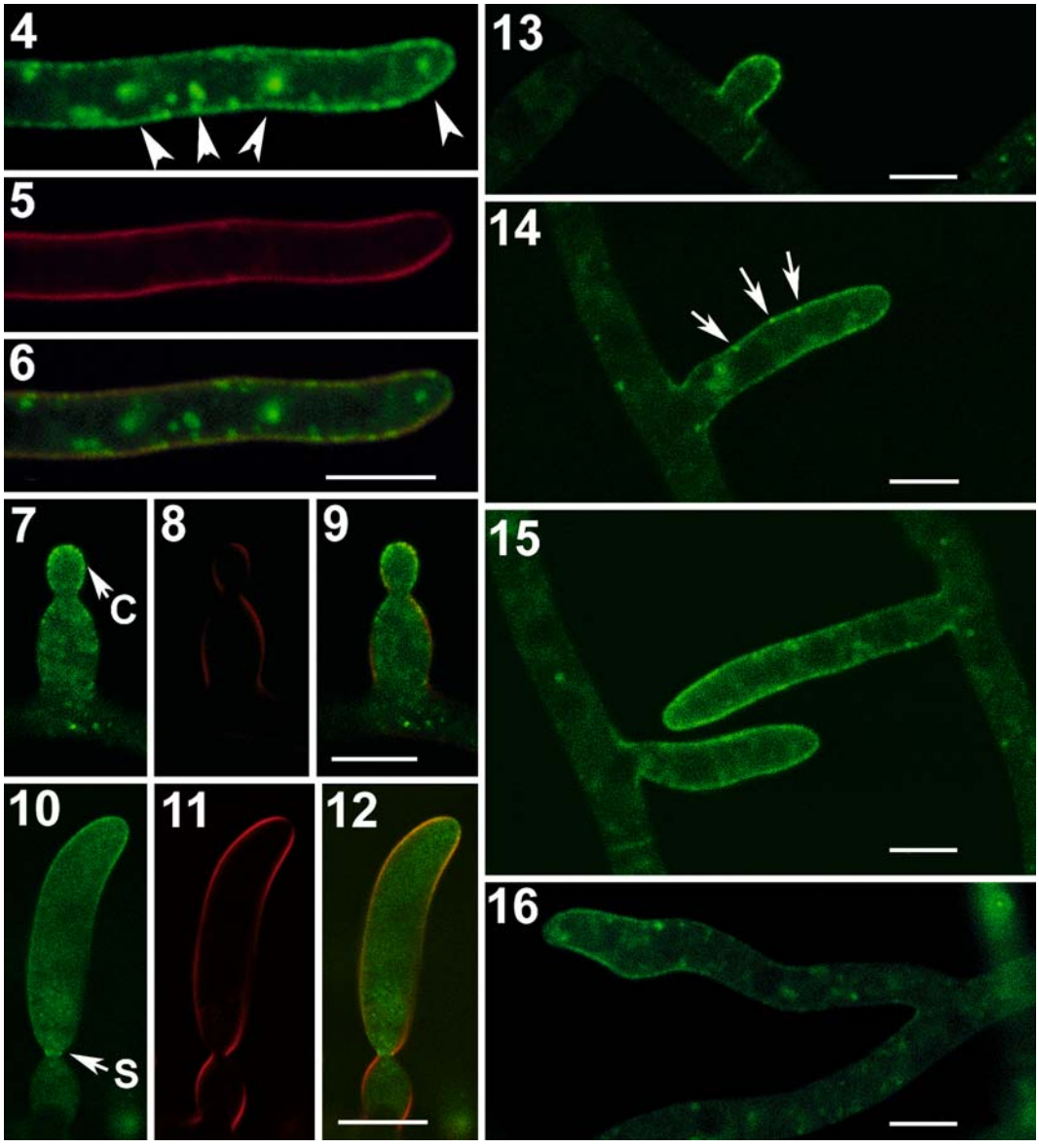


Fig. 1. Diagram of the construct pTxA-sGFP-ChsA with the constitutively expressed *toxA* promoter and the *C. graminicola* chitin synthase A gene (*chsA*) fused to a green-fluorescent protein gene (s*gfp*). The construct was used to transform *C. graminicola* T30, a strain with a disrupted *chsA*

Fig. 2. The wild-type *C. graminicola* transformed with pCT74, a construct with the constitutively expressed *toxA* promoter fused to s*gfp*. Note that when the sGFP is not fused to ChsA, the sGFP is distributed throughout the cytoplasm and nucleoplasm but is not present in the vacuoles. *V* Vacuoles. Bar: 10 μm

Fig. 3. As in all remaining figures, *C. graminicola* T30 (*chsA*Δ) that was transformed with the plasmid shown in Fig. 1, a *chsA-sgfp* fusion driven by the *toxA* promoter. Bars: 10 μm



Figs. 4–16. *Colletotrichum graminicola* ChsA fused to sGFP. The strongest fluorescent signal from ChsA-sGFP is in the growing apex of the cell. Bars: 5 μm

Figs. 4–12. *Colletotrichum graminicola* ChsA-sGFP was incubated in the plasma membrane stain FM 4-64 and then viewed by confocal microscopy. Images show the signals from sGFP (Figs. 4, 7, and 10), FM 4-64 (Figs. 5, 8, and 11), or both fluorochromes (Figs. 6, 9, and 12)

Figs. 4–6. In the tip of a hyphal branch, ChsA-sGFP is localized in the plasma membrane, and in particles in the cytoplasm, as indicated with arrowheads

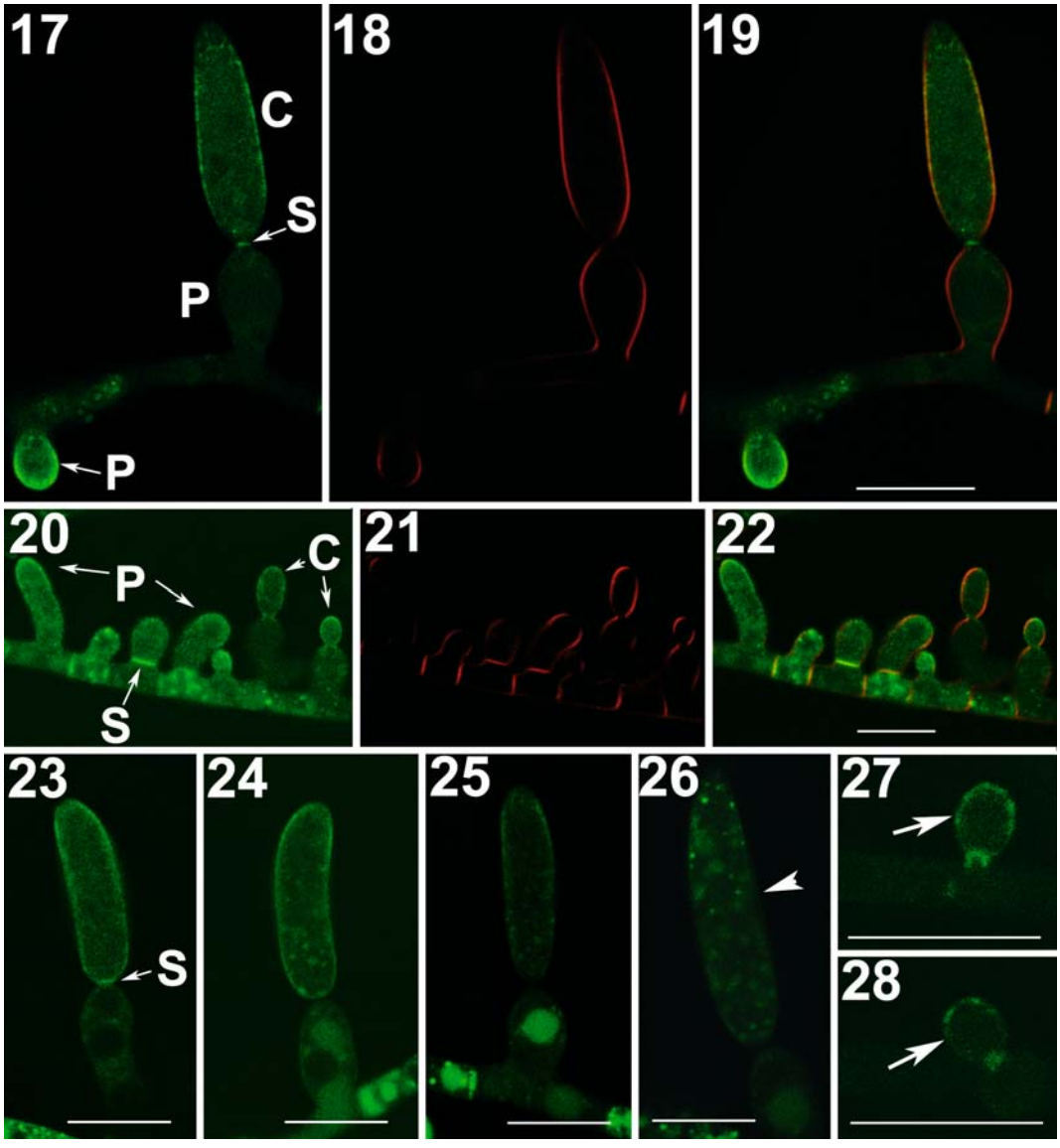
Figs. 7–9. In a developing conidium, ChsA-sGFP is localized in the plasma membrane of the apex of the nascent conidium (*C*)

Figs. 10–12. In an almost mature conidium, ChsA-sGFP is localized in the plasma membrane of the conidium and in the septum (*S*) between the conidium and the conidiophore

Figs. 13–16. Confocal micrographs of *C. graminicola* ChsA-sGFP branches of hyphae at various distances from the tip. Note the ChsA-sGFP is most pronounced at the hyphal tips and becomes weaker in older hyphae

Overall, the ChsA-sGFP was most pronounced in the plasma membrane adjacent to sites of de novo wall synthesis in multiple, but not all, cell types. Interestingly, the ChsA-sGFP signal was never observed in germ tube tips or in the tips of hyphae at the advancing edge of the colony even though growth was occurring (data not shown). In contrast, ChsA-sGFP was expressed in nascent hyphal branches. In hyphal branches, the ChsA-sGFP expression was strongest in the plasma membrane at the tips (Fig. 13); the signal was progressively weaker in the older

portions of the branches (Figs. 14–16). Similarly, the GFP-labeled intracellular particles were associated with growing apices, but only in cells with label in the plasma membrane. In Figs. 17–19, the strongest ChsA-sGFP signal is in the plasma membrane of a developing conidiophore (on the left side of Fig. 17); the older conidiophore (on the right side of Fig. 17) has no discernible signal, but its comparatively younger conidium and the septum be-



Figs. 17–22. *Colletotrichum graminicola* with ChsA fused to sGFP was incubated in the plasma membrane stain FM 4-64 and then viewed by confocal microscopy. Images show the signals from GFP (Figs. 17 and 20), FM 4-64 (Figs. 18 and 21), or both fluorochromes (Figs. 19 and 22). Bars: 10 μm

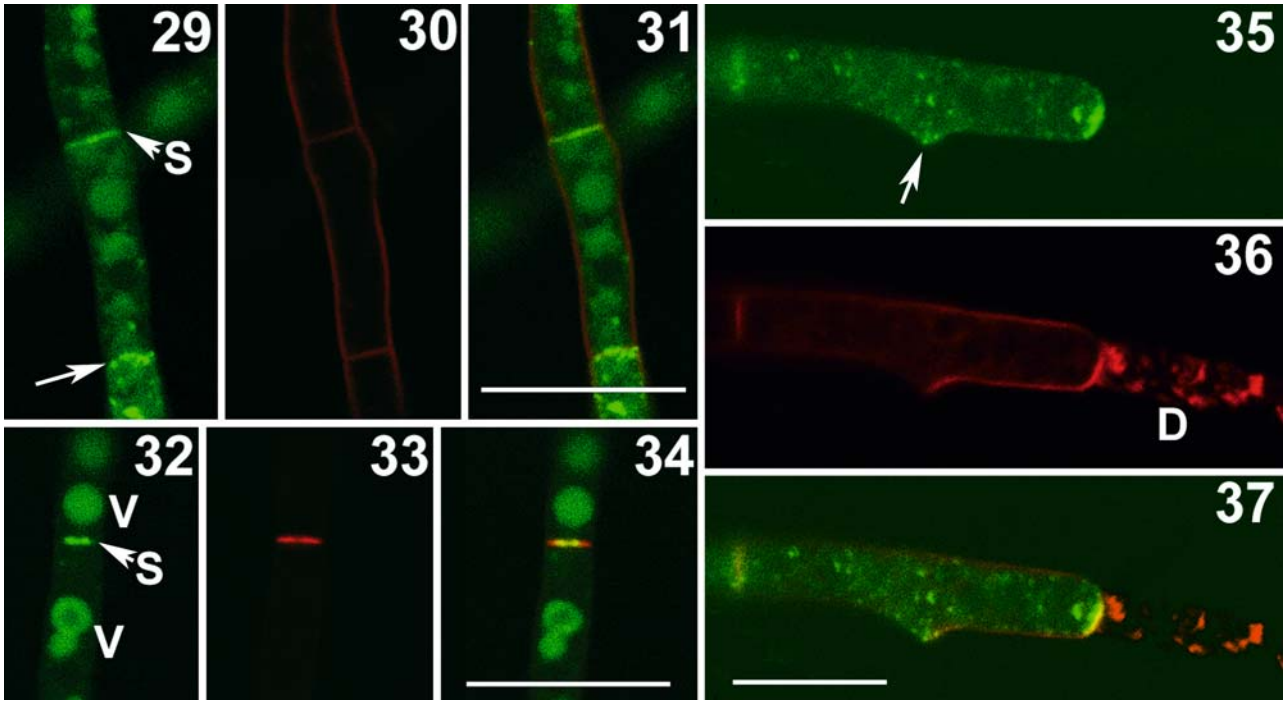
Figs. 17–19. ChsA-sGFP is expressed more in the developing conidiophore (*P*) on the left than in the mature conidiophore (*P*) on the right. In comparison to the mature conidiophore on the right, there is a stronger signal in the plasma membrane with the most recently produced wall: at the conidial (*C*) apex and at the septum (*S*) between the conidium and the conidiophore

Figs. 20–22. ChsA-sGFP is expressed most in the plasma membrane where new wall is produced: in a septum (*S*) and in tips of expanding conidiophores (*P*) and conidia (*C*)

Figs. 23–28. *Colletotrichum graminicola* with ChsA fused to sGFP was viewed by confocal microscopy. Bars: 10 μm

Figs. 23–26. Whereas an almost mature conidium that is still attached to its conidiophore expresses ChsA-sGFP at both the basal septum (*S*) and the apex (Fig. 23), there is less ChsA-sGFP expression as the conidium ages and dehisces, as indicated with an arrowhead in Fig. 26

Figs. 27 and **28.** During synthesis of oval conidia, ChsA-sGFP is expressed in the plasma membrane where nascent wall is produced, as indicated with arrows



Figs. 29–37. *Colletotrichum graminicola* with ChsA fused to sGFP was incubated in the plasma membrane stain FM 4-64 and then viewed by confocal microscopy. Images show the signals from GFP (Figs. 29, 32, and 35), FM 4-64 (Figs. 30, 33, and 36), or both fluorochromes (Figs. 31, 34, and 37). Bars: 10 μm

Figs. 29–34. In older cells, ChsA-sGFP is localized in the plasma membrane of the septa (*S*), and occasionally in particles (see arrow) adjacent to a nascent septum. In older cells, the GFP signal is not present either in the plasma membrane or in particles in the cytoplasm. Rather, the GFP is diffused in the vacuoles (*V*)

Figs. 35–37. In a broken cell, the ChsA-sGFP is expressed in the plasma membrane of the previously formed septum adjacent to the hyphal break; here, the septum is converted to an end wall. The membranous contents in the damaged (*D*) cell are stained by FM 4-64 (Fig. 36)

tween the conidium and its conidiophore have a moderately strong signal.

In Figs. 20–22, the ChsA-sGFP is shown in a variety of cell types, including conidiophores and conidia in different stages of development. ChsA-sGFP also is strongly expressed in nascent septa (Fig. 20). In keeping with the pattern in which ChsA-sGFP is most strongly expressed in nascent conidia (Fig. 7), as the conidium forms a septum (Figs. 10, 17, and 23), and finally dehisces (Figs. 23–26), the ChsA-sGFP signal decreases. Here, we have referred to the lunate-shaped "falcate" conidia as conidia. However, *C. graminicola* also produces oval-shaped conidia, which are not produced on distinct conidiophores (Panaccione et al. 1989). The pattern of ChsA-sGFP expression in oval conidia is the same as in the falcate conidia (Figs. 27 and 28) in that a strong signal is produced in the plasma membrane when the conidium is producing a nascent wall.

As indicated above, ChsA-sGFP is expressed in the plasma membrane during septum formation between conidiophores and conidia. ChsA-sGFP also is expressed in plasma membranes associated with nascent septum formation within hyphae (Figs. 29–31 and 32–34). Intracellular particles of ChsA-sGFP were sometimes observed near the nascent septum. When cells were damaged by breakage (Figs. 35–37), ChsA-sGFP was concentrated in the plasma membrane of a previously formed septum near the breakage; this septum became a new end wall of the hyphae.

As indicated above, intracellular particles (diameter of $<$500 nm) of ChsA-sGFP were observed adjacent to the plasma membrane (Fig. 14). The particles were also viewed in the cytoplasm (Figs. 4 and 35), although less frequently than adjacent to a plasma membrane. In older cells, the sGFP signal was often present in vacuoles (Figs. 25, 26, 32, and 34) and occasionally diffused throughout the cytoplasm (data not shown); this is consistent with eventual cleavage of ChsA and sGFP.

Discussion

The ChsA-sGFP fusion complemented the *chsA*-disrupted mutant T30 for the phenotype associated with the *chsA* disruption: the bursting of conidia and the swelling of hyphal

tips. Thus, *chsA* fused to an sGFP reporter gene appeared to function as the wild-type *chsA*. We believe that this is the first report of localization of any class Vb CS. Here, we localized the class V CS (ChsA) in *C. graminicola* in the plasma membrane of the growing apex of multiple cell types: tips of hyphal branches, conidiophores, falcate conidia, and oval conidia. In addition, ChsA was localized in the plasma membrane of septa under two conditions: in nascent septa, and in septa that due to hyphal breakage were apparently being made into new end walls. Thus, CgChsA-sGFP was localized near the site of wall breakage and at the apparent site of de novo wall synthesis, suggesting that it is involved in wound repair, as is a CS class IV in *S. cerevisiae* (Osmond et al. 1999). Previously, CSs were localized in *S. cerevisiae* and *Schizosaccharomyces pombe* (DeMarini et al. 1997, Holthuis et al. 1998, Matsuo et al. 2004, Valdivia and Schekman 2003). Sietsma et al. (1996) localized a class II CS in chitosomes in *Neurospora crassa* by immunolocalization with antibodies to the yeast class II CHS2. Recently, Takeshita et al. (2005) used immunolocalization to show that the CS Va *A. nidulans* CsmA is localized in the plasma membrane of apical tips and hyphal branches.

Although ChsA-sGFP is expressed in many cell types, we did not observe expression in all cell types. We visualized ChsA in almost all growing tips of nascent hyphal branches but only rarely in the growing tip of hyphae at the advancing colony margin. In addition, ChsA-sGFP was not observed in actively growing germ tubes. This may be due to different rates of growth in various tips.

Our confocal images support the classic view that CS is localized in the plasma membrane (Leal-Morales et al. 1988) of actively growing hyphal tips (Gooday 1971). It is improbable that the sGFP signal in the plasma membrane is from an sGFP-myosin motor-like domain that was cleaved from its chitin synthase domain. In *A. nidulans*, the class Va CsmA was cleaved between the N-terminal myosin motor-like domain and the C-terminal CS domain, but only after two days (Takeshita et al. 2002). Similarly, after a 4-day incubation (data not shown) and in older cells on slides that were incubated for shorter time periods, we observed GFP in the vacuoles and sometimes in the cytoplasm. This is consistent with cleavage of ChsA-sGFP, but only after de novo wall synthesis is completed.

We are unaware of information on the intracellular movement of CS Vb to the plasma membrane. Takeshita et al. (2005) demonstrated that the CS Va *A. nidulans* CsmA binds to actin. Most of the studies on intracellular movement of CS either have utilized *S. cerevisiae*, which does not have a class V CS, or have examined total CS without regard to class. *Saccharomyces cerevisiae* Chs3p, a class IV CS, is present in an inactive form in possibly Golgi compartments, and as an active enzyme in other intracellular membrane-bound vesicles and the plasma membrane (Bulik et al. 2003, Valdivia and Schekman 2003). Older biochemical evidence indicates that CSs are transported to the plasma membrane in chitosomes (Ziman et al. 1998), microvesicles with a diameter of approximately 60 nm (Leal-Morales et al. 1988) that aggregate in the Spitzenkörper and serve as a vesicle supply center for tip growth (Riquelme et al. 1998). After incorporation into the plasma membrane, there may be recycling of CSs via endocytosis of vesicles (Holthius et al. 1998). Here, we did not visualize a Spitzenkörper in *C. graminicola*, even with longer incubations with FM 4-64 (data not shown). Regardless, we sometimes viewed ChsA-sGFP particles in a region that was consistent with the Spitzenkörper (Figs. 4 and 35). Interestingly, Takeshita et al. (2005) did not immunolocalize AnCsmA, a CS Va, in a Spitzenkörper-like structure in *A. nidulans*.

Because isolates with an ectopic copy of *chsA* on a heterologous promoter express the sGFP reporter gene in the plasmalemma, but isolates with the same heterologous promoter express a nonfused sGFP in the cytoplasm, *chsA* either directly or indirectly has the information for its localization. *Colletotrichum graminicola* ChsA ORF has a C-terminal region with at least nine significant transmembrane domains that could contribute to its localization. In addition, all ascomycete CSs V, including CgChsA, contain an N-terminal myosin motor-like domain that might contribute to its movement. Indeed, Takeshita et al. (2005) demonstrated that the *A. nidulans* CS Va CsmA binds actin. However, whereas CSs Va have some features of a myosin motor (i.e., a P loop, the ATP-binding sites GESGAGKT or GESGSGKT, and switch I and II motifs), the CSs Vb only have a homologous amino acid sequence, with a homology less than that of the CSs Va. Here, we did not determine if the ChsA-sGFP and F-actin were colocalized; we were unable to label the F-actin in *C. graminicola* isolates with Texas Red-X phalloidin (Molecular Probes, Eugene, Oreg.) (data not shown), possibly because the microfilament network is labile after fixation (Heath 1990).

Here, *chsA* was driven by a constitutively and highly expressed promoter from *Pyrenophora tritici-repentis*, *toxA* (Ciufetti et al. 1997). In preliminary experiments, we constructed a plasmid containing *egfp* (Clontech, Palo Alto, Calif.) fused in-frame to the N terminus of *chsA* and driven by the *C. graminicola chsA* promoter. However, *C. graminicola* T30 transformants did not emit sufficient fluorescence for microscopic examination (data not shown). We did not determine whether the difference between the

results with this plasmid and pTxA-sGFP-ChsA were due to differences between expression of the reporter proteins eGFP and sGFP (Cormack 1996) or to differences in the strength of the endogenous promoter for *chsA* versus the heterologous *toxA*. Nonetheless, it is reasonable to assume that the *toxA* promoter overexpressed *chsA*. Indeed, in transformants with the ChsA-sGFP fusion, we sometimes observed background green fluorescence in cytoplasm. We also observed intense fluorescence in vacuoles, particularly in mature cells in which chitin synthesis was no longer required. Chung et al. (2002) observed a similar background fluorescence in *Cercospora nicotianae* that was transformed with the cercosporin resistance gene *pdx1* fused to GFP. They observed more background fluorescence with the PDX-GFP fusion under the control of the constitutively expressed *gpdA* promoter than with the fusion driven by the native *pdx1* promoter. Regardless, although there was no visible phenotype associated with any overexpression of ChsA, constructs with *chsA* driven by its own promoter should be utilized in future work.

Colletotrichum graminicola produces two types of conidia in culture and in planta. The lunate-shaped falcate conidia, which are produced on the surface of infected plants and are critical for splash-dispersed dissemination, are produced on distinct conidiophores and are shown in most of the figures, (e.g., Figs. 12 and 23). Oval-shaped conidia (Figs. 28 and 29) are not produced on distinct conidiophores and appear to serve in in planta dissemination (Panaccione et al. 1989). Previous phenotypic data indicated that *chsA*Δ reduces wall strength of both types of conidia (Amnuaykanjanasin and Epstein 2003; Epstein et al. 1998, 2001). Here, the localization of ChsA-sGFP supports the hypothesis that ChsA is involved in CS activity in both oval and falcate conidia. In addition, ChsA is involved in the production of the nascent wall between the conidiophore and its conidium before dehiscence (Figs. 17 and 23).

Genomic data indicate that only filamentous fungi have class V CSs, and that early in the evolution of the ascomycota, an ancestral CS V was duplicated and that there are two extant families: a better characterized CS V, which we have called CS Va, and a lesser characterized CS Vb. Determination of whether CS Va and CS Vb have redundant or unique functions remains to be determined. However, we have never observed phenotypes in CgChsAΔ reported in some CS Va disruptants: proliferation of intracellular new hyphae in AnCsmAΔ and AoChsYΔ (Horiuchi et al. 1999, Müller et al. 2002a) and periodic subapical swellings along the length of the hyphae in AnChsDΔ, AoChsYΔ, AfChsEΔ, and FoClass VΔ (Aufauvre-Brown et al. 1997, Madrid et al. 2003, Müller et al. 2002a, Specht et al. 1996). Overall, the CS Vb CgChsA appears to be primarily expressed in spores and the CS Va appears to be primarily expressed in hyphal tips.

Because chitin is not present in either mammals or plants, there has been interest in the development of fungicides that inhibit CS in order to control infections of plants, animals, and humans (Munro and Gow 2001). Consequently, fungicides that inhibit CS V might be of value because deletion mutants of these isozymes have such deleterious and pleiotropic phenotypes. Inhibition of CS Vb might be particularly useful, but too little is known about its biology to predict.

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