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Physiological, biochemical, and microscopic analyses of weakened walls of conidia in *Colletotrichum graminicola* with a disrupted class V chitin synthase gene, *chsA*

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Abstract

Colletotrichum graminicola (teleomorph, *Glomerella graminicola*) mutant T30 has a disruption in *chsA*, which is a class V or a class VI chitin synthase (class VI if recognized). The mutant conidia were significantly easier to fracture with glass beads than conidia of the wild type and two T30-derived strains that were complemented with *chsA*. Similarly, after extraction in alkali, the walls of significantly more mutant conidia collapsed in comparison to the wild type and the two *chsA*-complemented strains. These data provide genetic evidence that the chitin synthesized by ChsA is critical for conidial wall strength and rigidity. The conidial wall compositions of the wild type and the T30 strains were compared. The *chsA*-disrupted conidia had a significantly lower percentage of chitin and an alkali- and trifluoroacetic acid (TFA)-insoluble glucan (30 and 15% less, respectively). The mutant had a correspondingly higher content of a TFA-hydrolyzable glucan than the wild type. Both strains had similar percentages of covalently bound protein and alkali-insoluble polysaccharide(s) containing TFA-hydrolyzable mannose, galactose, arabinose, xylose, and rhamnose. The significant reduction in both chitin and the TFA-insoluble glucan supports the hypothesis that chitin is present in walls as a chitin-glucan complex and that the glucan's insolubility is due to its linkage to chitin.

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1. Introduction

Conidia of fungi such as *Colletotrichum graminicola* (teleomorph, *Glomerella graminicola*) are splash-dispersed in rain water and require free water for germination [9]. Consequently, the spores are exposed to an environment with a higher water potential than present inside the spore; cell walls are critical for preventing the explosion of cells under turgor pressure. Chitin, a microfibrillar, β-1,4 linked polymer of *N*-acetylglucosamine (GlcNAc) provides the structural skeleton for fungal cell walls and is considered to be indispensible for wall strength, even though the quantity of chitin varies between fungal species and cell types [27].

Models of the fungal wall generally posit the following: (1) chitin is localized in the inner layer of the wall [20] and is embedded in an amorphous matrix consisting primarily of polysaccharide with some glycoprotein [27]; (2) nascent walls, including the hyphal tips, become increasingly insoluble and rigid as the chitin forms crystalline microfibrils [32]; and (3) chitin is covalently linked to β-glucan [21].

The polymerization of GlcNAc into chitin is catalyzed by a variety of chitin synthases (CSs) [27]; most, if not all, filamentous fungi have multiple CSs [26]. Fungi with disrupted CSs have a variety of phenotypes that range from non-viable to apparently normal [24,26]. The CSs are currently classified into five classes based on their deduced amino acid sequences. Recently, Chigira et al. [5] suggested that class V is sufficiently divergent that a new class (VI) that is related to class V should be constructed. Mellado et al. [17,18] also suggested that there are six classes of CSs, but

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their class VI only contains a highly divergent CS-like protein encoded by *Aspergillus fumigatus chsD*; it is not related to the class V CSs. Regardless, no sixth class has been officially accepted, and all of the class V and their related putative VIs will be referred to here as class V CSs; previously, we classified the originally designated class V [30] as class V subgroup A [2] and Chigira et al.'s [5] class VI as class V subgroup B [2].

In contrast to the other four CS classes, all class Vs have homology to 'single-headed myosins' (also known as 'Myosin I'), except for *Ustilago maydis* Chs6, which lacks a myosin-like domain. Class V CSs apparently are only found in filamentous fungi because *Saccharomyces cerevisae* and other characterized yeasts do not have them. In the dimorphic fungus, *Paracoccidiodes brasiliensis*, there is more transcription of the class V *chs4* in hyphal than in yeast cells, even though yeast cells in this organism contain more chitin [25].

Currently, class V CS genes have only been disrupted in six fungi, primarily in *Aspergillus spp*. With one exception [2], all of the disruptions are in genes from class V subgroup A [2]. Our previous study is the only report of the disruption of a class V subgroup B [2].To date, the disruptions indicate that the class V CS genes are involved in the production of 20–70% of the hyphal chitin [3,13,17,30]. The disruptions further indicate that class V CSs are often critical for normal fungal growth and development [2,3,13,16,22,30]. Interestingly, the particular phenotypes of the disruptants vary but include swollen hyphal tips [2,30], periodic subapical swellings along the length of the hyphae [3,16,23,30], proliferation of intracellular new hyphae (called intrahyphal hyphae) [13,22], irregularly positioned septa [13], an increased number of subapical hyphal branches [23], abnormally swollen conidiophore vesicles that lyse [30], reduced conidiation [3,8,22], excessive conidial swelling and then lysis [30], conidial rupture without swelling [2], reduced virulence in plants [16] and animals [15], and increased sensitivity to plant-produced anti-fungal compounds [16]. Transcripts of both *Aspergillus nidulans csmA* [31] and *Fusarium oxysporum chsV* [16] are upregulated in conditions of hyperosmotic stress; expression in the latter is also upregulated in the presence of the plant-produced antifungal compound α-tomatine.

After using transformation to insertionally mutagenize *C. graminicola*, we isolated a mutant (T30) with a dramatically lethal phenotype of conidial rupture in media with high water potential [7,8]. The T30 phenotypes of conidial rupture and swelling at some hyphal tips, is annuled by addition of osmotica to the media. After sequencing the insertionally mutagenized gene in T30, the wild type gene was cloned and used to complement T30. Reverse-transcriptase PCR indicated that T30 did not express any mRNA in the presumed catalytic region of ChsA. Sequence analysis indicated that the disrupted gene encodes a CS that would be classified as either a class V or class VI, depending upon whether some current members of class V are placed

in a new class VI. The wild type and five strains derived from T30 that were complemented with *chsA* had conidia that did not rupture, no swollen hyphal tips and approx 21% chitin in the conidial walls. In contrast, the *chsA*-disrupted T30 and two non-complemented strains had conidia that ruptured, some hyphae with swollen tips, and significantly ($P < 0.001$) less chitin in the conidial walls [2]. The data indicated that ChsA directly or indirectly produces $29 \pm 6\%$ of the conidial chitin (95% confidence interval).

Here, to further examine the ramifications of the presence or absence of ChsA, we compared conidia of the *chsA*-disrupted strain T30 to two T30-derived strains that were complemented with *chsA*, and to the wild type. Condia were either shaken with glass beads or incubated in alkali to remove the alkali-soluble portion of the wall, and then examined microscopically for fragmentation and collapse, respectively. The results support the conclusion that ChsA is critical for wall integrity. To examine aspects of the current model of walls of filamentous fungi, the composition of the walls of the wild type and the T30 mutant were compared biochemically, with an emphasis on the alkali-insoluble fraction of the walls, which is responsible for wall strength and rigidity [11]. The results demonstrate that the decreased chitin content in T30 is accompanied by a significant decrease in trifluoroacetic acid (TFA)-insoluble glucan and an increase in TFA-soluble glucan. Finally, the TFA-insoluble fraction of the wild type was further characterized. Overall, the data indicate that the chitin produced by a class V CS is covalently linked with glucan, and that the insolubility of the glucan is at least partly due to its association with the chitin produced by ChsA.

2. Material and methods

2.1. Strains

The wild type M1, the *chsA*-disrupted T30, and two strains that were derived from T30 but complemented with *chsA* (Ch1510A and Ch59A) were described previously [2].

2.2. Assessment of the strength of conidial walls

Conidia were harvested from Petri dishes and washed as previously described [7]. The concentration of conidia was determined with a hemacytometer. First, to compare the mechanical strength of the walls in the mutant and the wild-type conidia, 3×10^8 conidia in 400 μl water were mixed in a 2 ml tube with 800 μl of 0.5 mm diameter glass beads and shaken in a Mini-BeadBeater™ (BioSpec Products, Inc., Bartlesville, OK, USA) for 40 s, and then again for another 20 s. Conidia were recovered from the glass beads by repeatedly suspending the beads in water and collecting the supernatant. Then, the conidia were concentrated by centrifugation and the concentration of unfragmented conidia was quantified in a hemacytometer.

As another measure of wall strength, conidia were extracted with ethanolic NaOH, and then assessed for wall rigidity. In a 15 ml tube, a suspension of 3×10^8 conidia in 400 µl water was mixed with 10 ml of 2:1 95% EtOH: 3 M NaOH and incubated at 85 °C for 1 h. The tube was inverted 3–4 times every 15 min. After incubation, the extracted conidia were collected by centrifugation at 1100 g for 8 min, rinsed once with 10 ml Tris–HCl, pH 7, and twice with sterile water. The conidia were re-suspended in 2 ml water and assessed microscopically for the percentage of collapsed conidia.

2.3. Chemical analysis of conidial walls

2.3.1. Preparation of walls and determination of dry weight

Culture conditions for the wild type and T30 were described previously [7,8]. Conidia were collected in water from an oatmeal agar plate (100 mm diameter), concentrated by centrifugation and transferred to a 2 ml microfuge tube. Equal volumes of 0.5 mm diam glass beads and conidial pellet were added to each tube. To disrupt the conidial walls, the suspension of beads and conidia was shaken with a Mini-BeadBeater™ for 40 s at 5000 rpm and then cooled on ice. The shaking-cooling cycle was performed until >99% of the conidia were broken (3–4 times). To separate the wall fragments from the beads, 1 ml of water was added to each tube, the tubes were vortexed and the supernatant was transferred to a 15 ml tube; the process was repeated five times. Conidial walls were collected by centrifugation at 1100 g for 8 min. After the supernatant was discarded, the wall fragments were cleaned of any remaining cytoplasm by incubation for 1 h in 10 ml 1 M NaOH at 85 °C. Walls were again concentrated by centrifugation, and rinsed once with 10 ml 1 M Tris–HCl, pH 7 and twice with water. Then, conidial walls were freeze-dried (BenchTop, Virtis Inc., Gardiner, NY, USA) for 48 h. Each dried wall preparation was weighed twice and the average mass was used for calculations. In all samples, the standard deviation for wall mass was 0.1 mg or less. There were three replicate wall preparations per strain, each of which was independently prepared.

2.3.2. Composition of the trifluoroacetic acid-soluble carbohydrates

Dried conidial wall preparations that weighed 5–7 mg were acid hydrolyzed with 5 ml 2 M TFA at 121 °C for 1 h in a 10 ml tube. One-half milligram inositol was included as an internal standard. The hydrolysate, excluding the remaining residue, was used for determination of sugars that were soluble in TFA (Fraction I in Fig. 3). After the hydrolysates were evaporated to dryness with N_2, the samples were resuspended in approx. 1 ml methanol and evaporated again. Alditol acetate derivatives of the monosaccharides produced in the hydrolysis step were prepared according to the procedures of Albersheim et al. [1] and Blakeney et al. [4], as described by Huysamer et al. [14]. Gas chromatographic separation and quantitation of alditol acetates was performed using an Autosystem Gas Chromatograph (PerkinElmer, Shelton, CT, USA) interfaced to a Perkin Elmer Sigma 10 data system as described by Huysamer et al. [14].

2.3.3. Analysis of the TFA-insoluble carbohydrates

After the TFA-hydrolysis as described above, the pellet was rinsed with 2 ml water three times. For initial quantification, the pellet was hydrolyzed in 72% (v/v) sulfuric acid at 80 °C for 1 h; no residue remained after hydrolysis. The supernatant (Fraction II in Fig. 3) was assayed with the anthrone-sulfuric acid assay [6] using glucose as a standard. Chitin was also hydrolyzed and was used to demonstrate that the anthrone assay does not detect GlcNAc.

The dried, TFA-insoluble residue was also analyzed by the University of Georgia Complex Carbohydrate Research Center. Glycosyl composition analysis was performed by GC-MS of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides [19,34]. Methyl glycosides were first prepared by methanolysis in 1 M HCl in methanol at 80 °C (18–22 h), followed by re-N-acetylation with pyridine and acetic anhydride in methanol, for detection of amino sugars. The samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce Chemical Co., Rockford, IL, USA) at 80 °C for 0.5 h. GC-MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD (Agilent Technologies, Wilmington, DE, USA), using a Supelco EB1 fused silica capillary column (Supelco, Bellefonte, PA, USA). Because only 25% of the mass of the sample was accounted for in this analysis, an aliquot of the original TFA-insoluble residue was again hydrolyzed in 2 M TFA at 121 °C for 2 h before methanolysis, re-N-acetylation, trimethylsilylation and GC-MS analysis as described above. Analyzed carbohydrates accounted for 92% of the mass of this sample.

For glycosyl linkage analysis, the sample was permethylated, depolymerized, reduced, and acetylated; the resultant partially methylated alditol acetates (PMAAs) were analyzed by GC-MS. Initially, an aliquot of sample was permethylated [34] with sodium hydroxide and methyl iodide in dry DMSO. The permethylation was repeated twice in order to aid complete methylation of the polymer. The permethylated material was hydrolyzed using 2 M TFA for 2 h in a sealed tube at 121 °C, reduced with NaBD₄, and acetylated using acetic anhydride/pyridine. The resulting PMAAs were analyzed on a Hewlett Packard 5890 GC interfaced to a 5970 MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30 m Supelco 2330 bonded phase fused silica capillary column.

To determine the ratio of alpha and beta linkages, approx. 1 mg of the sample was deuterium-exchanged by lyophilization from D_2O and dissolved in 0.5 ml DMSO-d6. One- and 2-D 1H NMR spectra were determined on a Varian Inova 500 MHz spectrometer at 298 K (25 °C). Proton chemical shifts were measured relative to the DMSO-d5 multiplett ($d = 2.49$ ppm).

2.3.4. Quantification of covalently-bound protein

To isolate walls for protein analysis, conidia were collected and cracked with glass beads, and the wall fragments were separated from glass beads as described above. Instead of the alkali treatment used to clean the walls for carbohydrate analysis, the conidial walls were washed as follows. The conidial wall fragments were washed four times with 10 ml of 1 M NaCl containing 1 mM phenylmethyl sulfonyl fluoride (PMSF), a protease inhibitor. Then walls were washed two times with 10 ml of water containing 1 mM PMSF. To remove non-covalently bound proteins, walls were boiled for 5 min in 50 mM Tris–HCl (pH 7.8), 2% SDS, 100 mM Na-EDTA, 40 mM β-mercaptoethanol, and 1 mM PMSF. After walls were collected by centrifugation, the washing was repeated once. Then walls were washed by centrifugation five times with water and freeze-dried for 48 h. After weight determination, covalently-bound wall proteins were released by boiling the walls in 1 M NaOH for 10 min (Fraction IV in Fig. 1), and quantified with the bicinchoninic acid reagent as previously described [29] using glycine as standard.
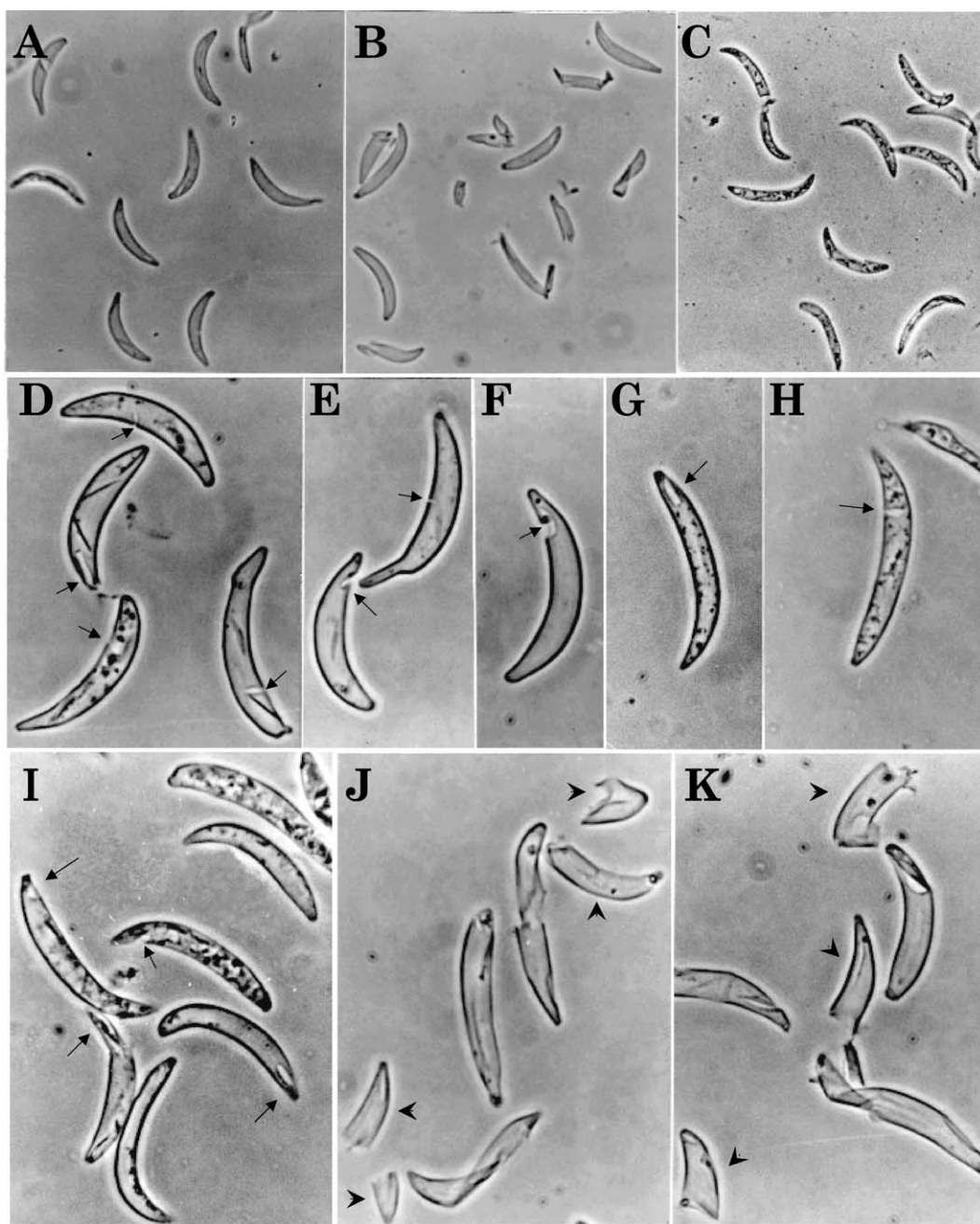

Fig. 1. After shaking with 0.5 mm diam glass beads for 60 s, micrographs taken at 160× (A–C) and 400× (D–K). Wild type M1 (A), *chsA*-disrupted T30 (B), and Ch59A, a strain derived from T30 that was complemented with *chsA* (C). Wild type (D–F) and complemented Ch59A (G–I), show small breakages in the walls, as indicated by the arrows. In contrast, mutant T30 (J–K) show fragmented walls, as indicated by arrowheads.

Table 1
Strength and rigidity of conidial walls in the *C. graminicola* wild type, the *chsA*-disrupted mutant T30, and two strains of T30 complemented with *chsA*

Strain	Derivation	Fragmented conidia after shaking with glass beads, % ± SEM	Collapsed conidia after extraction with NaOH, % ± SEM
M1	Wild type	8 ± 2 a	4 ± 2 a
T30	Transformant with disrupted *chsA*	51 ± 2 b	50 ± 3 b
Ch1510A	T30 complemented with *chsA*	16 ± 5 a	4 ± 1 a
Ch59A	T30 complemented with *chsA*	11 ± 4 a	7 ± 2 a

Data were analyzed by an ANOVA for a completely randomized design. Within a column, values followed by the same letter were not significantly different ($P \geq 0.05$) by Tukey's hsd test.

2.4. Analysis of experimental data

For each experimental trial, there were three replicates per strain and all experiments were repeated at least once with similar results. For the protein quantification, data were analyzed by an an analysis of variance (ANOVA) for a randomized block design using four independent trials as blocks. For the other experiments, data from a single trial were analyzed by ANOVA for a completely randomized design. In the text, 'significant' refers to $P < 0.05$.

3. Results

3.1. Wall strength of conidia

For a test of mechanical strength of conidial walls, conidia were shaken with glass beads for 60 s. Whereas only 8% of the wild type fragmented, significantly more (51%) of the *chsA*-disrupted T30 conidia were broken apart (Table 1, Fig. 1). The percentage breakage in two strains of T30 that were complemented with chsA was indistinguishable from the wild type. In the unfragmented conidia of either the wild type (Fig. 1E and F) or a *chsA*-complemented strain (1G), approx. 90% had a single tiny physical breakage of the walls that resembled a split or a hole. For the unfragmented T30 conidia, the splits in the walls were more extensive; in some, there were big tears that almost separated each conidial end in a transverse direction. The edges of the tears of T30 conidial wall fragments were often tattered in comparison to the smoother tears in the fragmented M1 walls.

In a test of rigidity of conidial walls, conidia were extracted in a solution of NaOH and EtOH and then examined microscopically. Whereas only approx. 5% of the wild type and the *chsA*-complemented conidia collapsed (Table 1, Fig. 2), 50% of the T30 conidial walls collapsed. Conidia with collapsed walls were often twisted or folded.
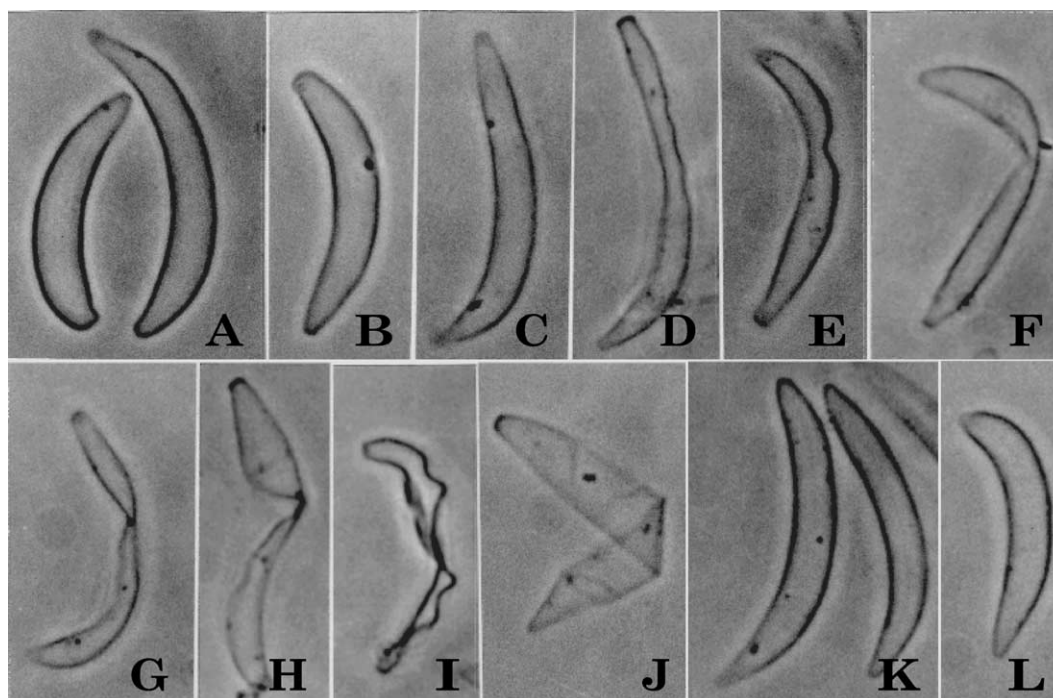

Fig. 2. Micrographs of conidia after extraction with NaOH in EtOH. Wild type M1 (A–B), *chsA*-disrupted T30 (C–J) in which panel (C) shows a non-collapsed conidium and panels (D–J) show a variety of collapsed conidia. Ch59A, T30 complemented with *chsA* (K,L).

In addition, some of the conidia in the mutant T30 appeared to have thinner walls (Fig. 2J); this was never observed in the wild type or the two *chsA*-complemented strains.

3.2. Conidial wall components

Here, we analyzed conidial wall components in four different fractions (Fig. 3), which accounted for essentially all (107%) of the mass of the conidial walls of the wild type. Chitin accounted for 20% of the mass of wild type conidial walls and covalently bound protein accounted for 16% (Table 2). The alkali-insoluble but TFA-soluble fraction in the wild type accounted for a total of 39% of the mass of the walls and contained six monosaccharides: primarily glucose (77% of the fraction), with comparatively small amounts of mannose (12%), galactose (9%), arabinose (1%), xylose (1%), and rhamnose (0.5%).

The alkali- and TFA-insoluble fraction in the wild type accounted for 26% of the mass of the wall, according to the anthrone-sulfuric acid assay. Glycosyl composition analysis of the TFA-insoluble fraction that was partially solubilized by methanolysis had 86 mol% glucose and 14 mol% *N*-acetyl glucosamine. After the TFA-insoluble fraction was rehydrolyzed in TFA before methanolysis, the sample was completely solubilized and contained 92 mol% glucose, 3% *N*-acetyl glucosamine, 2% rhamnose, 2% galactose and 1% mannose. Linkage analysis indicated that the TFA-insoluble fraction contained primarily 1,3-linked glucopyranosyl residues (94%) and much lesser amounts of 1,4-linked (4%) and terminal (2%) glucosyl residues. *N*-acetyl glucosamine was detected in a trace amount as 1,4-linked *N*-acetyl glucosamine. H NMR and TOCSY NMR suggested that the ratio of β to α-linked glucose is 1.3:1.

In the *chsA*-disrupted T30, we accounted for approx 101% of the mass of the conidial wall. F-tests of individual components indicated that T30 conidial walls contained significantly less of two components: 31% less chitin, and 15% less of the TFA-insoluble glucan. Statistically, T30 walls contained the same percentages of mannose, galactose, the three trace monosaccharides, and covalently bound protein as the wild type. However, the *chsA*-disrupted
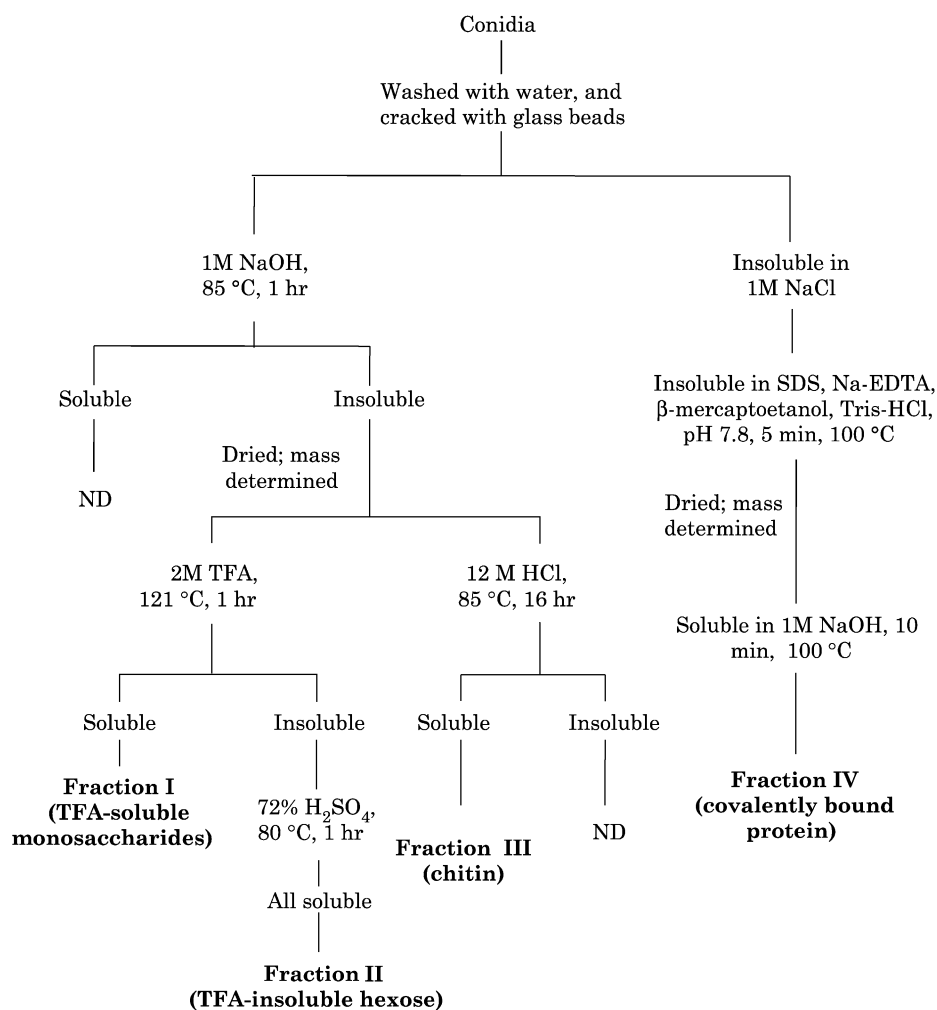

Fig. 3. Fractionation of *C. graminicola* conidial walls for the quantification shown in Table 2. ND, not determined.

Table 2
Composition of conidial walls in the *C. graminicola* wild type and the T30 mutant, which is disrupted in *chsA*

Compound	μg compound mg^{-1} dry weight of walls $\pm$ SEM of strains		*P*-value[a]
	Wild type M1	Mutant T30	
N-acetylglucosamine[b]	212 ± 10	147 ± 10	0.011
TFA-soluble glucose	306 ± 3	387 ± 12	0.016
TFA-soluble mannose	46 ± 0	43 ± 1	NS[c]
TFA-soluble galactose	34 ± 2	32 ± 3	NS
TFA-soluble trace monosaccharides[d]	10 ± 0.2	10 ± 0.2	NS
TFA-insoluble glucan[e]	278 ± 5	237 ± 7	0.024
Covalently-bound protein	170 ± 4	158 ± 7	NS
Total	1066	1014	–

Sample preparation and analysis is summarized in Fig. 3.
[a] Data were analyzed by an ANOVA with three and four replicates for the carbohydrates and protein, respectively.
[b] Results for *N*-acetylglucosamine were reported previously [2]. To quantify glucosamine, dried conidial walls were hydrolyzed in 12 M HCl at 85 °C for 16 h.
[c] NS, not significant, $P \geq 0.05$.
[d] Fraction had arabinose, xylose and rhamnose.
[e] Data were quantified as glucose equivalents by the anthrone-sulfuric acid assay. Further characterization of this fraction is described in the results section.

conidia contained significantly (27%) more TFA-soluble glucose than the wild type.

4. Discussion

4.1. The chsA-disrupted mutant has significantly less of both chitin and a TFA-insoluble glucan in its conidial walls: genetic evidence of a TFA-insoluble chitin-glucan complex

Our data indicate that the *chsA*-disrupted T30 contains significantly less chitin in conidial walls than the wild type. This is not surprising given that *chsA* encodes a CS. In comparison to the wild type, the reduction in chitin in T30 was accompanied by a significant reduction in glucose in the TFA-insoluble fraction. Wessels and his colleagues postulated that chitin in fungal walls is present as a chitin–β-glucan complex and that complexed glucan is less soluble in alkali than its uncomplexed form [12,21,33]. More specifically, in *Saccharomyces cerevisae* and *Aspergillus fumigatus*, chitin is linked via a β-1,4 linkage to β-1,3-glucan [10]. Our findings support the hypothesis that some GlcNAc residues are covalently linked with 1,3-glucose, and suggest that the complexing of glucan with chitin contributes to glucan's insolubility in TFA. This hypothesis is supported by the following data for *C. graminicola*. (1) The TFA-insoluble fraction in the wild type contained GlcNAc (14 mol% or less depending on how the fraction was prepared) and glucose. (2) In T30, the decrease in chitin content was accompanied by a decrease in the TFA-insoluble glucan. (3) In T30, the decrease in TFA-insoluble glucan was accompanied by a significant increase in TFA-soluble glucan. (4) The monosaccharide compositions of the TFA-soluble and the TFA-insoluble fractions in the wild

type were similar; both contained primarily glucose with some mannose, galactose and rhamnose.

Although the data indicate that the *chsA*-disrupted strain contained significantly more TFA-soluble glucan than the wild type, it is important to note that the wall components were determined on a per unit mass of walls, not a per spore basis. Thus, given that the proportion of chitin is less in the *chsA*-disrupted strain, the proportion of another compound(s) in the wall mass must be greater, whether or not there is a larger quantity on a per spore basis. Because the simplest hypothesis is that the disruption of *chsA* would not induce de novo synthesis of a TFA-soluble glucan, we postulate that the 27% increase in glucose in the TFA-soluble fraction is partly due to the decrease in T30 chitin and partly due to recovery of some of the normally TFA-insoluble glucan in a more soluble fraction. Mellado et al. [17] produced a mutant of *Aspergilus fumigatus* with disruptions in two CSs, a class III (*chsG*) and a class V (*chsE*). Their strain has half the mycelial GlcNAc of the parental strain, and a significant increase in α-1,3-glucose in the alkali-soluble fraction. They suggested that the reduction of chitin was compensated for by an increase in α-glucan. However, this may be an artifact of calculating relative composition on a unit mass basis, as discussed above. Nonetheless, we note that our carbohydrate analysis was limited to the alkali-insoluble fraction of the wall, which has been classically used for fungal wall preparations because of concerns about cytoplasmic contamination.

Using the wild type, after we isolated the alkali-insoluble fraction of the conidial walls, we partially characterized the TFA-insoluble fraction. More than 90% of the glucose residues in this fraction were 1,3-linked with only small amounts of 1,4-linked and terminal residues. No 1,6-linkages were detected. Fontaine et al. [10] characterized the total alkali-insoluble component of hyphal walls of

Aspergillus fumigatus and noted the following: (1) β-1,6-glucan was absent; and (2) chitin, a linear β-1,3/1,4-glucan, galactomannan, and a β-1,3-glucan with β-1,6 branches were present. Chitin, the linear β-1,3/1,4-glucan, and galactomannan were covalently linked to the nonreducing ends of β-1,3-glucan side chains. Their data were consistent with a model in which β-1,6 branches from β-1,3 glucan are formed early in wall synthesis and provide acceptor sites for chitin, galactomannan, and the linear β-1,3/1,4-glucan [10]. Although we fractioned *C. graminicola* walls differently and conducted fewer biochemical analyses, our results are consistent with theirs.

4.2. A class V chitin synthase, chsA, is essential for the assembly of a strong conidial wall

Previous microscopic analysis indicated that the T30 walls ripped open under turgor pressure whereas the wild type did not [7]. Here, our data demonstrate that conidia of the *chsA*-disrupted mutant T30 have inherently weaker walls than conidia of either the wild type or two *chsA*-complemented strains. The T30 conidia were significantly less resistant to fragmentation with glass beads. When conidia were shaken with glass beads, approx. 90% of the conidia in the three strains with the wild type phenotypes had a minor tear or a hole in them but were not fragmented, whereas in T30, half of the conidia were fragmented. Also, T30 conidia with a tear or a hole were observed in a lower frequency than in the wild type. This suggests that when mechanically stressed, walls of T30 are more likely to completely tear apart than in the wild type, indicating the mutant walls have less tensile strength than the wild type walls. Confocal microscopy of conidia that were stained with fluorescently labelled wheat germ agglutinin and conconavalin A had no apparent differences in the width of the stained walls between T30 and the wild type (data not shown).

In comparison to the three strains with wild type phenotypes, significantly more of the *chsA*-disrupted conidia collapsed after cytoplasmic and wall contents were extracted with ethanolic NaOH. That is, after extraction, T30 conidial walls were significantly less rigid and more deformable. Some of the T30 walls that were extracted with ethanolic NaOH appeared thinner than their wild type counterparts (Fig. 2J). However, it is not clear whether the post-extraction collapse is due to the lower quantity of chitin and its complexed glucan in T30 walls, or whether there was more material extracted from T30 compared with wild type walls. Historically, fungal wall components have been divided into two groups based on their solubility in hot alkali. The alkali-insoluble fraction localized on the inner layer of the wall contains chitin, cellulose (when present), and β-glucans that are complexed to chitin [28]. The alkali-soluble fraction on the outer layer contains glycans, e.g. α-1,3-glucan and α-1,4-glucan, and mannoproteins.

Although chitin content in mycelial cell walls was not determined, the pronounced phenotype of conidial rupture in comparison to occasional swellings in the hyphal tips suggests that ChsA is expressed to a greater extent during conidiogenesis than in hyphal tip growth. In addition, preliminary microscopic analysis of a strain with green fluorescent protein fused to ChsA indicates that *chsA* is expressed more during conidiogenesis than during hyphal tip growth (data not shown). Although only the falcate conidia were studied here, which are epidemiologically the important spore, the other spore types in *C. graminicola* T30 appear to be adversely affected too, and more adversely affected than the mycelia. A skewed segregation ratio in random ascospores versus complete tetrads from crosses of T30 and the wild type indicates that fewer T30 ascospores germinate compared to the wild type [8]. Oval conidia, which are apparently involved in intraxylem movement, were more readily protoplasted in T30 than in the wild type [8]. Although *Aspergillus nidulans* with a disrupted class V CS (*chsD*) have condia that lyse [30], the *chsA* disruption in *C. graminicola* is unique amongst the current class V disruptants in having a comparatively spore-specific phenotype. It is unknown whether this is due to the fact that walls of a cylindrically shaped conidium, as in *C. graminicola*, have less tensile strength compared to walls of spherical conidia as in *Aspergillus spp.* [7], the class VI designation has biological significance and too few CS class VI disruptants have been characterized, or that CS class in general is not necessarily correlated with function or phenotype. Regardless, *chsA* is an essential gene for *C. graminicola* survival in conditions in which nutrient rich-conidia are in solutions with high water potential such as rainwater.

Acknowledgements

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