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Chitin in the fungal cell wall: Towards valorization of spent biomass of Aspergillus niger

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CHAPTER 7

General Discussion

Filamentous fungi are intensively used in industrial biotechnology for the production of various enzymes, antibiotics, organic acids and other metabolites (Meyer et al., 2020). As exploitation of fungi to produce these (natural) products will continue to expand, so will the demand for large-scale fermentations. To make these high-value products, fungi are fed a diet of sugars, nitrogen and other essential organic elements such as phosphorous, potassium, sulfur and magnesium along with trace elements of other metal ions. In turn, the fungus grows to form biomass and secretes of large amounts of a desired product that can be harvested. During biomass formation, the supplied sugars are partially converted into glucan and chitin, the two main polymers that constitute the fungal cell wall. The cell wall is an essential structure for the fungus as it provides mechanical strength to withstand turgor pressure, but is also important under natural circumstances for the protection against external penetration and protection against environmental attack (Gow et al., 2017). In terms of mass, the cell wall accounts for approximately 50-80% of the mycelial dry weight, depending on fungal species (Isaza-Pérez et al., 2020). As such, the cell wall takes up a considerable part of the supplied sugars that are not available for the production of the desired fermentation product (e.g. acids or proteins). Post-fermentation, the mycelial biomass that has accumulated during the production process is generally disposed of and potentially precious polymeric sugars are lost. To recover and recycle these polymeric sugars from post-fermentation biomass waste into an added-value product, the fungal chitosan (FunChi) project set out to optimize extraction and production of the polymeric sugars chitin and chitosan from the fungal cell wall. The research described in this thesis addressed both production of chitin and provided insight into the way that chitin is attached to the cell wall. The findings from this work are discussed in this chapter and help to lay the foundations for profitable utilization of chitin and chitosan from fungal cell walls.

Chitin and its de-acetylated derivative, chitosan, are of high industrial interest due to the wide range of applications. The field of application of chitin/chitosan depends their properties such as molecular weight, oligomer length, degree of acetylation and patterns of acetylation (Dhillon et al., 2012; El Gueddari et al., 2014). As chitin/chitosan is not encountered in plant or human tissue, it often acts as an elicitor to plant and animal immune responses in order to fight off possible impending fungal infections. The application of both chitin and chitosan oligomers have been shown to prime plants against infection. Additionally, chitin and chitosan have been shown to benefit healthy plants in drought protection and may also promote total biomass accumulation (reviewed in Das et al., 2015; Hidangmayum et al., 2019; Orzali et al., 2017). During this project, the FunChi partners in Germany and Spain have focused on the chitin and chitosan

composition from fungal cell walls and their effects on the plant immune response, respectively. Our contribution to the FunChi project focused on i) increasing production of chitin in fungal cell walls and ii) removal of covalent cross-links of chitin to the cell wall that would allow for easier, environmentally friendly and cheaper extraction. of chitin We have taken a fundamental approach to tackle these two applied research challenges. To increase chitin production, we have screened an existing *Aspergillus niger* cell wall mutant strain library for mutants with a high chitin content. The research that is presented in this thesis both unravels the previously unknown involvement of three genes that dictate the amount of chitin deposition (described in **Chapters 4, 5 and 6**). Furthermore, we assessed the impact of removing the covalent cross-link between chitin and glucan (**Chapters 2 and 3**). In an attempt to modify chitin cross-linking, we set out to disrupt the seven-membered chitin cell wall crosslinking enzyme encoding gene family in *A. niger* that was considered to be responsible for the covalent cross-links between β-1,3-glucan and chitin. In the yeast *Saccharomyces cerevisiae*, the deletion of the genes encoding these glucan-chitin crosslinking enzymes result is a Congo Red hypersensitive (*CRH*) phenotype which have given the name to this gene family. The *A. niger* mutant in which all seven *crh* genes were deleted, was screened for phenotypic and transcriptomic alterations as a proxy for cell wall integrity.

Because the cell wall is such an important, complex and dynamic structure, many paralogues are found in the gene families that contribute to its construction, modification, fortification and degradation/recycling (Muszewska et al., 2018; Pel et al., 2007). The *crh* gene family in *A. niger* has putative involvement in cell wall construction and/or fortificaton and constitutes seven paralogues that have not been studied before. In contrast, the *S. cerevisiae* genome harbors three *CRH* genes, only two of which are active during vegetative growth. The role of these *CRH* genes have been extensively studied in relation to cell wall biosynthesis (Blanco et al., 2015; Cabib et al., 2008, 2007; Rodríguez-Peña et al., 2000a). Because redundancy in cross-linking of chitin to β-1,3-glucan was demonstrated for *CRH1* and *CRH2*, a likely scenario of redundancy in *A. niger* required the knockout of all seven *crh* genes. The construction of a seven-fold knockout presented a practical constraint with the available state-of-the-art transformation techniques at the start of the FunChi project.

1. Dealing with technical challenges for strain construction: CRISPR/Cas9 for gene editing

Over the years, advances in molecular techniques have allowed easier and more efficient construction of single gene knockouts in *A. niger* (Arentshorst et al., 2012; Carvalho et al., 2010). A major step in gene knockout efficiency has been through disruption of the Non-Homologous End-Joining (NHEJ) DNA repair pathway, leaving only homologous recombination (HR) as the default DNA repair pathway. In the wild type *A. niger* strain, the efficiency of HR is approximately 7% using the bipartite or "split marker" approach for gene knockouts, whereas disruption of the NHEJ pathway (through a *kusA* gene knockout; *ku70* homolog) boosted to HR efficiency to >80% (Arentshorst et al., 2015; Meyer et al., 2007). Two downsides of *kusA* disruption are (i) the

reduction in the ability to repair broken DNA and (ii) the increase in sensitivity towards radiation (Meyer et al., 2007a). To avoid permanent disruption of *kusA*, a clever *amdS*-based loop-out mechanism was devised to allow restoration *kusA* after transformation (Arentshorst et al., 2012).

The construction of an entire gene family knockout, such as the seven-membered *crh* gene family, poses additional challenges to knockout efficiency. To analyze the impact of a single gene deletion, the split marker technique relies on the integration of a selection marker at the gene of interest (GOI) which is easy to use and quick to generate. However, when multiple knockouts are desired in a single strain, the split marker technique can become time-consuming and problematic in its use. In the first place, the number of available selection markers for *A. niger* is limited (Niu et al., 2016b). Additionally, the use of integrative selection markers restricts the use of any selection markers that may be required for complementation studies. Some of the available markers are recyclable (Arentshorst et al., 2012; Carvalho et al., 2010), but this approach is laborious and requires counter-selective compounds that can be of mutagenic nature, such as 5'-FOA (Wellington et al., 2006). Moreover, even more problematic is that the expression of (auxotrophic) selection markers can be affected by the site of integration, described as "position effects", that compromise conclusions of gene function (Miki et al., 2009). This has specifically been shown for *pyrG/pyr4/URA3* auxotrophic markers in *Aspergillus nidulans*, *Aspergillus flavus*, *Neurospora crassa* and *Candida albicans* (Bok et al., 2006; Greenstein et al., 2006; Lay et al., 1998; Luo et al., 2016; Miki et al., 2009; Oestreicher et al., 2008; Robellet et al., 2010; Staab and Sundstrom, 2003). In this thesis, we also observed a similar "position effect" for the *Aspergillus oryzae pyrG* (*AOpyrG*) selection marker in a split-marker knockout experiment for both *crhE* and *crhG* (**Chapter 2**). Integration of the *AOpyrG* selection marker gene at these loci showed uridine-dependent growth-retardation phenotype, most likely due to aberrant expression. Similar to *crhG*, we observed

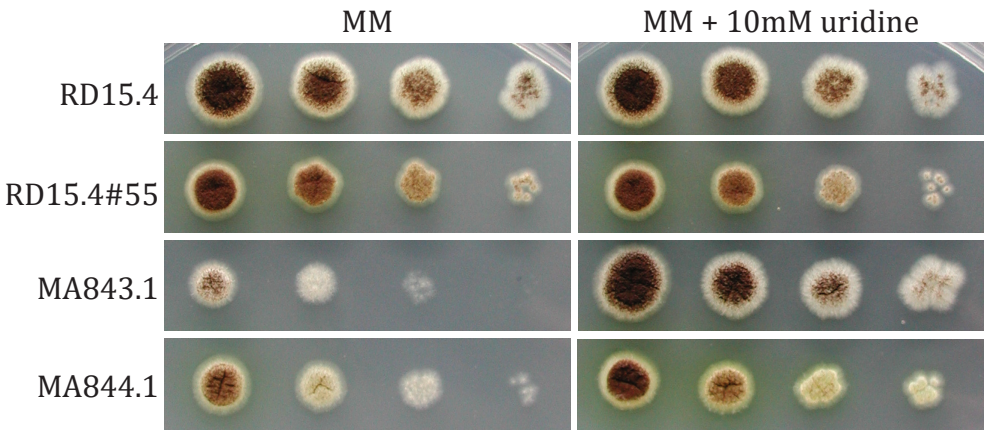

Figure 1. Growth on either minimal medium (MM) or MM with 10mM uridine. Parental strain and mutant (RD15.4#55) and single knockouts of *agsC* (MA843.1) and *cwcA* (MA844.1). MA843.1 and MA844.1 show a reduced growth phenotype on MM, but the growth phenotype is completely complemented upon supplementation of uridine. This indicates that the phenotype on MM in MA843.1 and MA844.2 is caused by uridine/uracil deficiency.

this "position effect" in **Chapter 4** for the knockout of *agsC* (MA843.1), and to a lesser extent for *cwcA* (MA844.1), where *AOpyrG* was used as in integrative selection marker to create single knockouts (Figure 1.) In both cases, a reduced growth phenotype was observed on selective medium, whereas these phenotypes were fully complemented by supplementation of uridine to the medium.

In **Chapter 2**, we describe a CRISPR/Cas9-based approach that circumvents the integrative selection marker problem mentioned above, by removing the reliance on integration of selection markers altogether. Instead, a gene knockout now requires a non-integrative plasmid, harboring a constitutively expressed Cas9 gene, a GOI-specific sgRNA and a hygromycin (*hph*) resistance cassette, along with a PCR-generated selection marker-free repair DNA fragment. This DNA repair fragment—homologous to flanks on either side of a GOI—was shown to successfully repair a Cas9-induced double strand break (DSB), effectively removing the gene through HR (**Chapter 2**). As such, CRISPR/Cas9-based gene editing still requires disruption of *kusA* for high HR efficiency. After desired gene editing, we showed that the Cas9/sgRNA plasmids can be easily cured by removal of selection pressure. This consequently permits the recycling of the same selection marker-containing plasmid—using a different GOI-specific sgRNA—for subsequent gene editing steps. Moreover, up to three plasmids—identical apart from the GOI-specific sgRNA—can be transformed to *A. niger* simultaneously, along with their respective PCR-generated repair DNA fragments, to generate a triple knockout. This strategy drastically improves the speed of which multiple knockouts can be made; in about one-third of the time of 5'-FOA induced *AOpyrG* recycling which is based on an estimation of the required construction time for a seven-fold knockout using either CRISPR/Cas9 recycling (73 days) or *AOpyrG* recycling (214 days), as mentioned in **Chapter 2**.

Next to the quick, easy and seamless construction of a seven-fold knockout strain in **Chapter 2**, we have also demonstrated that our CRISPR/Cas9 procedure works well to carry out precise gene-editing of single nucleotides, as described in **Chapter 4** and **Chapter 5**. In these chapters, we describe the identification of phenotype-responsible SNPs for two separate mutants and, importantly, prove single SNP involvement. This was done by re-creating these mutations in wild type backgrounds, at the endogenous locus of the relevant alleles in both RD15.8#16 (*gdiA* and NRRL3_05482) and RD15.4#55 (*cwcA*), a feat that is more difficult using traditional gene editing methods. Traditional methods either introduce a mutant allele ectopically, through HR with a selection marker (Arentshorst et al., 2015) or as large DNA constructs that contain mutant alleles fused to a selection maker that require to fully integrate into the genome at the endogenous locus. In either case, the expression levels of ectopically introduced genes have the potential to bring out "position effects" as described above. These possible side-effects can now be avoided altogether by using CRISPR/Cas9-based gene editing.

Aside from the advancements in multiplex gene editing, the current state-of-the-art CRISPR/Cas9-based gene editing leaves some to be desired. In particular, we encountered that in some cases (as in the case of NRRL3_05482) sgRNA design was somewhat limited by the lack of suitable

PAM sites to guide the Cas9 for gene editing. Many efforts have been made to design strategies that allow (almost) unrestricted use of Cas9-based gene editing to counter this constraint in the future. These efforts include protein engineering to alter PAM-recognition sites of current Cas9 enzymes (Liu et al., 2019; Miller et al., 2020; Nishimasu et al., 2018), usage of alternative Cas9-like enzymes that naturally contain different PAM-recognition sites, such as Cpf1 (Zetsche et al., 2015) (PAM site on 5' end of the guide RNA as 5' – TTTN – 3'), MAD7 (Inscripta, Inc, Boulder Colorado) and engineered variations thereof (Bin Moon et al., 2018; Gao et al., 2017) or engineered sgRNA structures (Kocak et al., 2019). Lastly, CRISPR/Cas9-based gene editing works very well in NHEJ deficient strains, but remains inefficient and unpredictable in existing strains with a wild type *kusA.* The inefficiency of CRISPR/Cas9-based gene restoration in strains with wild type *kusA* became evident in **Chapter 4**, where the *cwcA* mutant allele was restored to a wild type allele in RD15.4#55. A possible way to negotiate this hurdle is by temporal knock down of *kusA* through RNA silencing mechanisms, such as with small interfering RNAs (siRNA). Ideally, these would either be co-transformed as siRNAs together with CRIPSR/Cas9 plasmids and repair DNA fragments or siRNA expressing constructs can be integrated and expressed from CRISPR/Cas9 plasmids directly. Short hairpin RNAs (shRNA) have effectively been shown to be expressed from a vector and silence genes in different fungi (Dang et al., 2011). As such, implementation of a *kusA* targeting shRNA cassette on Cas9/sgRNA expressing vectors may provide an elegant solution absolve dependability on NHEJ deficient mutants by default in the future. Such an approach may be of particular relevance if transformation of industrial strains is desired, as these strains are usually not NHEJ deficient. The findings listed above, ongoing work and future plans on CRISPR systems continue to expand the genome editing toolkit and, in the foreseeable future, are likely enable any type of genetic alteration, anywhere in the genome.

2. Chitin attachment in the fungal cell wall

Chitin is known to be cross-linked to the fungal cell wall. Based on *S. cerevisiae* studies it was shown that Crh enzymes facilitate chitin cross-linking to both β-1,3-glucan and β-1,3-glucan branches of β-1,6-glucan (Cabib et al., 2008, 2007; Kollár et al., 1997; Rodríguez-Peña et al., 2000b). Absence of the Crh enzymes Crh1p and Crh2p resulted in complete loss of these covalent links between chitin and β-1,3-glucan, and an increased susceptibility towards Congo Red (CR) and Calcofluor White (CFW). In **Chapter 2,** we describe the construction of an *A. niger* strain in which all seven *crh* genes have been deleted. Surprisingly, we found that this seven-fold *A. niger* mutant did not show increased susceptibility to either CR or CFW and only showed slightly more compact colonies on plates under all tested conditions. Neither did we find differences in sensitivity towards other cell wall disturbing compounds nor in growth rate in batch-fed cultivations. Even more strikingly, both extensive in-depth analysis on transcriptomic level and cell wall composition of the seven-fold *crh* deletion strain revealed only minor differences compared to the wild type strain (**Chapter 3**). Consequently, we conclude that the effect on the cell wall of *CRH* gene family knockout in *S. cerevisiae* does not translate to the effect of a *crh* gene family knockout in *A. niger*. When looking

into detail, the relative cell wall composition of *A. niger* is different from *S. cerevisiae* and contains additional polysaccharides, such as α-glucan and galactomannan. In **Chapter 3**, we present evidence suggesting that these polymers compensate for the loss of *crh* gene function. Due to the interactions that exist between chitin and α-1,3-glucans—the core structure of what provides rigidity to the cell wall of filamentous fungi (Kang et al., 2018)—we hypothesized that the chitin cross-links and α-glucan act redundantly in tethering chitin to the cell wall. In correspondence to that, we have found that partial removal of *A. niger* α-glucan synthesis (by deleting *agsA* and *agsE*), in addition to the seven-fold *crh* family knockout, resulted in disturbed cell wall integrity that was demonstrated by increased sensitivity towards CR and CFW. Separately, knockouts of either *agsA* and/or *agsE* or *crhA-G* did not show the same effect and suggests that the interaction

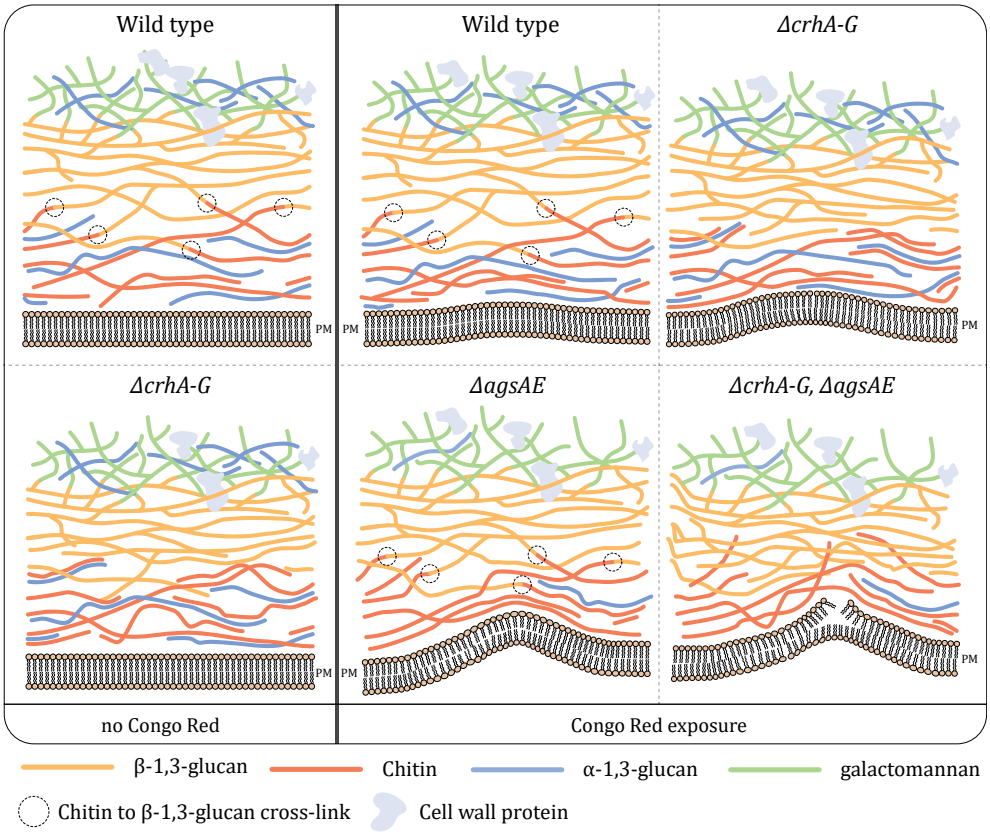

Figure 2. Schematic representation of the cell wall and the role of chitin crosslinks and α-glucan during cell wall stress in filamentous fungi. The left panel shows both the wild type and *ΔcrhA-G* cell wall and plasma membrane (PM) under normal conditions. Congo Red (CR) CR is thought to disrupt chitin and β-glucan assembly by which it weakens the cell wall (Klis et al., 2007), the effect of which is shown for both the wild type and three mutants (*ΔcrhA-G*, *ΔagsAE* and *ΔcrhA-G* + *ΔagsAE*) in the right panel. In the wild type, addition of CR causes slight bulging of the PM. Similarly, removal of the *crh* genes (*crhA-G*) does not affect cell wall integrity under CR-induced cell wall stress. Disruption of alpha-glucan synthases *agsA* and *agsE* reduces the total cell wall alpha-glucan and, in the presence of CR, weakens the cell wall even more, shown by the effects on cell membrane deformation. A combined knockout of the *crh* gene family and *agsAE* causes significant weakening to the cell wall resulting in lysis of the PM when CR is added.

between these two polymers acts as a redundancy mechanism for chitin-to-cell-wall anchorage. A schematic representation of how the knockouts of *crhA-G* and *ags* genes may affect the cell wall when exposed to CR-induced cell wall stress is shown in Figure 2.

Because the levels of cell wall α-glucan were not assessed in the *ΔcrhA-G*, *ΔagsAE* and *ΔcrhA-G* + *ΔagsAE* mutants, it remains unknown to what extent cell wall α-glucan is reduced, as *agsB*, *agsC* and *agsD* are still present. As α-glucan is present in two parts of the cell wall, namely the mobile outer layer and the hydrophobic inner layer, our hypothesis would suggest that the remaining α-glucan of the *ΔagsAE*-mutants resides in the more mobile outer layer, not interacting with chitin. A five-fold α-glucan synthase knockout would be required to study this in more detail. In case of other *Aspergillus* species, α-glucan synthase family knockouts were not found to be lethal, but do show phenotypic effects, such as reduced conidial aggregation, resulting in more dispersed submerged growth (Yoshimi et al., 2017). These are likely effects that result from loss in α-glucan from the outer mobile layer of the cell wall. However, it is worth noting that in *Aspergillus fumigatus* the loss of all α-glucan synthases resulted in a compensatory increase of both β-glucan and chitin (Henry et al., 2012), suggesting that total loss of α-glucan synthases affects more than the outer layer of α-glucan. With special regard to the FunChi project goals, it would be highly interesting as a future project to obtain both an α-glucan synthase family knockout and a α-glucan synthase family knockout combined with the seven-fold *crh* deletion strain, to assess cell wall composition and chitin extraction efficiency.

In the light the reported findings, it remains difficult to explain why there are so many *crh* genes in *A. niger* and other filamentous fungi (Arroyo et al., 2016). Apparently, natural selection has allowed the diversification and maintenance of multiple *crh* genes in the genome. It is not the first encounter where an attempted gene family knockout—orthologous to yeast—did not result in a significant phenotype in a filamentous fungus. Deletion of 11 mannosyltransferases responsible for establishing α-1,6 and α-1,2-mannose linkages in *A. fumigatus* did not affect mannan content in the mycelial cell wall, but only the conidial cell wall, despite highly conserved protein sequence with yeast (Henry et al., 2016). Later it was found that two other orthologs, with a different function in yeast, were important for this mannan polymerization in mycelial walls (Henry et al., 2019). In another study of cell wall enzymes, the GH76 family, responsible for the insertion of galactomannan into the cell wall, was recently investigated in *A. fumigatus*. Here, a disruption of only one out of the seven genes *dfg3* (*defective in filamentous growth*) displayed significant growth phenotypes and loss of cell wall galactomannan, whereas both single and multiple knockouts of other *GH76* members did not show any aberrant growth phenotypes (Muszkieta et al., 2019). Similar to the eleven-membered mannosyltransferases, seven-membered *dfg* family, the number for other cell wall biosynthesis genes found in *A. niger* is also high, e.g. five alpha-glucan synthases, ten chitin synthases, seven β-1,3-glucanosyltransferases, thirteen chitinases and ten mannosyltransferases (Pel et al., 2007). A similar "lack of effect" as in the studies described above could actually be the case for the GH16 *crh* gene family. A recent study characterized the five-membered *crh* gene family in *A. fumigatus.* The authors revealed the chitin to glucan cross-linking ability of those Crh enzymes, but single and multiple knockouts failed to show a

clear cell wall sensitivity phenotype (Fang et al., 2019), similar to our own observations for *A. niger*. Apparently, the cell wall permits the loss of cell wall biosynthesis related gene families with high paralog numbers, without a clear phenotype.

If filamentous fungi possess an α-glucan-chitin-interaction redundancy mechanism that renders that *crh* gene family dispensable, why do filamentous fungi contain more *crh* copies than yeast-like fungi that do not possess α-glucan? What is the evolutionary advantage that drove the duplication to attain so many *crh* genes? A possible explanation for the high number of *crh* paralogs is the diversity in developmental stages of filamentous fungi compared to unicellular growth. It is true that multi-condition expression data analysis showed differential expression of *crh* genes during different developmental conditions (Table 4, **Chapter 3**). Nevertheless, lack of Crh enzymes did not seem to affect cell wall integrity, vegetative growth, growth rate, sporulation or even formation of sclerotia. Therefore, it is difficult to see the morphological differentiation of filamentous fungi as an argument to explain the existence of so many *crh* genes, especially if α-glucan interactions with chitin suffice for structural cell wall integrity. However, it is worth noting that the evolution of cell wall α-glucan is considered to have emerged after the introduction of the *crh*-facilitated β-glucan to chitin transglycosylation (Kiss et al., 2019); possibly allowing selection pressure to drive the diversification of Crh enzymes during different growth conditions before the occurrence of α-glucan. The subsequent evolution of α-glucan—already recognized as an important virulence factor for pathogenic fungi and conidial aggregation factor in non-pathogenic species (Yoshimi et al., 2017)—may thus have gained an additional benefit as a chitin-interacting polymer that creates an additional layer of cell wall integrity.

If α-glucan provides such a beneficial role, this would mean one of two things for the *crh* gene family: i) The *crh* genes have become obsolete in all filamentous fungi that also possess α-glucan in their cell walls. Therefore, one would expect either gene loss or detrimental effects such as digression into pseudogenes, but no such detrimental effects are observed as active sites and substrate binding pockets are conserved. It is therefore likely that Crh enzymes still perform a relevant function. ii) There is still a benefit of having the *crh* gene family, but the benefit may be unclear under laboratory testing conditions. Singular phenotypic assessments may hamper the interpretation of the biological relevance of this multi-paralog gene family. In other words, we might fail to see how the disruption of (what we see as) one process, is actually not just a standalone component. Instead, the process of cell wall cross-linking has become an integral part of cell wall construction in conjunction with its other elements. This means that selection pressure for cell wall integrity may not occur at the level of individual components per se, but rather on the cell wall as a whole. To put this in context, the natural habitat of *A. niger* is the soil which poses a nutrient poor and competitive environment that is filled with hostile organisms competing over nutrients. These competitors possess an arsenal of various biological weapons that include chitinases and other putative cell wall destructive enzymes, and may attack the cell wall at structurally different levels. As such, having multiple layers of redundant mechanisms to maintain cell wall integrity makes a bit more sense from a biological perspective.

3. Exploitation of cell wall mutants for increased chitin production

Chitin consists of poly-β-1,4-linked *N*-acetyl-glucosamine that makes up a significant fraction (~16%) of the cell wall of *A. niger* (Isaza-Pérez et al., 2020). To synthesize chitin, UDP-*N*-acetyl-glucosamine monomers are assembled into a chitin chain by chitin synthases. At the cytosolic side of the plasma membrane, chitin synthases add UDP-*N*-acetyl-glucosamine monomers to the non-reducing end of an existing chitin chain in a β(1→4) fashion, a process in which UDP is cleaved off. Because chitin synthases reside in the plasma membrane, the chitin chain is protruded from the cytosol, through the chitin synthase, into the periplasmic space where it becomes part of the cell wall. Once in the cell wall, chitin can either be cross-linked to β-1,3-glucan or remain un-linked as "free" chitin. Chitin can also be remodeled through change of acetylation patterns or degree of polymerization by chitin deacetylases and chitinases/chitosinases, respectively. Break-down can either lead to chitooligosaccharides (COS) or *N*-acetylglucosamine/glucosamine monomers that can be reused in the chitin biosynthesis pathway (Figure 3). Because the length of and rate

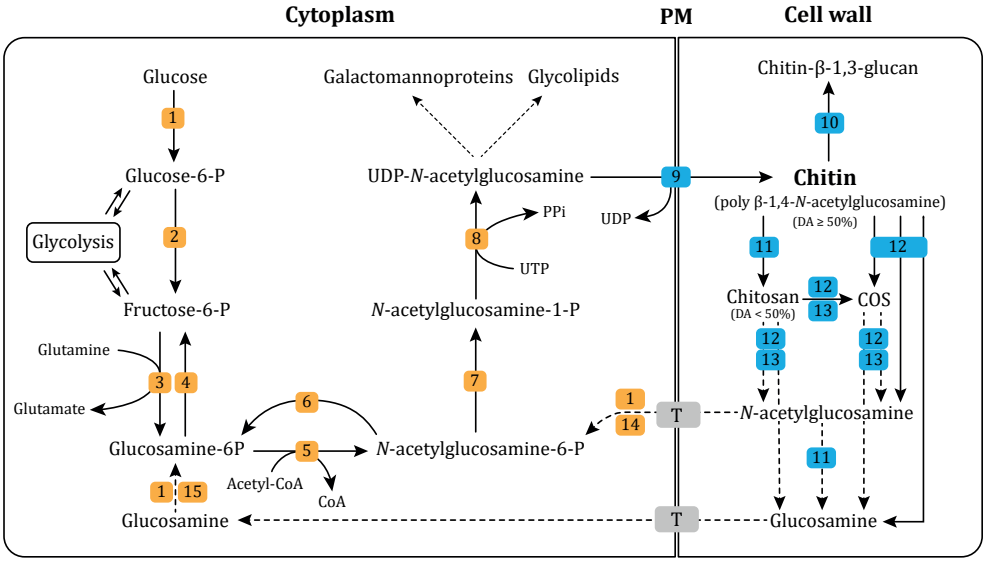

Figure 3. Chitin synthesis, cell wall incorporation and recycling in filamentous fungi. Glucose is converted to glucose-6-phosphate by a hexokinase (1) and is either used for glycolysis or converted to fructose-6-phosphate by glucose-6-phosphate isomerase (2). Fructose-6-phosphate can also either be used in glycolysis or branches off to the chitin biosynthesis pathway. Next, fructose-6-phosphate receives an amino group from glutamine that results in glucosamine-6-phosphate (GlcN-6-P), catalyzed by a glutamine-fructose-6-phosphate amidotransferase (3). Reversion can be facilitated by a glucosamine-6-phosphate deaminase (4). Chitin synthesis continues by the addition of an acetyl group to GlcN-6-P via donation of acetyl-CoA, catalyzed by glucosamine-6-phosphate acetyltransferase (5), resulting in *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P). Also, the reverse reaction is possible here following the deacetylation of GlcNAc-6-P via the *N*-acetylglucosamine deacetylase (6). To continue chitin synthesis, GlcNAc-6-P goes on to be altered to *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) by a phosphoacetylglucosamine mutase (7). GlcNAc-1-P is then uridinylated by UDP- *N*-acetylglucosamine pyrophosphorylase (8), using UTP as a donor molecule, yielding UDP- *N*-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is the precursor molecule that is used by the transmembrane-spanning chitin synthases (9) that remove UDP and add GlcNAc to the non-reducing end of a nascently formed β-1,4-linked GlcNAc polymer, called chitin. As such, chitin synthases protrude chitin chains

across the plasma membrane (PM) into the cell wall. Subsequently, cell wall chitin can either be cross-linked to β-1,3-glucan by chitin transglycosylases (10) or remain as "free" non-covalently interacting polymer in the cell wall. Chitin remains defined as chitin as long as the degree of acetylation (DA) is equal to or higher than 50% (i.e. GlcNAc ≥ GlcN). Below 50% acetylation (i.e. GlcNAc < GlcN), the polymer is referred as chitosan. Chitin deacetylases (11) actively contribute to the deacetylation of chitin and facilitate formation of chitosan. Both chitin and chitosan can be degraded by endo- and exo-acting chitinases (12) and chitosinases (13), resulting in either monomers of GlcNAc/GlcN or chitooligosaccharides (COS). Both GlcNAc and GlcN can putatively be transported into the cell via either specific GlcNAc/GlcN transporters or by promiscuous hexose transporters (T). Here, the GlcNAc/GlcN building blocks can be reintegrated into the chitin biosynthesis pathway via phosphorylation by hexokinases (1) or specific GlcNAc and GlcN kinases, respectively (14, 15). Solid lines define established in literature routes, dashed lines represent putative routes and frequently interspaced dashed lines depict alternative biosynthetic pathways using UDP-GlcNAc. Respective genes in *Aspergillus niger* that correspond to steps 1-8, 14 and 15 (yellow) are shown in Table 1, and steps 9-13 (blue) are shown in Supplementary table 1.

of which the chitin polymers are synthesized is dictated by the available concentration of UDP-*N*-acetyl-glucosamine (Kang et al., 1984; Keller and Cabib, 1971; Orlean and Funai, 2019; Peter, 1987; Sburlati and Cabib, 1986), the synthesis of UDP-*N*-acetyl-glucosamine is considered a primary rate-limiting step in chitin biosynthesis (Merzendorfer, 2011). UDP-*N*-acetyl-glucosamine synthesis shares part of the glycolysis pathway and splits off when fructose-6-phosphate is converted to glucosamine-6-phosphate via a glutamine-fructose-6-phosphate-amidotransferase, at the expense of glutamine (donating an amino-group which converts glutamine to glutamate). Glucosamine-6-phosphate is then acetylated to *N*-acetyl-glucosamine-6-phosphate, using acetyl-CoA as a substrate, and converted to *N*-acetyl-glucosamine-1-phosphate prior to becoming UDP-*N*-acetyl-glucosamine via donation of a UTP molecule. The UDP-*N*-acetylglucosamine is used for the synthesis of chitin. As mentioned above, chitin is remodeled and can be broken down in the cell wall. The resultant products, glucosamine and *N*-acetylglucosamine, can be taken up by hexosamine transporters into the cytosol. Glucosamine and *N*-acetylglucosamine can either be reused for chitin synthesis or they are catabolized to fructose-6-phosphate where they can be re-routed into glycolysis to provide energy for the cell. The required proteins for chitin catabolism, a *N*-acetylglucosamine transporter, *N*-acetylglucosamine deacetylase and a glucosamine-6-phosphate deaminase, are all found in the same gene cluster. This cluster also harbors two putative transcription factors (An16g08990 (*farB*) and An16g09150), a glucose-methanol-choline (GMC) oxidoreductase (An16g09050), a dehydrogenase (An16g09060), a protease (An16g09010) and a *N*-acetyl transferase (An16g09090). All enzymes that are involved in the chitin biosynthetic and catabolic pathways of *A. niger* are shown in Table 1 (and in more detail in Supplementary table 1), and are schematically shown in Figure 3.

The expression of *A. niger gfaA* (glutamine-fructose-6-phosphate-amidotransferase A) was shown to be first rate-limiting step in chitin synthesis (Ram et al., 2004). Additionally, *gfaA* expression was increased together with an elevated levels of chitin content upon activation of the cell wall integrity (CWI) pathway in *A. niger* (Ram et al., 2004). The CWI pathway s highly conserved among fungi, triggered by either disruption or misconstruction of the cell wall (Dichtl et al., 2016). Next to *A. niger*, activation of the CWI pathway has also been shown to cause increased cell wall chitin deposition in other fungi (Fortwendel et al., 2010; Heilmann et al., 2013; Walker et al., 2015,

2008). Aside from expression of *gfaA*, *agsA* (α-glucan synthase A) has also been shown to be induced upon cell wall stress (Damveld et al., 2005b) in *A. niger*, suggesting that simultaneous expression of both *agsA* and *gfaA* upon cell wall stress is relevant for fortifying the cell wall structure. This evidence was recently corroborated by a detailed study of intact *A. fumigatus* cell walls. It was shown that chitin and α-glucan tightly interact, non-covalently, deep inside the cell wall, forming a hydrophobic core that is of structural importance for cell wall integrity (Kang et al., 2018).

In a previous study of our lab, a positive screening method was constructed in an attempt to

Table 1. Enzymes involved in the chitin biosynthetic and catabolic pathways of *Aspergillus niger*. Enzymes are related to steps in Figure 3. In those cases where the corresponding genes have not been named and/or linked to a gene identifier in *Aspergillus niger,* n.a. (not available) is indicated.

Step	Enzyme action	Enzyme entry	gene	CBS513.88 ID	NRRL3_ID
1	Glucokinase	EC 2.7.1.1	*glkA*	An12g08610	NRRL3_03068
	Hexokinase		*hxkA*	An02g14380	NRRL3_05100
	Putative hexokinase		-	An13g00510	NRRL3_01532
	Putative hexokinase		-	An06g00380	NRRL3_11729
2	Glucose-6-P isomerase	EC 5.3.1.9	*pgiA*	An16g05420	NRRL3_06888
3	Glutamine-fructose-6-phosphate amidotransferase (GFAT)	EC 2.6.1.16	*gfaA*	An18g06820	NRRL3_10711
			gfaB	An03g05940	NRRL3_08347
4	Glucosamine-6-P (GlcN-6-P) deaminase	EC 3.5.99.6	-	An16g09070	NRRL3_06624
5	GlcN-6-P acetyltransferase	EC 2.3.1.4	*gnaA*	An12g07840	NRRL3_03114
6	*N*-acetylglucosamine (GlcNAc) deacetylase	EC 3.5.1.25	-	An16g09040	NRRL3_06627
7	Phosphoacetylglucosamine mutase	EC 5.4.2.3	*pcmA*	An18g05160 An18g05170	NRRL3_10581
8	UDP-GlcNAc pyrophosphorylase	EC 2.7.7.23	*ugnA*	An12g00480	NRRL3_09173
9	Chitin synthase	EC 2.4.1.16	10	**	**
10	Chitin transglycosylase	EC 3.2.1.-/ 2.4.1.-	7	**	**
11	Chitin deacetylase/chitooligo deacetylase	EC 3.5.1.41/3.5.1.-	8	**	**
12	Chitinase (endo)/GlcNAc glucosaminidase (exo-chitinase)	EC 3.2.1.14/ 3.2.1.52	13*	**	**
13	Chitosinase (exo/endo)	EC 3.2.1.132	2	**	**
14	GlcNAc kinase	EC 2.7.1.59	n.a.	n.a.	n.a.
15	GlcN kinase	EC 2.7.1.8	n.a.	n.a.	n.a.
T	Putative GlcNAc transporter	n.a.	n.a.	An16g09020	NRRL3_06628
	Putative UDP-GlcNAc transporter	n.a.	n.a.	An03g06940	NRRL3_08261

*Number of (putative) chitinases is 13 for CBS513.88 and 14 for NRRL3. Between the two strains, five chitinases are not shared, i.e. there are no corresponding orthologues (and gene IDs) present.

**See Supplementary table 1 for details on gene IDs

identify genes that are required for correct cell wall biosynthesis, lack of which would cause loss of cell wall integrity. At the time, the reported 20-fold induction of *agsA* expression during cell wall stress was exploited in a UV-mutagenesis screen to find mutants with a continuous state of cell wall stress (Damveld et al., 2008). This system yielded 240 cell wall mutant strains with a constitutively activated CWI pathway, based on *agsA* expression. Several mutants from this collection been characterized over the last few years and include a *ΔtupA* (general transcription repressor) mutant (Schachtschabel et al., 2013), the *ΔugmA* (galactofuranose synthesis) strain (Damveld et al., 2008), a deletion of which absolves cell wall galactofuranose and results in increased chitin content (El-Ganiny et al., 2008 and own observations), along with a verified upregulation of *gfaA* during transcriptomic analysis (Park et al., 2016). Other mutants that were isolated and characterized from this UV-screen are *vmaD* (vacuolar H(+)-ATPase (V-ATPase) subunit d) and *ugeA* (galactofuranose synthesis), but were not tested for cell wall chitin content, though *ΔvmaD* did not show increased CFW staining (Park et al., 2014; Schachtschabel et al., 2012).

Using the same cell wall mutant library as listed above, we screened for mutants with increases in cell wall chitin content, as described in **Chapter 4**. From this screen two candidates were selected with an increased chitin content, RD15.4#55 and RD15.8#16, which were separately characterized in **Chapter 4** and **Chapter 5**, respectively. Characterization of both mutants RD15.4#55 and RD15.8#16 resulted in the identification of two genes that were previously unknown to be involved in the CWI pathway, *cwcA* and *gdiA*. The former gene was named after the cell wall chitin (*cwcA*) increase and encodes a putative repressor of which we postulate that, under non-inducing conditions, represses the activation of the CWI pathway (**Chapter 4**). The latter gene (*gdiA*), Rab GDP dissociation inhibitor (GDI) A, regulates the balance of GTPase cycling between GDP and GTP bound states. GTP-bound, a GTPase is active and interacts with effector proteins, transports vesicles, assists in cargo selection, forms vesicles from membranes and assist fusion of vesicles with the target membranes (Oesterlin et al., 2014). Post-effector interaction, the active GTP-bound GTPase is hydrolyzed to its inactive GDP-bound form, a process which prevents further effector-interaction. Rab GDIs specifically bind the GDP-bound form and solubilize the inactive GTPases from membranes into the cytosol, preventing direct turnover of GDP to GTP (Pfeffer and Aivazian, 2004; Schalk et al., 1996; Ullrich et al., 1993). As such, GDIs act as negative regulators of GTPase cycling between active and inactive form. We identified that a full knockout of the *gdiA* gene is lethal, but that reduced levels of correct transcript result in a chitin increase in the cell wall (**Chapter 5**).

The genes *cwcA* and *gdiA* can, together with other identified mutated genes from the cell wall mutant library, *tupA*, *ugmA*, *ugeA* and *vmaD*, be effectively categorized into three specific functional groups:

i) Both *ugmA* and *ugeA* are involved in the **synthesis** of galactofuranose (Gal*f*), a principle component that is important for the integrity of the cell wall. Gal*f*-conjugates can be found in parts of the galactomannan fraction, *N*-glycan moiety of extracellular glycoproteins and in glycosylinositolphosphoceramides (Tefsen et al., 2012). It remains unknown to what extent

disruption of either one these elements contributes to the activation of the CWI pathway specifically or whether they all contribute equally.

ii) Enzymes VmaD and GdiA are both involved in the **transport** of other enzymes through the secretory pathway. The subunit d of the V-ATPase complex that *vmaD* encodes, is required for a functional V-ATPase that helps acidify intracellular compartments, including vacuoles, endosomes and late Golgi compartments. Organelle acidification has been shown to be important in protein sorting, in biosynthetic and endocytic pathways, proteolytic activation of zymogen precursors and transmembrane transport (Forgac, 2007; Nishi et al., 2003). A full gene disruption of *vmaD* showed a more severe phenotype than the original conditional mutant (RD6.13#15). Similarly, as described above, for *gdiA*, only a conditional mutant with reduced transcription levels was viable, whereas a full knockout was lethal, suggesting that both VmaD and GdiA play an important role in the secretory pathway, possibly for the transport of cell wall biosynthesis proteins and/or the synthesis and secretion of galactomannan or (galactomannoproteins) in the secretory system.

iii) A final class of enzymes acts on a higher regulatory level, encompassing *cwcA* and *tupA*, which both act as **transcriptional repressors** of the CWI pathway. Both deletions of *cwcA* and *tupA* caused pleiotropic effects which can be expected for regulatory genes, among which is the production of differently colored pigments that often related activation of secondary metabolite clusters. It is known that secondary metabolite gene clusters can be activated by the CWI pathway (Valiante, 2017), but does not appear to be universally induced—at least not to the same extent—in the cell wall mutants studied in our work.

Together, the different classes of cell wall mutants derived from this screen are involved in **precursor synthesis, protein transport** or **transcriptional repression**, but no enzymes directly involved in the synthesis of cell wall elements have been identified, such as chitin or glucan synthases. Clearly, the recurrent observations of 17 *tupA* mutants (Schachtschabel et al., 2013) and 4 *ugmA* mutants (Damveld et al., 2008) suggests saturation of this screen. Contrarily, only one mutant was identified for *ugeA*, also involved in Gal*f* synthesis and showing a similar colony phenotype to *ΔugmA*. This suggests that certain mutations may be more likely to occur in this screen even if they are part of the same (synthesis) process.

In **Chapter 6** we assessed the effects of a *kexB* deletion which also resulted in a higher chitin content and found transcriptional upregulation of both *agsA* and *gfaA* for the *ΔkexB* strain. The *ΔkexB* strain was selected separately from the UV-mutant screen based on its morphology and thicker cell walls (Jalving et al., 2000; Punt et al., 2003). Cell wall analysis showed increased chitin deposition which corroborated the observed expression levels for *agsA* and *gfaA*. The KexB enzyme is present on the membranes of the secretory pathway—mainly inside the Golgi apparatus by means of a Golgi retention signal (Jalving, 2005)—and may be categorized as a **transport** related class of cell wall mutants, similar to VmaD and GdiA. Its function is related the correct sorting or zymogenic activation of certain (cell wall biosynthesis) proteins. However, whether lack of KexB is directly related to activation of the CWI pathway as a consequence for the incorrect processing and sorting of cell wall biosynthesis proteins remains unknown.

4. Outlook and future perspectives

The FunChi project set out to optimize the process of chitosan extraction from fungal biomass waste. In order to increase the total yield, two different strategies were employed: removal of covalent cross-links of chitin to the cell wall and increase overall chitin composition. To this end, we analyzed an existing cell wall mutant collection that was previously obtained using a forward genetics screen for mutants with increased alpha-glucan synthase A (*agsA*) expression. The approach has led to the identification of a GdiA mutant strain that produces approximately 60% more cell wall chitin (26% of the cell wall dry weight) than its parental strain (16% of the cell wall dry weight), shown in **Chapter 5**. However, it remains unknown if this is the maximum attainable increase in cell wall chitin content and, if this would already represent an economic feasibility. If further increase is desired, it is also important to gauge the effect on primary product yields. The primary purpose of large-scale fermentations is generally to produce a desired compound in the most efficient way possible with an optimal conversion of energy (glucose) into the production of, for example, citric acid. Because glucose is also required for the synthesis of the cell wall, including chitin (Figure 3), an increase in chitin content directly competes with the available sugars for the desired product, which could be considered as an unfavorable outcome. Consequently, it may only be feasible to increase chitin content if, for example, cell wall α-glucans and β-glucans levels are reduced to compensate for increased chitin content. Alternatively, the extra chitin yield may provide additional value that offsets the loss in e.g. citric acid production. These considerations are outside the scope of this thesis, but are worth investigating if the commercial production of fungal-derived chitosans is to take off in order to compete with the current crustacean chitosan supply.

Instead of focusing on increasing chitin production, it may actually also be very interesting to find clever and cheaper ways to extract chitins and chitosans from fungal mycelial waste. Part of the work described in this thesis (**Chapter 2** and **Chapter 3**) set out in this direction by removing the Crh enzymes that were expected to facilitate covalent links of chitin to the cell wall. Our discoveries have led to the conclusion that removal of these Crh enzymes does not appear to weaken the cell wall at all. These observations can be explained by the possibility that chitin also interacts with α-glucan. We showed that only the knockout of the entire *crh* gene family combined with α-glucan synthase knockouts weakened the cell wall, whereas either knockouts of α-glucan synthases or all *crh* genes alone did not. Therefore, a lot more may be gained by removing all α-glucan synthases in conjunction with the removal of the *crh* gene family to enhance extraction of chitin from cell walls. In addition, removal of α-glucan is expected to reduce both mycelial clumping—causing more dispersed growth as seen in other *Aspergilli* (Yoshimi et al., 2017)—and reducing stirring viscosity, another problem in large scale fermentations (Cai et al., 2014; Papagianni, 2004). In fact, the disruption of *agsE* has already been explored for industrial purposes to increase production yields (van Peij et al., 2014). Reduction in stirring viscosity is also worth exploring further for the *kexB* deletion strain. The *kexB* disruption showed some of these reduced viscosity characteristics next to an increased cell wall chitin content, and the *kexB* deletion has already been combined

with the *crh* gene family knockout (**Chapter 6**), and now requires further investigation for its economic feasibility in industrial application.

There are alternatives ways to screen for mutants with increase chitin content in the cell wall of fungi. A logical experiment would be to create a similar reporter strain for a forward genetic screen that does not rely on *agsA* expression, but rather on chitin precursor synthesis directly, i.e. *gfaA* (or its paralog *gfaB*, Table 1). In this way, one looks directly to gene expression for the synthesis of UDP-*N*-acetyl-glucosamine instead of coincidental expression with *agsA*, which does not occur in all cell wall mutants as seen in **Chapter 4**. The use of the *gfaA* promoter was initially considered for the construction of the UV-mutant library, but was less favorable due to the relatively high baseline expression under non-cell wall stress conditions (Damveld et al., 2008). However, *gfaB* shows relatively low expression levels under non-cell wall stress conditions, similar to those of *agsA* (expression data of wild type in **Chapter 3** and **Chapter 6**) and was strongly induced in the *ΔugmA* strain (FC 119) (Park et al., 2016), and induced in the *ΔkexB* strain (FC 6.21), described in **Chapter 6**. In addition to reporter-based screening, one could also employ adaptive evolution in the presence of cell wall disturbing compounds such as caspofungin, a known echinocandin to induce cell wall chitin deposition through activation of the CWI pathway (Walker et al., 2015, 2008). Because both adaptive evolution and reporter-based screening techniques typically yield loss of function mutations, one might also consider a more directed strain engineering approach to increase chitin content in fungal cell walls. As such, overexpression of both of *gfaA* and *gfaB* may improve the total concentration of cytosolic UDP-*N*-acetyl-glucosamine. Additionally, again with the rise the CRISPR/Cas9 era, it will be much easier to generate strains with overexpression of all genes involved in the chitin biosynthesis pathway from the first hexokinase step, down to last UDP-*N*-acetyl-glucosamine pyrophosphorylase reaction (Table 1). Overexpression of putative regulatory elements for chitin synthesis or chitin synthase genes may be less favorable, as chitin synthases are under different transcriptional regulation and the mRNA transcript levels do not necessarily correlate to chitin synthase activity or chitin content due to post-transcriptional and post-translational modifications (Rogg et al., 2012). In similar multi-gene strategies, one could include the selective elimination of chitinases, with a possible caveat that disruption of all chitinases may hamper germination or hyphal growth (Takaya et al., 1998).

The way forward in either screening or designing mutant strains that produce more cell wall chitin ultimately depends on the requirements versus the trade-offs attached to each approach. The issues imposed by the FunChi project were to both increase chitin production and enhance extractability of chitin from fungal cell walls, through strain optimization. In this thesis, three mutants were described with increased cell wall chitin deposition as a result of cell wall stress. Next to determination of cell wall chitin content, the entire cell wall composition of all three mutants, *cwcA, gdiA* and *kexB,* is currently being analyzed in detail, using the monosaccharide analysis as described in **Chapter 3**. Additionally, fractionated cell wall samples of the three chitin mutants are being tested for their effect on the plant immune response. Both these cell wall content and efficacy tests are absolutely essential to assess whether the cell walls of these mutants

can be turned into an applied plant bio-stimulant. Additionally, it is required to know how these mutations would translate to the phenotype of an industrial strain and how they affect production parameters. With the exception of *ΔkexB*, it remains unknown whether these mutations affect growth rate. During the FunChi project, it was opted to introduce cell wall mutations in industrial strains which are notoriously difficult to transform. Successful knockouts have been introduced in an industrial strain using the CRISPR/Cas9 approach described in this thesis, demonstrating the system's effectiveness. Lastly, as mentioned above, we have obtained new insights in chitin to cell wall cross-linking. We showed evidence that suggests chitin-α-glucan interactions may act as a level of redundancy in cell wall integrity for α-glucan containing filamentous fungi. These novel observations pave the road towards directed strain design that would allow easier extraction from fungal cell walls in the future. The findings presented here and the ongoing work by the FunChi partners all contribute to the common project goal of valorizing spent fungal biomass.

Supplementary table 1. Enzymes involved in the chitin biosynthesis pathway.

Step	Enzyme action	Enzyme entry	CAZy entry	Gene	Annotation	CBS513.88_ID	NRRL3_ID
1	Hexokinase	EC 2.7.1.1	n.a.	*glkA*	Glucokinase	An12g08610	NRRL3_03068
				hxkA	Hexokinase	An02g14380	NRRL3_05100
				-	Putative hexokinase	An13g00510	NRRL3_01532
				-	Putative hexokinase	An06g00380	NRRL3_11729
2	Glucose-6-P isomerase	EC 5.3.1.9	n.a.	*pgiA*	Putative glucose-6-phosphate isomerase	An16g05420	NRRL3_06888
3	Glutamine-fructose-6-phosphate amidotransferase (GFAT)	EC 2.6.1.16	n.a.	*gfaA*	GFAT	An18g06820	NRRL3_10711
				gfaB	GFAT	An03g05940	NRRL3_08347
4	Glucosamine-6-P (GlcN-6-P) deaminase	EC 3.5.99.6	n.a.	-	GlcN-6-P deaminase	An16g09070	NRRL3_06624
5	GlcN-6-P acetyltransferase	EC 2.3.1.4	n.a.	*gnaA*	GlcN-6-P acetyltransferase	An12g07840	NRRL3_03114
6	*N*-acetylglucosamine (GlcNAc) deacetylase	EC 3.5.1.25	n.a.	-	Putative GlcNAc deacetylase	An16g09040	NRRL3_06627
7	Phosphoacetylglucosamine mutase	EC 5.4.2.3	n.a.	*pcmA*	Phosphoacetylglucosamine mutase	An18g05160 An18g05170*	NRRL3_10581
8	UDP-GlcNAc pyrophosphorylase	EC 2.7.7.23	n.a.	*ugnA*	UDP-GlcNAc pyrophosphorylase	An12g00480	NRRL3_09173

Supplementary table 1. Enzymes involved in the chitin biosynthesis pathway. (Continued)

Step	Enzyme action	Enzyme entry	CAZy entry	Gene	Annotation	CBS513.88_ID	NRRL3_ID
9	Chitin synthase	EC 2.4.1.16	GT2	*chsA*	Chitin synthase	An07g05570	NRRL3_04653
				chsB	Chitin synthase class III	An09g04010	NRRL3_00331
				-	Chitin synthase class II	An08g04350	NRRL3_11077
				chsD	Chitin synthase class IV	An09g02290	NRRL3_00179
				chsE	Chitin synthase class III	An12g10380	NRRL3_02932
				chsG	Chitin synthase class VI	An08g05290	NRRL3_11152
				chsL (csmB)	Chitin synthase class VII/class V myosin motor domain	An02g02340	NRRL3_06067
				chsM (csmA)	Chitin synthase class VII/class V myosin motor domain	An02g02360	NRRL3_06066
				chsF	Chitin synthase class III	An03g06360	NRRL3_08320
				chsC	Chitin synthase class I	An14g00650 An14g00660*	NRRL3_00641
10	Chitin transglycosylase	EC 3.2.1.-/2.4.1.-	GH16	*crhA*	GH16_2	An11g01540	NRRL3_10021
				crhB	GH16_2	An07g07530	NRRL3_04809
				crhC	GH16_2	An07g01160	NRRL3_04315
				crhD	GH16_2	An01g11010	NRRL3_02532
				crhE	GH16_2	An13g02510	NRRL3_01365
				crhF	GH16_2	An16g02850	NRRL3_07085
				crhG	GH16_2	An15g05350	NRRL3_03998

Supplementary table 1. Enzymes involved in the chitin biosynthesis pathway. (Continued)

Step	Enzyme action	Enzyme entry	CAZy entry	Gene	Annotation	CBS513.88_ID	NRRL3_ID
11	Chitin deacetylase/chitooligo deacetylase	EC 3.5.1.41/3.5.1.-	CE4	*cdaA*	Putative chitin deacetylase	An04g07110	NRRL3_07680
				cdaB	Putative chitin deacetylase	An12g04480	NRRL3_03397
				cdaC	Putative chitin deacetylase	An11g00920	NRRL3_10065
				cdaD	Putative chitin deacetylase	An18g04560	NRRL3_10540
				cdaE	Putative chitin deacetylase	An02g13530	NRRL3_05169
				cdaF	Putative chitin deacetylase	An08g09290	NRRL3_11464
				cdaG	Putative chitin deacetylase	An01g13340	NRRL3_02724
				cdaH	Putative chitin deacetylase	An15g00280	NRRL3_03591
12	Chitinase (endo)	EC 3.2.1.14	GH18	*cfcA*	Putative endochitinase, class V	An02g07020	NRRL3_05709
				chiD/ cfcB	Putative chitinase	An08g09030	NRRL3_11445
				cfcC	Putative chitinase	An04g04670	NRRL3_07862
				cfcD	Putative chitinase	An01g05360	NRRL3_02063
				cfcE	Putative chitinase	An15g00840	NRRL3_03643
				cfcF	Putative chitinase	An11g01160	NRRL3_10047
				cfcG	Putative chitinase	An19g00100	NRRL3_01224
				cfcH	Chitinase; endochitinase	An14g07420	n.a.
				cfcI	Endochitinase	An02g13580	NRRL3_05164
				cfcJ	Hydrolase activity	An11g05860	NRRL3_09665
				cfcK	Putative chitinase	An12g05330	n.a
				ctcA	GPI-anchored chitinase	An09g06400	NRRL3_00523
				-	Putative chitinase	n.a.	NRRL3_01212
				ctcB	Putative chitinase	An09g05920	NRRL3_00478
				-	Putative chitinase	n.a.	NRRL3_04221
				-	Putative chitinase	n.a.	NRRL3_09653

Supplementary table 1. Enzymes involved in the chitin biosynthesis pathway. (Continued)

Step	Enzyme action	Enzyme entry	CAZy entry	Gene	Annotation	CBS513.88_ID	NRRL3_ID
12	GlcNAc glucosaminidase (exo-chitinase)	EC 3.2.1.52	GH20	*nagA*	Putative *N*-acetylglucosaminidase	An09g02240	NRRL3_00174
				-	Putative *N*-acetyl glucosaminidase	An03g02960	NRRL3_08564
				-	Putative *N*-acetylglucosaminidase	An01g01920	NRRL3_01790
13	Chitosinase (exo/endo)	EC 3.2.1.132	GH75/ GH46	-	Putative chitosinase	An03g05260	NRRL3_08400
				-	Putative chitosinase	An04g04530	NRRL3_07870
14	GlcNAc kinase	EC 2.7.1.59	n.a.	n.a.	n.a.	n.a.	n.a.
15	GlcN kinase	EC 2.7.1.8	n.a.	n.a.	n.a.	n.a.	n.a.
T	(UDP-)GlcNAc transporter	n.a.	n.a.	n.a.	Putative GlcNAc transporter	An16g09020	NRRL3_06628
				n.a.	Putative UDP-GlcNAc transporter	An03g06940	NRRL3_08261

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